This PDF is available from The National Academies Press at http://www.nap.edu/catalog.php?record_id=19916

FROM THE ARCHIVES

Integrity of Frozen Spermatozoa (1978)

Pages
349

Size
5 x 9

ISBN
0309026458

U.S. National Committee for the International Institute of Refrigeration; Commission on Sociotechnical Systems; National Research Council

Visit the National Academies Press online and register for...

- Instant access to free PDF downloads of titles from the
  - NATIONAL ACADEMY OF SCIENCES
  - NATIONAL ACADEMY OF ENGINEERING
  - INSTITUTE OF MEDICINE
  - NATIONAL RESEARCH COUNCIL
- 10% off print titles
- Custom notification of new releases in your field of interest
- Special offers and discounts

Distribution, posting, or copying of this PDF is strictly prohibited without written permission of the National Academies Press. Unless otherwise indicated, all materials in this PDF are copyrighted by the National Academy of Sciences.

To request permission to reprint or otherwise distribute portions of this publication contact our Customer Service Department at 800-624-6242.

Copyright © National Academy of Sciences. All rights reserved.

NATIONAL ACADEMY OF SCIENCES
1863–2013
Celebrating 150 Years of Service to the Nation

The Integrity of Frozen Spermatozoa

Proceedings of a Round-Table Conference
Held on April 6-7, 1976, at the National Academy of Sciences, Washington, D.C., under the auspices of the U.S. National Committee for the International Institute of Refrigeration, in conjunction with the Food and Drug Administration and the Naval Medical Research Institute

U.S. National Committee for the International Institute of Refrigeration
Commission on Sociotechnical Systems
National Research Council

NATIONAL ACADEMY OF SCIENCES
Washington, D.C. 1978

NAS-NAE
FEB 1 1978
LIBRARY

8-0005
.1

NOTICE: The project that is the subject of this report was approved by the Governing Board of the National Research Council, whose members are drawn from the Councils of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine. The members of the Committee responsible for the report were chosen for their special competences and with regard for appropriate balance.

This report has been reviewed by a group other than the authors according to procedures approved by a Report Review Committee consisting of members of the National Academy of Sciences, the National Academy of Engineering, and the Institute of Medicine.

International Standard Book Number 0-309-02645-8

Library of Congress Catalog Card Number 77-940301

Available from

Printing and Publishing Office
National Academy of Sciences
2101 Constitution Avenue
Washington, D.C. 20418

Printed in the United States of America

Order from
National Technical
Information Service,
Springfield, Va.
22161
Order No. PB 279-522/7

PREFACE

For the past few years the U.S. National Committee for the International Institute of Refrigeration has been conducting short conferences devoted to the presentation and discussion of research papers in the field of low-temperature phenomena as they relate to the life sciences and the application of the results. At these conferences the presentation of each paper is followed by a discussion period during which the conferees comment on the presented paper or offer additional information. As a service to the scientific community and the general public the National Academy of Sciences is publishing the papers and the pertinent discussions. It should be noted, however, that neither the National Academy of Sciences, the U.S. National Committee, nor the conferees approve or disapprove opinions or results presented in this publication.

Nelson T. Grisamore
Staff Executive
U.S. National Committee for
the International Institute
of Refrigeration

PARTICIPANTS

CONFERENCE CO-CHAIRMEN

BERNARD LASALLE, Animal and Plant Health Inspection Service, Department of Agriculture, Room 831, Federal Bldg. No. 1, Hyattsville, MD 20782

ARTHUR P. RINFRET, Union Carbide Corporation, Tarrytown Technical Center, Old Saw Mill River Road, Tarrytown, NY 10591

SESSION CHAIRMEN

April 6 Session

E. F. GRAHAM, Department of Animal Science, University of Minnesota, St. Paul, MN 55108

April 7 Session

JOHN C. PETRICCIANI, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

JOHN O. ALMQUIST, Department of Dairy Science, Pennsylvania State University, University Park, PA 16802

MICHAEL BARILE, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

DAVID E. BARTLETT, American Breeders Service,
DeForest, WI 53532

AARON BENDICH, Sloan-Kettering Cancer Center,
1275 York Avenue, New York, NY 10021

ROLLAND R. CONTE, Vice President, R&D, Minnesota Valley
Engineering, 407 7th Street, N.W., New Prague, MN 56071

A. H. DARDIRI, Plum Island Animal Disease Center, ARS,
USDA, Northeastern Region, P.O. Box 848, Greenport,
Long Island, NY 11944

RICHARD DIGIOIA, Veterans Administration Hospital,
50 Irving Street, Washington, DC 20422

RICHARD DONOVICK, Director, American Type Culture
Collection, 12301 Parklawn Drive, Rockville, MD 20852

ELIZABETH EARLEY, Bureau of Biologics, Food and Drug
Administration, 8800 Rockville Pike, Bethesda, MD 20014

RUNE ELIASSON, Karolinska Institute, Stockholm, Sweden

BENNETT ELISBERG, Bureau of Biologics, Food and Drug
Administration, 8800 Rockville Pike, Bethesda, MD 20014

F. I. ELLIOTT, Director, Laboratories and Research,
American Breeders Service, DeForest, WI 53532

JOSEPH FELDSCHUH, Medical Director, Idant Corporation,
645 Madison Avenue, New York, NY 10022

WILLIAM FISHBEIN, Chief, Biochemistry Division, Room 3001
Armed Forces Institute of Pathology, Washington, DC 20306

R. H. FOOTE, Department of Animal Science, Cornell
University, Frank B. Morrison Hall, Ithaca, NY 14850

GARY FRIEDLAENDER, Naval Medical Research Institute,
National Naval Medical Center, Bethesda, MD 20014

FREDERICK GRIGSBY, Bureau of Drugs, Food and Drug
Administration, 8800 Rockville Pike, Bethesda, MD 20014

E. S. E. HAFEZ, Wayne State Medical School,
540 E. Canfield Avenue, Detroit, MI 48201

JAMES HILL, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20014

JOYCE JOHNSON, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

L. A. JOHNSON, Reproduction Laboratory, Building 200, Agricultural Research Center, Beltsville, MD 20705

RUDOLF KADEN, Free University, Hautkinik im Klinikum Steglitz, 1 Berlin 45, Germany

DAVID LANG, Duke University Medical Center, Durham, NC 27710

J. ROBERT LODGE, Animal Science Laboratory, Department of Dairy Science, University of Illinois, Urbana, IL 61801

RONALD MAYNOR, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

J. W. MCVICAR, Plum Island Animal Disease Center, ARS, USDA, Northeastern Region, P.O. Box 848, Greenport, Long Island, NY 11944

JULIE MILSTEIN, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

KAMAL MITTAL, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

VICTOR H. MULLER, Central Indiana Regional Blood Center, 2859 North Meridian, Indianapolis, IN 46208

J. T. MULLHAUPT, Senior Research Scientist, Linde Division, Union Carbide Corporation, Tarrytown Technical Center, Old Saw Mill River Road, Tarrytown, NY 10591

PHILIP NOGUCHI, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

JOSEPH O'MALLEY, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

PAUL PARKMAN, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

W. T. PENTZER, 13216 Ingleside Drive, Beltsville, MD 20705

B. W. PICKETT, Director, Animal Reproduction Laboratory, Colorado State University, Ft. Collins, CO 80523

VERNON G. PURSEL, Reproduction Laboratory, Building 200, Agricultural Research Center, Beltsville, MD 20705

G. W. SALISBURY, Director, Agricultural Experiment Station, College of Agriculture, University of Illinois, Urbana, IL 61801

MORRIS SCHAEFFER, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20014

KENNETH SELL, Naval Medical Research Institute, National Naval Medical Center, Bethesda, MD 20014

J. K. SHERMAN, Department of Anatomy, College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72201

ROBERTA WHITE, R.N., Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

LEON ZUSSMAN, Director, Human Sexuality Center, Department of Community Medicine, 410 Lakeville Road, New Hyde Park, NY 11040

NAS STAFF:

Nelson T. Grisamore, *Staff Executive*, USNCIIR

Joan M. Sieber, *Administrative Secretary*

FOREWORD

This conference, like those preceding it, has brought together participants of diverse professional backgrounds who have contributed to the science and technology of the subject or whose current interests, scientific or otherwise, bear significantly thereon. Unlike the earlier conferences, this is a joint undertaking of two separate organizations, the USNCIIR and the Bureau of Biologics of the Food and Drug Administration. The reasons for this combined program were made clear to all participants in the invitations sent to them and in due course, as the published proceedings are made available, these reasons should become evident to all who refer to that volume.

For more than a quarter century, geneticists and reproductive physiologists have sought to take advantage of the fact that bovine spermatozoa, and that of other economically important domestic animals, can be frozen and cooled to very low temperatures and can subsequently be recovered in functional form. Among us today are several investigators who have contributed to the science and technology underlying the widespread artificial insemination operations at present carried on in this country and abroad. It is inevitable that obvious success with cryopreservation of sperm among lower mammals would have led to studies in man. We are also fortunate to have with us individuals who have devoted much of their professional careers in this field to discuss and interpret their observations. Human artificial insemination, which encompasses entities defined as biologic products, is of major interest to the Bureau of Biologics, FDA. On earlier occasions, having discussed with Drs. Petricciani and Noguchi the content of the conference program, I have come to understand the

basis for the Bureau's concern with product quality and other aspects of regulatory responsibility in this field of medicine. I believe that as our conference proceeds, all its participants will arrive at a similar understanding.

The first day of the conference, as in preceding USNCIIR round-table programs, is centered around a group of themes, each presented by a professional of recognized competence in the subject. At the conclusion of each presentation, the other participants are free to comment on, criticize, or challenge any or all material included therein. All present at round-table sessions are here by invitation, with the understanding that each is expected to contribute to the proceedings, at least from the basis of his or her own experience. None are invited merely to audit. All are free to contribute at greater length, and as a matter of record, for inclusion in the published proceedings.

These conferences, although based on subjects primarily of scientific and technological interests, are designed to include participants other than scientists whose professional activities are affected by such matters. Thus, members of pertinent federal agencies, practicing physicians, university faculty, attorneys, business executives, and others can be expected to bring points of view not likely to be encountered at a meeting consisting entirely of experts in a tightly defined field of science. Although research is often reported, a conference of this type is not intended to deal exclusively, or even comprehensively, with recent original investigation. Rather, its purpose is to regard a subject of current scientific or technological interest from a number of, hopefully, closely reasoned points of view.

The common thread throughout the prior conferences sponsored by the National Research Council and organized by the USNCIIR, as well as in those contemplated for the future, lies in that area of science concerned with the influence of physical processes, temperature and pressure, on living systems and other materials of biological origin. Special emphasis has been placed on temperature below the freezing point of water, and as a consequence, the pressures and other phenomena induced by changes of state. The present conference is no exception. A large and growing body of scientific knowledge, as such of limited significance to society, is being implemented in an expanding body of technology that holds, for good or otherwise, much of major significance. Needless to say,

this is not an unusual situation in many fields of science. Our round-table conference format is a simple device for assembling people who regard such matters from varying vantage points.

The second session, which is being co-sponsored by the Naval Medical Research Institute and the Bureau of Biologics of the Food and Drug Administration, was organized with the interest and help of the National Academy of Sciences and the USNCIIR, in such a way that it complements the first session. The success in obtaining outstanding speakers for this session is in no small way due to the support provided by the Naval Medical Research Institute in meeting the travel expenses of some of the speakers.

In many ways it is strange that in the year 1976 we should be sitting down to discuss issues that have a direct bearing on the possible regulation of human sperm banks. It is strange because the product--human sperm--is a biologic with which we have had experience for hundreds of thousands, if not millions, of years. And in that sense it is hardly a new or novel biologic, nor is it being considered outside the context of its usual role in reproductive biology. But we must bear in mind that there is a distinction between what occurs during the natural course of human reproductive interactions and those cases in which human semen is used in a therapeutic setting such as in the treatment of infertility. Human sperm could easily be considered the ultimate biologic product. Other than ova, which are neither as accessible nor as abundant as sperm, there is no biologic that has the enormous potential of sperm. Nevertheless, concern has been expressed that this unique biologic may be associated with health hazards. Dr. Sherman has been especially active in bringing this issue to the attention of many of us. Our meeting today is meant to focus attention on human sperm banking with the overall aim of developing both data and comments that will be useful to a regulatory agency faced with the questions of whether or not to initiate regulatory activity, and if so, which type would be the most appropriate. As we proceed through today's presentations, I think it will become clear that the regulation of sperm banking is a complex issue, and that there are many areas in which our basic knowledge is grossly deficient. We hope that as a by-product of this meeting, major funding agencies such as the National Institutes of Health and National Science Foundation will begin to recognize the importance of

supporting studies to answer some of the fundamental questions in this field.

The general situation in sperm banking has been characterized by some as an enterprise where we know very little about the donor and even less about what happens to the semen specimen after the deposit in the bank. What we want to do today is try to define what is reasonable to know about the donor and his sample before it is used in artificial insemination.

A. P. Rinfret
John C. Petricciani

CONTENTS

Introduction
Bernard LaSalle 1

Fundamentals of the Preservation of Spermatozoa
E. F. Graham 4

Summary of Research at The Pennsylvania State University
J. O. Almquist 45

Techniques for the Cryopreservation and Field Handling of Bovine Spermatozoa
W. E. Berndtson and B. W. Pickett 53

Banks for Frozen Human Semen: Current Status and Prospects
J. K. Sherman 78

Gametic Integrity During Storage at Low Temperatures--Evidence for an Aging Effect
G. W. Salisbury and J. R. Lodge 92

Molecular Factors Affecting Gametic Integrity During Storage at Low Temperatures
J. T. Mullhaupt 126

Maintenance of Fertility of Spermatozoa at -196°C
R. H. Foote 144

Theoretical and Practical Significance of Frozen Semen Banking in the Animal Breeding Industry
F. I. Elliott 163

Microbial Contaminants in Frozen Semen
David E. Bartlett 173

Current Clinical Applications of Sperm Banking
Joseph Feldschuh 180

A Community's Human Tissue Resources and the Role of the Community Blood Bank--Custodial or Developmental?
Victor H. Muller 188

Semen Banking Today: A Review of Current Technology in Equipment for the Storage of Semen at Liquid Nitrogen Temperature
R. R. Conte and K. A. Rensberger 191

History of Artificial Insemination and the Development of Human Semen Banking
J. K. Sherman 201

A Plan for the Controlled Growth and Development of Tissue Banks
K. W. Sell and G. E. Friedlaender 208

Artificial Insemination in Clinical Practice
Joseph Feldschuh 212

The Conjoint Artificial Insemination Technique
Leon Zussman 214

Microbial Contamination of Human Semen
Philip D. Noguchi, Joseph E. Kiss, and John C. Petricciani 216

Mycoplasma Contamination of Human Spermatozoa
Michael F. Barile 226

Viruses in Human Semen
David J. Lang 237

Motility and Viability of Human Spermatozoa
Rune Eliasson 250

Physiomorphological Parameters of Human Spermatozoa
E. S. E. Hafez 262

INTRODUCTION

Bernard LaSalle

Semen preservation includes selection of donors, collection, addition of cryoprotectant, stabilizer, diluent and preservatives, freezing, thawing, macroscopic and microscopic examinations, microbiological tests, immunobiological tests, genetic tests, insemination, and evaluation of fertility and offspring.

It is interesting to recall that artificial insemination was first practiced in horses by the Arabs, in the fourteenth century. In the last half of the eighteenth century, Jacobi, Spallanzani, and Rossi experimented on fish, frogs, silkworms, and dogs. In the nineteenth century, several papers on artificial insemination were published by gynecologists. It is only since the end of the nineteenth century, and the beginning of the twentieth century, that the technique of artificial insemination was developed and extensively applied to livestock.

Russian scientists, led by Ivanov, contributed greatly to the improvement of the livestock industry in their country, through artificial insemination. They were the first, in 1907, to begin writing extensively on its application in horses, cattle, sheep, swine, fowl, furbearers, and wild animals. Scientists of Europe, America, and Asia studied and pursued the Russians' work.

Interesting reviews on the subject of this conference may be found in

- *The Veterinary Record,*
- *The Technique of Artificial Insemination,* Imperial Bureau of Animal Genetics, 1933
- *Revue Internationale d'Agriculture,* 1934
- *Recueil de Medecine Veterinaire,* 1935
- *Germ Plasm Resources,* American Association for the Advancement of Science, 1961

J. K. Sherman presented "State of the Art of Banking Frozen Human Semen" at our 1972 USNCIIR round-table conference, "A Review of Problems Associated with Biological Integrity at Low Temperatures." From the beginning of artificial insemination in the fourteenth century, through the first three decades of the twentieth century, semen was generally preserved at body temperature, and protected against cold shock, for use immediately or within one-half hour after collection. The need for improving semen preservation was obvious.

The Russian workers developed and used buffered sugar solutions as diluents, to increase the volume and prolong the life of semen. These diluents preserved semen for a few days at room temperature, but did not protect spermatozoa against cold shock. In 1932, 560,000 mares, 2,000,000 cows, 3,000,000 ewes, and 200,000 sows were artificially inseminated in Russia. The practice was then generally nonexistent or only emerging in most other countries.

To transport semen to our widely distant farms, the American workers developed egg-yolk-phosphate buffer-citrate diluents, which made it possible to slowly cool diluted semen from body temperature to $7^{\circ}C$ in 90 min. and to stop motility and reduce the loss of fertility to 5 percent daily for 3 or 4 days.

Ivanov reported, in 1923, "Experiments on the Disinfection of Sperm in Mammals, Especially in Relation to Dourine in Horses." He mixed infected semen with salvarsan (arsphenamine) and neosalvarsan (neoarsphenamine), 1:10,000, to kill *Trypanosoma equiperdum,* without affecting fertility.

The American scientists pioneered the use of antibacterial agents, such as sulfanilamide, penicillin, and streptomycin. They also pioneered, in their own way, the use of diluents containing glycerol, glycine, sugars, milk, whole egg, skim milk, buttermilk, dried milk, CO_2, dimethyl sulfoxide, ethylene glycol, and others.

Although attempts to preserve semen by freezing were made toward the end of the nineteenth century, they were not successful until the late thirties and early forties of this century, when only human semen was found to survive freezing at the low temperatures of liquid gases. Semen freezing became routine after the breakthrough of the French and British scientists who discovered the protective effects of glycerol. Their initial work with frog and fowl semen was soon extended to semen of other animal species including man.

Pretreatment of bovine semen with glycerol, slow cooling, freezing, and storage in liquid nitrogen at -196°C has since been used extensively and routinely, with great success. Several breakthroughs have been reported for the preservation of the fertility of boar semen through freezing and thawing. All this has made possible great progress in livestock improvement by increasing several thousandfold the potential progeny of better-selected sires or donors and by allowing ample time for extensive evaluation and testing for genetic qualities, freedom of genetic defects and agents of infectious diseases, for international transport, and for long-range breeding programs.

The history of semen preservation, the current literature, and this conference prompt me to share with you the expectation that greater emphasis will be placed on:

- Selection and care of donors
- Long-range breeding and livestock breeding programs
- Testing donors for all diseases common to the species
- Aseptic collection and handling of semen
- Testing semen for all known pathogens and undesirable contaminants
- Improved cryoprotective diluents and cryogenic preservation
- Addition of antibacterial, antifungal, antimycoplasmal, and other preservatives
- Cleaning, disinfection, and sterilization of all facilities, equipment, instruments, containers, freezers, and liquid nitrogen tanks.
- Sterile filtration of liquid nitrogen
- Recordkeeping
- Regulations
- Other precautions to prevent the use of contaminated, infectious, dangerous, or worthless semen.

FUNDAMENTALS OF THE PRESERVATION OF SPERMATOZOA

E. F. Graham

From time eternal the mysteries and intricacies of sexual reproduction have been pondered. Enter man, the manipulator, into the normal process of reproduction of self or the beasts that serve him; enter artificial means of semen collection; enter collected semen; enter preservation in the liquid state; enter cryopreservation; enter artificial insemination; and enter challenges, opportunities, problems, and questions. Accomplishment in the field of physiology of reproduction has been made to an extent that would stagger the imagination of those not working in the area. But the immense perturbation that man has introduced into the process is as unprecedented as it is relentless and cannot now be checked save ignoring several centuries of hard-won understanding. We cannot reverse this trend of events, but we should have the power of discernment to use appropriately the fruits of our research in proper perspective. Goals in animal research have been directed to animal improvement for the betterment of mankind. Many of our findings are, however, directly applicable to the human, although we have no control at present over their use in this context either for good or ill. Research is turning intensively to the very basics, propelled headlong by applications beyond the limits of our current understanding. Our only recourse is to endeavor to recognize very early the scope of our impact, seek true answers, and apply knowledge cautiously with wisdom and vision for the future.

The purpose of this paper is to list and review several of the factors, steps, problems, and questions that may be important either to animal or man in the process of reproduction from sperm collection through fertilization. During this conference, drawing from the principal speakers and discussants, it is hoped that

definitive answers with appropriate data will shed insight to the many questions raised, and at the same time pose questions to guide future research important to reach our goals. Only selected references will be used to illustrate some diversity of opinion.

SEMEN COLLECTION

The artificial vagina is the preferred method of semen collection in most species. It provides for the proper temperature, pressure, and lubrication to simulate natural mating.[79] Care should be taken to maintain sanitary conditions. Electroejaculation of semen is used when animals are not trained for the artificial vagina or are physically unable to mount.[47] Larger volume, resulting in lower sperm concentrations with comparable or somewhat decreased fertility of bull semen, is obtained. Electroejaculated ram semen has less uniform ejaculates, lower quality, and lower fertility than semen collected with the artificial vagina.[126,182]

Collecting semen too frequently causes a decrease in concentration and volume per ejaculate, but increases the total number of sperm collected per unit time. Variations do exist from species to species.[79] The maximum potential daily sperm output has been estimated as 12.8 billion (mature Holstein bulls), 5.3 billion (young shorthorn bulls), and 31.3 billion (2-yr-old Yorkshire boars).[34]

Sexual preparation and stimulation by allowing a single false mount increases sperm output by 50 percent, while three false mounts increases production by 100 percent. Prolonged restraint before mounting has a similar effect. Changing teasers and environment also increases sexual interest and semen quality and quantity.[189]

INITIAL SEMEN QUALITY

Volume of semen varies with age, species, and individual animal and is used along with sperm concentration and motility to determine final dilution rate for freezing. One study showed a significant ($r = -0.170$ and -0.111) correlation between volume and fertility.[24]

Many researchers have investigated the relationship between initial sperm concentration and fertility.

Initial sperm-rich cell concentration of boar semen gave the highest correlation to fertility of all characteristics studied (r = 0.61).[196] Similar results have been reported for the stallion.[218] However, no significant correlation between sperm concentration and fertility in ram semen has been reported.[97,233] Several investigators [24,25,172] reported significant correlations between initial sperm concentration and fertility (r = 0.216 to 0.353) for bull semen. However, others [39,40,50,92] reported no significant correlation between concentration and fertility. In general, initial concentration does not appear to be a practical method for predicting fertility, but definitely influences the chemical environment that may be important to the cell.

Viability is often assessed by estimating percentage progressively motile cells using a microscope or closed-circuit television. Other types of electronic and photographic equipment have been used. Abnormal motion patterns indicate cold shock or chemical shock.[79] Observations of sperm velocity, survival, and swimming patterns using photoelectric methods have been correlated to fertility.[220] Percentage of initial motility has also been correlated to fertility (r = -0.218).[24] Significant correlations between post-thaw motility and fertility have also been reported.[155] Various vital stains and morphology tests have been widely used to assess semen quality. In general, when there are less than 20 percent abnormal sperm cells, fertility is not affected, but as the percentage of abnormal sperm cells increases, fertility decreases.[34] More recently, acrosome morphology of thawed, incubated semen has been shown to have a high correlation with fertility (r = -0.81) based on 90-day nonreturn rates.[180] Several metabolic tests have also been utilized, such as methylene blue reduction, oxygen uptake, pH change, fructose utilization, and lactic acid production. Investigators [24] reported significant correlations with oxygen uptake (r = -0.267) and methylene blue reduction time (r = -0.227) with fertility, while others [172] reported no significant correlations with these tests.

Semen Chemistry

The constituents of semen are numerous and varied--choline, citric acid, fructose, inositol, ergothioneine,

various ions, and other substances not found in large quantity in the rest of the body.[34] The main constituents which have been studied, which vary greatly, include inorganic phosphorus, Na, K, Ca, Cl, total protein, reducing substances, and phosphatases.

Minerals

Inorganic phosphorus is used in most bovine semen extenders, but has been found to depress motility and oxygen consumption in higher concentrations.[121] Early studies indicated that fertility was not correlated with levels of inorganic phosphorus in the bovine,[55] but later work[53] established that levels of inorganic phosphorus were correlated with combined levels of Na and K and were not significantly related to fertility.

Many mineral substances are found throughout the male tract and in semen, and come from both the seminal plasma and spermatozoa.[121] Sodium, potassium, and calcium are the bulk cations, with many trace elements (boron, magnesium, iron, copper, etc.) existing in very small concentrations.[189] In an early review,[121] concentrations of Na and Ca were believed to be higher in seminal plasma and K concentrations higher in the sperm cell, with the latter being confirmed by others.[235]

Potassium was found to depress motility in bull semen,[22,199] and a higher concentration was found in epididymal semen than in ejaculated semen (K:Na ratio 1:1 in epididymal plasma, 1.0:2.5 in seminal plasma). This accounted for decreased motility in K-rich medium and epididymal sperm. The concentration appeared to shift in the ejaculation process with added seminal fluids, and the increased Na concentration initiated motility in ejaculated semen.[121,199] High concentrations of electrolytes in semen extenders were found to have a damaging effect on sperm.[21,96] In ram semen, the addition of K increased metabolism. Calcium depressed O_2 uptake and fructose oxidation. However, the addition of 0.5-5.0 mm KCl stimulated the metabolism of both recently ejaculated and thawed spermatozoa.[142]

Some attempts[73] have been made to correlate minerals to fertility. The data suggests that in bull, boar, and turkey spermatozoa, a positive correlation exists with K and P and a negative correlation exists with Ca concentrations found in seminal plasma and fertility.

Total Protein

Many studies have involved the total protein content in semen. It is estimated that 90 percent of the total N present in seminal plasma is protein.[189] Seminal plasma protein was found to be approximately 14.4 percent N.[104] Various figures have been given for the actual protein content in bovine semen, and they all were very close: whole semen, 7.32 g percent; seminal plasma 7.04 g percent.[223]

It was shown that total protein content of bovine seminal plasma was not significantly correlated with fertility. It was, however, related to reducing substances, Ca and Cl concentrations,[53] and semen volume, but not to sperm cell concentration.[100] Those species (bull, ram, goat, and man) with higher seminal plasma protein concentrations responded to the freezing procedures more readily than those species with low seminal plasma protein concentrations (boar, stallion, and turkey).

Reducing Substances

The reducing power of sperm has long been used as an indicator of semen quality,[121] and several reducing substances have been found in semen: fructose, ascorbic acid, sulfhydryl compounds, and sulfite.[189] Fructose is the principal reducing compound and is actively metabolized by sperm cells. This reducing substance is derived solely from the seminal vesicles,[16] with various estimations made as to its concentration: 376-1,062 mg percent,[121] 280-779 mg percent, and 26-872 mg percent.[177] A significant correlation between initial fructose level and fertility was reported.[49]

Immotile sperm cannot use fructose, so a positive correlation between fructolysis rate and motile sperm concentration was expected and confirmed.[18,51]

A formula for predicting fertility level using fructolysis was devised, and it was found that bulls with a high fructolysis index had a conception rate 5.9 percent higher than bulls with a low index.[164] However, negative correlations between reducing substances in seminal plasma and fertilizing capacity has also been reported.[53,73] Sperm cell concentration and volume are inversely related to concentration of seminal fructose.[51] Therefore, a

high fructose concentration may not indicate fertilizing ability.[121]

Enzymes

The presence of phosphatases was first demonstrated in human semen in 1935 and bovine semen in 1945.[119] Acid and alkaline phosphatase activity is intermediate in the bull, while acid is high and alkaline low in the human.[14] Both alkaline and acid phosphatases were positively correlated with sperm cell concentration, volume, and motility.[84,173,179,200] Only alkaline phosphatase was correlated positively with fertilizing capacity,[200] while both alkaline and acid phosphatase showed a positive correlation in another study.[73] Several enzymes have been added to semen extenders, and it has been suggested that their inclusion enhances fertility.

Certain cellular components are released when cells are damaged. Measurements of GOT (glutamic oxaloacetic transaminase) and LDH (lactic dehydrogenase) are two constituents that can be used as a possible indicator of fertility or damage.[70,73,143]

BUFFERS OR EXTENDER MEDIA

An ideal extender for semen should:

1. maintain proper osmotic pressure and electrolytic balance to ensure minimal salt effects;
2. have constituents with metal-complexing properties that could be effectively utilized to bind heavy metals harmful to sperm cells;
3. have a pK between 6 and 8, preferably 7, and have buffering capacity so deleterious shifts in pH can be prevented as lactic acid is formed and cooling occurs;
4. contain constituents that provide protection against the harmful effects of cooling and freezing;
5. be stable, resisting enzymatic and nonenzymatic degradation;
6. contain antibiotics that inhibit bacterial growth;
7. substantially increase the volume of semen so that multiple inseminations can be performed; and
8. provide an environment in which the metabolic activities of the sperm can continue.

Ions and Zwitterionic Buffers

The increase in intracellular salt concentration due to the removal of water from the cell during ice formation is a major cause of sperm mortality during freezing. The protective action of cryoprotectants such as glycerol is largely attributed to their salt "buffering"capacity, thus electrolytic damage as the water freezes out is minimized. It has also been reported that extracellular salts have a deleterious effect on spermatozoa.[121] Potassium and sodium have been shown to decrease the motility of bull spermatozoa.[37,236] Excess or insufficient calcium ions also adversely affect motility.[237] The heavy metals, such as Cu, Zn, Ag, Pt, Ni, Al, and Au (in decreasing order of potency), also decrease the motility of human spermatozoa.[99]

The zwitterionic buffers MES,[118] PIPES, TES, HEPES,[78] BES,[89] MOPS, and TRICINE[77] have been used extensively as buffers for spermatozoa.[6,7,8,9,12,25,41,42,57,60,70,236] The effect of binding heavy metals by BES and TES may explain their efficacy in decreasing the release of GOT, as is possibly the beneficial effect of sodium citrate and phosphate.[69]

Since the development of the phosphate,[147] sodium citrate,[187] carbon dioxide bicarbonate,[213] and trisbuffers,[22,41,42,57,60,236] little development of other buffers for preservation of bull spermatozoa has been reported. Many attempts have been made to improve these basic buffers by the inclusion of various additives such as gelatin, peptone, glycerol, glycine, glucose, and many other materials. Undefined materials, such as honey, coconut milk, tomato juice, alfalfa, and guandu leaves, have also been used with varying degrees of success.

The discovery that egg yolk in semen extenders has a beneficial effect on fertility[147,148] led to its widespread use in bull semen extenders. Early researchers reported that egg yolk aided the sperm cell in resisting cold shock.[21,106,107] However, the discovery[163] that bull semen could be successfully frozen in extenders containing glycerol diverted researchers from studying the protective properties of egg yolk. Further research indicated that glycerol was not essential for freezing spermatozoa under certain conditions[15,65] and actually decreased fertility when added to buffers for chickens, turkeys, and boars.[26,36,71,158,160]

Subsequent studies have shown that the lipoprotein fraction of egg yolk may be responsible for the protection

of spermatozoa against cold shock. This is accomplished by minimizing the loss of lipids from the sperm during freezing.[34]

While amino acids do not provide significant protection to spermatozoa,[13] peptides in certain concentrations were found to prevent damage to cells.

Antibiotics

Semen extenders are a good culture medium for bacteria, which may reduce fertility or adversely affect the inseminated female. Microbial contaminants have been linked to low fertility and conception rates, abortion, reproductive failure, endometritis, salpingitis, and other reproductive disorders.[83] Until recently, penicillin, sulfanilamide, streptomycin, and neomycin were the main antibiotics used to control bacteria that contaminate semen ejaculates.[5,6,46,48,238] Recently these have been replaced by Linco-Spectin, due to its superiority in controlling mycoplasma, and Vibrio.[83] The increased efficiency in controlling microbial contaminants by using Linco-Spectin is obtained without loss of fertility.[9]

pH - pK

Changes in pH may occur in addition to an increase in salt concentration during freezing, due to precipitation of either ice or salt. To date, there is virtually no quantitative information available on the measurement or change in pH at or below the freezing point for a mixed solvent system (an extender for biological material) incorporating a cryoprotective nonelectrolyte such as glycerol or DMSO.

Measurement of pH at low temperatures is difficult. As early as 1932,[54] Finn, using a glass electrode, showed that as the temperature of muscle tissue was lowered from $+15^{o}$ to $-4^{o}C$, its pH increased on the order of 0.02 units per degree Celsius. He also stated that when freezing commenced, concentration of the aqueous phase took place, producing a further change in pH. He showed that the pH of this liquid portion of frozen muscle tissue decreased with temperature below the freezing point.[210]

A solution of concentrated milk, collected by

centrifuging an ultrafiltrate, showed a pH shift from 6.7 to 5.9 during freezing.[210]

The pH of freezing aqueous salt solutions[214,217] showed that sodium phosphate solutions with a pH of 7.0 at 25°C decrease in pH during cooling (approaching a pH of approximately 3.5 near the eutectic point, -9.9°C), while potassium phosphate solutions increase in pH during cooling (approaching a pH of approximately 7.5 near the eutectic point, -16.7°C). The differences in pH are due to low solubility.[215]

Van den Berg[215] described a modified electrode for direct measurement of pH in frozen materials at temperatures as low as -30°C. Electrodes that can be used to measure pH at temperatures as low as -20°C are commercially manufactured today.

The pH of bull semen was estimated to be between pH 7.0 and 7.5.[226] Later studies[85] stated the pH range for semen of fertile bulls may be quite wide and lie between 6.4 and 7.8 with normal semen slightly acidic and never more alkaline than pH 7.3.[127] Present-day bovine semen extenders vary in their pH specification, ranging between 6.5 and 7.2.[58,62,69]

It has been speculated that quick freezing and thawing may not allow the biological material to be exposed to critical pH and salt concentration long enough for inactivation to occur.[24] It is also possible that at these low temperatures biological material may be less sensitive to pH changes than at body temperatures.

DILUTION

Time and temperature of dilution vary with species and are influenced by whether the sperm are easily cold-shocked, life expectancy of the cells, and biochemical changes of the cells in seminal plasma. Turkey semen does not cold-shock and has a life expectancy of only a few hours *in vitro*, so it can be diluted at 5 to 37°C immediately after collection. Semen of many species, such as bull, ram, stallion, and goat, is routinely extended immediately after collection at 37°C and cooled slowly to prevent cold shock. Bull semen held in its own seminal plasma for 20 min at 26°C before dilution resulted in higher fertility than semen extended 0 min at 26°C.[171] Several reports[71,72] show that slow cooling (1-2 h) to 22°C is important to maintain fertility in the use of frozen boar semen. A protein layer, important to

fertility, is developed on the sperm head from the seminal plasma during this period of time.[146]

The dilution ratio for freezing varies with species, freezing container and technique, thawing and insemination techniques, and volume and sperm concentration needed for fertility. High and low concentrations of sperm cells may be frozen successfully, depending on technique and species.

It has been shown[58] that the extender with the lowest pH consistently gave nonreturn rates equal to or higher than the rates for the control. Studies demonstrated[201] that the least damage before freezing occurred in 0.20 M medium (containing tris) of pH 6.5, and the highest percentage of post-thaw motile spermatozoa was observed in an extender adjusted to pH 6.50 and containing 0.20 M tris, 6.4 percent glycerol, and 1 percent fructose.

The effect of pH upon motility, velocity, and metabolism of bull sperm has been demonstrated by numerous works.[19,37,103,136,188,190,208,219,234] The observed responses varied markedly with temperature, media composition, and from sample to sample.

In selection of proper pH of an extender, one should be aware of the changes that occur during cooling and freezing. As concentration increases and the eutectic point is reached, the sample is approaching the pK value reflecting large changes in pH.

Although the osmolarity of semen and seminal plasma is similar to that of other biological fluids, sperm can tolerate considerable deviation in osmotic pressure. Motility may actually be enhanced in hypotonic solutions; however, fertility is maximal when the extender is isotonic.[34]

Concentration and Dilution Effects on Post-Thaw Survival and Fertility

Ram semen frozen at very high concentrations (1.5×10^9 cells/ml) did not survive as well as semen frozen at lower concentrations.[109,110] Likewise, semen frozen at an extension of 1:4 has better fertility than semen frozen 1:2.[209,221] Highest fertility was reported for cell suspensions frozen at a concentration of 666×10^6 cells/ml.[108]

A concentration of 1×10^9 cells/ml had a reported fertility of 76 percent[134] and post-thaw motility for

samples frozen at 3-5 x 10^8 cells/ml was enhanced if seminal plasma was removed.[35]

Frozen chicken semen at concentrations of 0.750 x 10^6 cells/ml resulted in high fertility.[225] Shaffner[192] reported 100 percent fertility for one treatment of semen frozen at 10^9 cells/ml. Post-thaw motility was depressed when cells were frozen at 0.330 x 10^6 cells/ml as compared to a concentration of 600 x 10^9 cells/ml.[224]

A concentration of 2 x 10^9 turkey sperm cells/ml has been frozen with a fertility of 50 percent.[239] Reports of previous work showed a uniformly low fertility for concentrations between 3.5 x 10^9 cells/ml[26] and 1 x 10^9 cells/ml.[117]

Bull semen has been frozen successfully at high or low concentrations. Three studies have reported successfully freezing bull semen at 500 x 10^6 cells/ml.[157,211] As extenders and methods have improved, reports of a dilution effect have diminished.

Boar semen showed depressed motility when frozen concentrated (500-1,000 x 10^6 cells/ml)[68] or dilute (15-30 x 10^6 cells/ml).[181] However, in one study, spermatozoa frozen in high concentration (600 x 10^6 cells/ml) resulted in 85 percent fertility.[186]

COOLING

Rapid decreases in semen temperature from 37° to 5°C cause cold shock, a damage to the sperm cell caused by changes in gas solubility, metabolism, fluid viscosity, and chemistry. However, prolonged exposure of semen to higher temperatures maintains greater metabolic activity and earlier death of spermatozoa.[189] Semen from most mammalian species is subject to cold shock. The optimum cooling rate is one that prevents cold shock but is fast enough to maintain healthy viable sperm cells. Lipoproteins and lecithins, such as found in egg yolk and milk, provide protection from cold shock when added to the semen before cooling.[189] Extender composition and pH have both been reported to influence the cold-shock susceptibility of boar spermatozoa as reflected by motility.[20,141,167,168] Changes occurred in acrosome structure in spermatozoa as a result of cold shock. A proper cooling rate to freezing temperature is important to preserve the viability and fertility of bovine semen.[189] However, human sperm are not susceptible to cold shock.[193]

CRYOPROTECTANTS

Since the discovery of glycerol as a cryoprotective agent for mammalian spermatozoa, much effort has been devoted to determining its protective mechanism. Several well-documented investigations have been conducted to determine its protective mechanism. These include: (a) decrease in high concentration of solutes, (b) maintenance of pH, and (c) change in liquid-ice phase. Several studies using highly sophisticated methods of evaluation and taking into consideration (a) molar solubility excess, (b) the volatility factor, (c) protection coefficient, (d) free energy of hydration, (e) hydrogen donors, (f) hydrogen binding, and (g) interference between donor and acceptor groups have been reported in an attempt to relate protective qualities, but with little success. Many small-molecular-weight penetrating compounds, referred to as colligative agents mimicking glycerol, have been studied along with other classes of materials, which include sulfoxides, N-oxides, and acetamides.

Some investigators reported that some polymeric solutes afforded protection to red blood cells. These include polyvinylpyrrolidone, polyethylene glycol, gelatin, starch, polyvinyl alcohol, poly-N-methyl glycine, and polyvinyl methyl sulfoxide.

The empirical application of colligative and/or polymeric protectors on freezing of cells becomes an endless endeavor. The effect of empirical application is difficult to interpret, because each compound seems to exert different effects in different concentrations, in different biological fluids, on different cell types, and by each on freeze rate. Also, there exist interactions between colligative and polymeric compounds that are difficult to separate. Polymeric compounds in this text refer to larger-molecular-weight proteins, lipids, or combinations. To this author's knowledge, there have been no reports of successful freezing of spermatozoa with any colligative compound in the absence of these materials. However, successful freezing has been demonstrated in the absence of colligative compounds, but is generally enhanced by their addition.[72] A total of 63 colligative and more than 100 polymeric compounds (either pure or dialyzed or nondialyzed from body fluids with or without further fractionation) have been tested in an appropriate buffer solution. Data showed that no colligative compound was effective in the absence of polymeric material, but several, including

glycerol, DMSO, 1,2-propanediol, 1,3-propanediol, ethylene glycol, triethylene glycol, 1,4-butanediol, and N-methyl acetamide, enhanced the recovery of spermatozoa in the presence of appropriate polymeric material. Egg yolk or its derivatives was the only polymeric material placed in an appropriate buffer solution that afforded appreciable protection in the absence of colligative agents. Other protein or lipoprotein materials, such as those in blood, milk, or other body fluids, were effective, but only in the presence of colligative materials.

Regardless, some success in the freezing of spermatozoa can be obtained by placing the cells in practically any type of isotonic solution containing lipoproteinaceous material with appropriate concentrations of several different colligative compounds. The time, temperature, rate of addition, and concentration of these compounds have been studied with many conflicting results. Addition of glycerol to bull semen at 5°C has been reported to be better than at 10 or 20°C,[30] while others[75] reported no difference between 5, 10, 20, and 37°C addition of glycerol. The same conflict of opinion is reported for ram spermatozoa.[33,108] Glycerol addition rates of 1 to 16 parts over periods of zero to several hours, as well as final concentrations of 2 to 20 percent (v/v), have been studied with conflicting results. In general, differences due to time, temperature, rate, and concentration of glycerol addition vary with species, extender, freezing, and thawing techniques. A general recommendation is to add glycerol to semen slowly with extender at 5°C: 7.0-7.5 percent in yolk citrate, 10-13 percent in heated skim milk, or 7-10 percent in heated homogenized milk.[189]

Semen equilibration time after addition of glycerol has been studied with conflicting results. Higher fertility has been reported[122] with bull semen equilibrated for 18 h rather than 2 h and the exact reverse reported by others,[124,138] while still others[75] reported no difference among 30 min, 4 h, and 18 h equilibration. In general, optimum equilibration time has not been established, but varies with species, extender, and techniques employed and is presumed to be shorter rather than longer.

PACKAGING OF FROZEN SEMEN

The three most common packages for frozen spermatozoa

are the ampule, the straw, and the pellet. Most of the relevant fertility data compares bovine spermatozoa frozen in ampules or in straws. The results from these studies indicate that fertility is virtually the same for these two packages. Good recovery can result from any of these three packages, and the choice between them is largely influenced by personal preference. Listed below are the advantages and disadvantages of the three common packages.

Ampule

Glass ampules are commonly available, they lend themselves to convenient flame sealing, and they are easily labeled. The thickness of the glass makes very high cooling or warming rates difficult, but these rates are seldom necessary. Because of their shape, ampules are not efficient in terms of storage space.

Straw

Plastic straws are not commonly used in general laboratories, but their use is widespread in the cattle-breeding industry. They can be readily labeled for identification and sealed to exclude contaminants. Straws lend themselves to high cooling and warming rates, if such rates are necessary. They also offer more efficient use of storage space than ampules, an important factor for long-term storage. The straws waste little extended semen because an insemination can be made directly from them, avoiding significant losses from transferring to an intermediate pipette before insemination.

Pellet

Pellets are formed by placing a drop of extended semen directly onto the surface of dry ice or, in rare instances where very high cooling rates are desired, on liquid nitrogen. The frozen spermatozoa are placed in suitable containers after freezing, so positive identification of each unit is more difficult than with ampules or straws. Special precautions are necessary to avoid undesirable contamination during storage. The cooling rate is very consistent, because the surface of

dry ice is always the same temperature. On the other hand, it is difficult to vary the cooling rate of pellets, although some variations of cooling rate can be achieved by varying the pellet volume.

STORAGE

Use of dry ice or liquid nitrogen storage temperatures has extended the life of frozen semen; these have been compared repeatedly. Early work indicated only a slight difference in nonreturn rate, favoring liquid nitrogen.[152] In later trials, nitrogen yielded higher nonreturn rates than dry ice and is believed to reduce possibilities of errors in handling.[155,212]

While semen stored in liquid nitrogen is more resistant to exposure than that in dry ice,[64,155] repeated exposure to warm temperatures and solar radiation is suspected of decreasing fertility of the semen.[149]

When dry ice was the common method of storing semen, the conception rate from ampules warmed to -55°C and recooled to -79°C showed a marked drop from 70 percent to 28 percent.[113] Others found severe damage if semen was allowed to partially thaw, then refreeze.[155] Rising storage temperatures, rather than original storage temperatures, are responsible for motility loss in human semen as well.[14] Researchers agree that exposure of any frozen semen should be kept to a minimum, whether stored in liquid nitrogen or dry ice.

Fertility

Early low-temperature storage allowed for only short-term use of bull semen. Once dry ice was used, semen was stored from 3 mo to 4 yr without a large decrease in conception rate.[52,113,114,130-132] Liquid nitrogen is a more recent advancement, and results have been extremely acceptable. Nitrogen was slightly favored over dry ice in early trials[95,152,156,202] and produced conception rates nearly as high as that from fresh semen.[154]

It has been suspected that some aging of the spermatozoa may occur if semen is stored for a long period of time and that this may be associated with embryo mortality and delayed returns.[184-186] In some studies, semen stored at -196°C was consistently high.[31,56,111,129,203]

Ram semen has been used with success after 5 yr storage,[183] and human semen has been preserved for up to 10 yr. Freezing and thawing procedures account for most of the loss of motility of human spermatozoa, but storage has also been considered to be the cause of significant declines in motility.[167]

Genetic Degradation

Now that human semen is being frozen and stored, the problem of possible genetic degradation becomes more pronounced. It is agreed that cryopreservation is genetically innocuous[195] and that the possibility of genetic damage is extremely remote.[194] When frozen and stored semen has been used, fewer abnormal babies and spontaneous abortions have occurred than in the general population. In commercial cattle breeding, no evidence of abnormal offspring or skewed sex ratios have been attributed to the use of semen stored at subzero temperatures.

RATE OF FREEZING AND THAWING

Smith and Polge[159,161,197] originally suggested that optimal recovery of bull sperm frozen in 1 cm^3 ampules requires an average cooling rate of 1 to 2°/min from +5 to -15°C and 4 to 5°/min from -15 to -79°C. While this became the "standard" rate for freezing semen, later experimenters found other rates worked well also.[12,17,27,32,43,63,88,91,98,105,112,139,161,178,222] Freezing in a liquid nitrogen freezing machine proved to be equal or superior to freezing at the "standard" rate with dry ice in alcohol.[32,178] Plunging samples from +5°C into a -20°C bath and subsequently cooling at 25°/min gave equal recovery to the standard rate,[17] as did immersion into baths held at -10, -20, and -30°C, but not -40°C,[88] or directly into a -40°C bath,[98] or -45°C bath.[105] Equally good recovery resulted from controlled liquid nitrogen freezing from 2.5 to 3.0°/min from +5 to 15°C and 5 to 6°/min from -15 to 50°C,[63] or 1 to 5°/min from zero to -10°C and 4 to 20°/min from -10 to -30°C.[27] Cooling above -15°C at rates of 15 to 50°/min had little detrimental effect if rate was reduced to less than 30°/min between -15 and -30°C,[91] although freezing faster than the standard rate caused rapid deterioration of semen after 24 h storage at 79°C.[207]

A critical temperature range, within which the greatest amount of damage occurs during "standard" cooling, is reported as -10 to -35°C.[139] The effect of damage within this range was reported to be minimized by equilibrating semen in diluent at 5°C for up to 18 h.[123,161] This critical range is also characterized by sensitivity of bull spermatozoa to high cooling velocities above -27 or -30°C, but very resistant to high velocities below -30°C.[42,161] Plunging into liquid nitrogen from -20°C results in practically no survival, but plunging from -27°C allows maximal survival.[112]

Thawing in ice water or water at 40°C or above is superior to thawing in 15 to 20°C water[140,153] using motility[153] or motility and acrosome retention,[140] while 60 to 90 day nonreturn rates show 5°C equal to 20°C and 40°C thawing bath,[81] or to 5°C thaw when the ice cap is removed 45 s after immersion.[23]

Freezing in smaller shapes, i.e., pellets and straws, has allowed much faster freezing and thawing rates due to a larger surface to volume ratio than that of ampules. The "standard" method for pellets is to drop extended semen on dry ice,[135] while for straws it is to suspend them singly on a rack in liquid nitrogen vapor.[94] Pellets and straws freeze much faster than ampules, and pellets never show supercooling. Pellets and straws frozen by standard methods give similar nonreturn rates.[66,128] Varying the freezing rate of pellets by using volumes of 0.019 to 0.095 ml has very little effect on motility.[227] Straws and ampules frozen by standard methods show similar motility when thawed in ice water, even though the 0.3 ml straw freezes at 108°/min from +5 to -15 to -60°C.[8] Semen frozen in 0.25 ml straws shows significantly better fertility than the same semen frozen in ampules.[133] Optimal freeze rate may depend on thaw rate used, since, with a 5°C thaw bath, straws frozen at 32°/min from +5 to -15° to -60°C showed significantly poorer recovery than straws frozen 43°/min from +5 to -15°C and 25°/min from -15 to -60°C, while thawing at 75°C eliminated freeze-rate differences and produced significantly better recovery.[6] Also, for straws frozen at 6°/min (+5 to -15°C) and 12°/min (-15 to -60°C) or 95°/min (+5 to -15°C) and 53°/min (-15 to -60°C), no freezing rate differences were seen.[7] For semen extended in heated skim-milk-glycerol extender and frozen in straws, an increased percent of normal acrosomes[230,232] and of progressive sperm motility[231,232] was found as thawing bath temperature was increased;

this was also true for egg-yolk citrate-glycerol extender.[175,176] Semen in straws survived better if frozen in dry ice-alcohol than in liquid nitrogen vapor when cooling rate is the same,[116] perhaps due to more efficient heat transfer. Thus, as a general rule, fast freezing of straws is superior to slow freezing[8,151,176] or shows no difference,[7,8] while fast thawing is superior to slow thawing.[1,2,7,8,151,174,175,230,232] The superiority of fast freezing is independent of thaw rate[176] or is significantly better only if a slower than optimal thaw rate is used.[8]

If rate within an applicable range is important (± few hundred degrees C per min), the only controllable method is one involving fast and efficient heat transfer. This can only be achieved by bath or thermal conductivity other than gas or vapor.

Freezing does not take place at a constant rate. The rate of cooling in a semen sample as it freezes is constantly changing due to two factors. The first factor is the change in phase from liquid to solid, occurring both abruptly at the freezing point and gradually until the eutectic point. The removal of the heat of fusion that allows this change in phase is aided by the greater thermal conductivity and smaller specific heat of ice than of water. The second factor, which can be controlled, is the way the sample is cooled. This includes the shape (geometry) of the sample, especially the surface/volume ratio; the contact to the cold surroundings; and control of temperature of the cold surroundings. Perhaps the disagreement in the literature as to the "best" method to freeze and thaw is due to repeatability problems, unreported differences in how the sample was cooled, or the effect of glycerol in widening the range of freezing rates giving acceptable results. Average rate of cooling over a wide temperature range is not an adequate description of how the sample was cooled.

Two basic ways of freezing by cooling in a bath are static freeze (bath fixed at a temperature colder than the sample) and dynamic freeze (bath cools, sample temperature follows bath temperature). In a dynamic freeze, the sample follows the bath until it crystallizes. During the freezing plateau the bath continues to cool. Once the heat of fusion has been removed, the rate of cooling is quite high until the sample temperature catches up with the bath. It is possible that this faster "catch-up" rate could injure the cells, especially if it occurs within the "critical temperature range" above -27°C.

There is no problem of the bath "getting ahead" of the sample when cooling in a static bath; however, the resulting cooling curve is nonlinear, while the cooling curve of a dynamic bath may be controlled.

One common "bath" is cold vapor of liquid nitrogen. It is used for static freezing by immersing samples in the static vapor above liquid nitrogen or for dynamic freezing by vaporizing liquid nitrogen at a controlled rate into a closed chamber in which nitrogen gas is circulated. Freezing curves produced by static vapor methods generally vary greatly from sample to sample within a freeze and especially from freeze to freeze. Since nitrogen gas has a low heat capacity per unit volume (1/1,500 of alcohol), a small amount of heat will warm the gas a great deal; the 80 calories of heat from freezing 1 cm^3 of water would warm 1 liter of gas approximately 260°C. Thus, static vapor equilibrium is easily disturbed. In addition, the poor thermal conductivity of nitrogen gas (1/9 that of alcohol) impedes fast, efficient heat transfer and uniform temperatures throughout the freezing chamber. Dynamic vapor methods only partially relieve the drawbacks of nitrogen gas by circulating the gas through the chamber at a high rate. The metal Cassou rack may be successful, because it conducts most of the heat from straws directly to the liquid nitrogen bath rather than depending on static vapor for cooling. Pellet freezing on dry ice provides fast and efficient heat transfer by using small samples, each with its own cold stream of gas, which at any point in this cooling process is controlled by each sample's temperature. Heat transfer is so efficient that supercooling of a pellet has never been observed. In general, liquid baths (e.g. alcohol) provide convenient, fast, repeatable, and efficient heat transfer for cooling semen, although high cooling rates are restricted to static liquid baths due to the high heat capacity of the liquid.

The Assay Problem

Although correlation to fertility should be the ultimate test of any assay, such correlation is difficult because fertility data have large variability. Also, different assays are possibly more sensitive to different types of damage that are of equal importance to fertility. We studied the sensitivity of a number of laboratory assays

on spermatozoa by adding known varying proportions of dead cells to live populations.[44] For bull spermatozoa, the 95 percent confidence limit of a single determination of percent undamaged cells was ±19 percent for motile cells. Motility of one representative field of one slide was estimated by three trained observers. Other methods may improve the accuracy of motility measurements. One is the cinematographic method of ABS, in which extended exposure photography is used to distinguish sperm that have moved between pictures. Another is the filter method, in which only the motile cells are able to traverse a column of Sephadex.[74] In some cases motility may not be very meaningful. For example, motility may be eliminated without noticably reducing fertility by adding a small amount of formaldehyde or this compound enhanced with caffeine. Among other assays, the 95 percent confidence limit was ±17.5 percent for normal apical ridges of acrosomes, ±23.5 percent for release of GOT, and ±14.1 percent for nigrosin-eosin live-dead staining. A new assay, the ability of a cell to swell in a hypotonic solution as measured by a specially modified Coulter counter, shows only a ±10.6 percent for a 95 percent confidence limit and appears very promising.[198] However, even with the best methods available today, only fairly wide differences may be distinguished on a single determination.

ARTIFICIAL INSEMINATION

With the use of AI increasing, studies of physiology, endocrinology, and biochemistry have aided scientists in determining the conditions necessary for optimum fertility results.

Time of Use after Thawing

Semen from various species is frozen by several methods, and thawing procedure is dependent on freezing procedure and species involved. Sperm cells, once thawed, do not remain viable as long as cells that have never been frozen. Therefore, regardless of method of freezing (in ampules, straws, or pellets) or thawing (warm versus cold), it is generally agreed that semen should be used very shortly or as soon as possible after thawing.[79,165,204]

Site of Deposition

Site of semen deposition is also a crucial factor, and again depends on species. In cows, a rectovaginal technique where semen is deposited into the uterus has yielded higher conception rates than other methods of insemination,[204] and insemination into the anterior cervix or uterine body is better than insemination at the external os.[59,115] Intrauterine insemination with frozen semen is a necessity in ewes, where intrauterine conception rates are nearly twice as high as cervical insemination.[3,110] Sows and mares are routinely inseminated deep in the cervix or into the uterus to make the most effective use of the sperm cells.[59,79,150,205]

Sperm Numbers and Dosage

In animals where a temporary locking occurs, a larger volume of semen is needed for maximum fertility. Horses require 500 million motile cells in a volume of 10-40 ml for best fertility results.[79,150,205] Sows require a minimum of 200 million motile cells in 40-60 ml if frozen semen is used.[165]

Cows and ewes do not require such a large dosage. When the ampule was the commonly used container for frozen bull semen, a volume of 1 ml containing at least 12 million motile cells was used.[79] Now that the French straw has evolved, a 0.25 ml dosage containing a smaller number of sperm cells can be used with equal success. Ewes can be inseminated with 200 million motile cells in a volume of 0.05 to 0.2 ml if frozen semen is used, 100 million cells if fresh semen is used.[79,109]

Time of Insemination

Considering changes some sperm cells may undergo in the female tract and coordinating insemination with the proper time pre- or postovulation are extremely important. Ovulation occurs after estrus in cattle. Insemination must take place before ovulation, preferably toward the middle or end of estrus.[79] Ovulation coincides with the end of heat in ewes, and inseminating too early will exhaust the sperm reserve. Inseminating 12-24 h[90,191] after first observations of heat will yield best results.

Sows ovulate 24-26 h after the onset of estrus and

fertilization takes place as early as 2-3 h after insemination. Best fertility is obtained when insemination coincides with ovulation.[79,165] Insemination must also be synchronized with ovulation in the mare. Ovulation occurs 4-6 days after onset of estrus, and fertility reaches a peak about 2 days before the end of estrus.[79] Daily inseminations beginning on day 2 of estrus have been recommended.[204]

Time of use after thawing, site of deposition, dosage, and cell numbers and timing of insemination must all be carefully controlled if viable cells are to be at the site of fertilization at the right time.

Fertility can be affected by production of autoantibodies in both males and females and by the production of antibodies against semen and cryoprotectants in the female.

Females show an immune response to semen.[206] Increases in serum titers of sperm agglutinating antibodies in cattle with large numbers of repeat breedings have been demonstrated.

Cryoprotectants such as egg yolk elicit an immune response[76] with increased frequency of egg yolk antibodies in uterine mucosa from tracts of cows receiving repeated inseminations. They also observed decreased fertility with increased uterine titers. Repeated inseminations of rabbit semen with egg yolk proteins result in a detrimental effect by egg yolk. An *in vivo* antigen antibody reaction releases histamine resulting in an anaphylactoid reaction.[86] It has also been shown that egg yolk antibodies significantly affected motility and percent intact acrosomes in cattle.[137]

Extensive research has been conducted on the many factors that influence the preservation and use of frozen semen, and the scientific and commercial applications have resulted in modern, sophisticated methods of animal production. These factors, however, are not always totally understood or easily controlled. Thus, we are still faced with opportunities and challenges to develop further our present knowledge and search for new ideas and principles so we may better manipulate the influencing factors for our own use.

DISCUSSION

R. H. FOOTE: With respect to your comment on the need for spermatozoa to be surrounded by protective

media, this is not a practical problem, considering that a concentration of 200 x 10^6 sperm/ml occupy only about 1 percent of the volume. With human semen, the original concentration is often less than this, thus more than 99 percent of the space would be occupied by cryoprotective media.

BIBLIOGRAPHY AND REFERENCES

1. Aamdal, J., and Anderson, K. 1968. Fast thawing of bull semen frozen in straws. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris, p. 154
2. Aamdal, J., and Anderson, K. 1968. Fast thawing of semen in straws (cattle). Zuchthygiene *3*:22-24.
3. Anderson, V. K., Aamdal, J., and Fougner, J. A. 1973. Intrauterine and deep cervical insemination with frozen semen in sheep. Zuchthygiene *8*:113-118.
4. Ackerman, D. R. 1968. Damage to human spermatozoa during storage at warming temperatures. Int. J. Fertil. *13(2)*:220-225.
5. Adler, H. C. 1960. The comparative efficiency of the intracervical and intrauterine insemination of cattle with streptomycin-treated and non-treated semen. Acta Vet. Scand. *1*:105-113.
6. Almquist, J. O. 1951. A comparison of penicillin, streptomycin, and sulfanilamide for improving the fertility of semen from bulls of low fertility. J. Dairy Sci. *34(8)*:819-822.
7. Almquist, J. O., and Wiggin, H. B. 1973. Effect of different combinations of freezing and thawing rates upon survival of bull spermatozoa in U.S. plastic straws. Artif. Insemination Dig. *21(9)*:10-11.
8. Almquist, J. O., and Zaugg, N. L. 1974. Fertility of bovine semen in milk diluent containing combinations of penicillin-neomycin and lincomycin-spectinomycin. J. Dairy Sci. *57*:1211-1213.
9. Almquist, J. O., and Wiggin, H. B. 1973. Survival of bull spermatozoa frozen and thawed by different methods in plastic straws. Artif. Insemination Dig. *21(4)*:12.
10. Anderson, J., 1942. J. Agric. Sci. *32*:298.
11. Bajpai, P. 1965. Some physiological immunological factors affecting the fertilizing capacity of turkey spermatozoa. PhD thesis, Ohio State University.
12. Bean, B. H., Pickett, B. W., and Martig, R. C. 1963.

Influence of freezing methods, extenders, and storage temperatures in motility and pH of frozen bovine semen. J. Dairy Sci. *46*:145-149.

13. Beljakov, S. P., and Aliev, N. 1966. Fertilizing ability of ram semen diluted with carbonated honey diluent and stored at 2°-18°C. Tr. Uzb. Nauchno-issled. Inst. Vet. *17*:264-267.
14. Bell, D. J., and Lake, P. E. 1962. A comparison of the phosphomonoesterase activities in the seminal plasmas of the domestic cock, turkey tom, boar, bull, buck, rabbit, and of man. J. Reprod. Fertil. *3*:363.
15. Berndtson, W. E., and Foote, R. H. 1972. The freezability of spermatozoa after minimal prefreezing exposure to glycerol or lactose. Cryobiology *9*:57.
16. Bernstein, A. D. 1937. Bull Biol. Med. Exp. URSS *4*:483.
17. Bialy, G., and Smith, V. R. 1957. Rapid rates for freezing bull semen. J. Dairy Sci. *40*:622.
18. Bishop, M., Campbell, R. C., Hancock, J. I., and Walton, A. 1954. Semen characteristics and fertility in the bull. J. Agric. Sci. *44*:227.
19. Blackshaw, A. W., and Emmens, C. W. 1951. The interaction of pH, osmotic pressure and electrolyte concentration on the motility of ram, bull and human spermatozoa. J. Physiol. 114:116.
20. Boenda, J. 1968. Morphological changes of the acrosome of boar spermatozoa during aging and cold shock. VI Int. Congr. Anim. Reprod. Artif. Insemination *2*:1217.
21. Bogart, R., and Mayer, D. T. 1950. The effects of egg yolk on the various physical and chemical factors detrimental to spermatozoan viability. J. Anim. Sci. *9*:143.
22. Bomstein, R. A., and Steberl, E. A. 1957. Preservation of washed bovine spermatozoa in synthetic medium at room temperature. Exp. Cell Res. *18*:217.
23. Boyd, L. J., and Hafs, H. D. 1968. Motility and fertility after rapid and slow thawing of semen in ice water. Artif. Insemination Dig. *16(12)*:8-9
24. Bratton, R. W., Foote, R. H., and Henderson, C. R. 1956. The relative usefulness of combinations of laboratory tests for predicting the fertility of bovine semen. J. Dairy Sci. *39*:1542.
25. Branton, C., Jones, C. B., Patrick, T. E., and Newson, M. H. 1951. The relationship between

certain semen quality tests and fertility and the interrelationship of these tests. J. Dairy Sci. *34*:310.

26. Brown, K. I. and Graham, E. F. 1971. Effect of some cryophylactic agents on turkey spermatozoa. Poult. Sci. *50*:832.
27. Bruemmer, J. H., Eddy, R. W., and Duryea, W. H. 1963. Temperature control in the low temperature preservation of spermatozoa. J. Cell. Comp. Physiol. *62(2)*:113-118.
28. Cassou, R. 1968. The evolution of the mini straw. VI Int. Congr. Anim. Reprod. Artif. Insemination.
29. Chilson, O. P., Costello, L. A., and Kaplan, N. O. 1964. Effects of freezing on enzymes. In: *Cryobiology*, pp. 5-55.
30. Choong, C. H., and Wales, R. G. 1964. The effects of glycerol addition and equilibration on the revival of bull spermatozoa frozen in reconstituted skim milk. Res. Vet. Sci. *5*:228.
31. Clegg, D. D., and Pickett, B. W. 1966. Effect of storage at -196°C and fertility. Artif. Insemination Dig. *14*:12-13.
32. Clegg, E. D., Pickett, B. W., and Gibson, E. W. 1965. Non-mechanical nitrogen vapor freezing of bull semen. Res. Rep. Storrs Agric. Exp. Stn. No. *5*:14.
33. Colas, G. 1975. Effect of initial freezing temperatures, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J. Reprod. Fertil. *42*:277.
34. Cole, H. H., and Cupps, P. T. 1969. *Reproduction in Domestic Animals*. Academic Press, New York or London.
35. Corteel, C. M. 1974. The use of progestogens to control the estrous cycle of the dairy goat. Colloquium on the control of sexual cycles in domestic animals. Noujilly, France.
36. Crabo, B. G., Bower, R. E., Brown, K. I., Graham, E. F., and Pace, M. M. 1971. Extracellular glutamic-oxaloacetic transaminase as a measure of membrane injury in spermatozoa during treatment. pp. 33-38. In: A. Ingelman-Sundberg and N. O. Lunell, eds. *Current Problems in Fertility*. Plenum Press, New York.
37. Cragle, R. G., and Salisbury, G. W. 1959. Factors influencing metabolic activity of bull spermatozoa. IV. pH, osmotic pressure and the cations, sodium, potassium and calcium. J. Dairy Sci. *42*:1304.

38. Cragle, R. G., Salisbury, G. W., and VanDemark, N. L. 1958. Sodium, potassium, calcium and chloride distribution in bovine semen. J. Dairy Sci. *41*:1267.
39. Cummings, J. N. 1954. Testing fertility in bulls. Univ. Minn. Agric. Exp. Stn. Technical Bulletin 212.
40. Cupps, P. T., Laben, R. C., and Mead, S. W. 1953. The relation of certain semen-quality tests to breeding efficiency and characteristics of semen from low-fertility bulls before and after hormone injection. J. Dairy Sci. *36*:422.
41. Davis, I. S., Bratton, R. W., and Foote, R. H. 1963. Livability of bovine spermatozoa at 5°C in tris-buffered and citrate-buffered yolk-glycerol extenders. J. Dairy Sci. *46*:57.
42. Davis, I. S., Bratton, R. W., and Foote, R. H. 1963. Livability of bovine spermatozoa at 5, -25 and -85°C in tris-buffered and citrate-buffered yolk-glycerol extenders. J. Dairy Sci. *46*:333.
43. DeGrott, B. 1956. The physiology of deep frozen bull semen. Fortpflanz. Zuchthyg. Haustierbesamung *6*:25-28.
44. Deibel, F. C. Jr., Smith, J. E., Crabo, B. G., and Graham, E. F. 1976. Evaluation of six assays of sperm quality by measurement of their accuracy, precision and sensitivity in separating known induced levels of damage. Submitted to VIII Int. Congr. Anim. Reprod. Artif. Insemination, Cracow.
45. DosReis, J. M. 1967. First results from the use of deep-frozen bull semen diluted in egg yolk-tomato (reto). Proc. 5th Pan-Am Congr. Vet. Med. Zootech, Venezuela *1*:451-457.
46. Dunn, H. O., Larson, G. L., and Willett, E. L. 1953. The effects of freezing bovine spermatozoa in extenders containing antibacterial agents. J. Dairy Sci. *36(7)*: 728-732.
47. Dzuik, P. J., Graham, E. F., and Peterson, E. W. 1954. The technique of electro-ejaculation of the bull. J. Dairy Sci. *37*:556.
48. Easterbrooks, H. L., Heller, P., Plastridge, W. N., Jungherr, E. L., and Elliott, F. I. 1950. Fertility of diluted bull semen containing 100 micrograms of streptomycin per milliliter. J. Dairy Sci. *33(10)*:737-740.
49. Ehlers, M. H., Flerchinger, F. N., and Erb, R. E. 1953. Initial levels of fructose and citric acid in bull semen as related to fertility. J. Dairy Sci. *36*:1020.

50. Erb, R. E., Ehlers, M. H., Mikota, L., and Schwartz, E. 1950. The relation of single semen quality tests to fertilizing capacity of bull semen. Wash. Agric. Exp. Stn. Tech. Bull. 2.
51. Erb, R. E., Flerchinger, F. H., Ehlers, M. H. and Glasnov, F. X. 1965. Metabolism of bull semen. II. Fructolysis relationships with sperm concentration and fertility. J. Dairy Sci. *39* :326.
52. Erickson, W. E., and Graham, E. F. 1959. Effect of storage on nonreturn rate of frozen spermatozoa. Artif. Insemination Dig. *7(5)*.
53. Fisher, G. R. 1965. Investigations on bovine semen extenders and their application. PhD. thesis, University of Minnesota.
54. Finn, D. B. 1932. Denaturation of proteins in muscle juice by freezing. Proc. R. Soc. B. *3:*396
55. Flerchinger, F. H., and Erb, R. E. 1955. Metabolism of bull semen. I. Inorganic and total phosphorus relationships. J. Dairy Sci. *38* :1028.
56. Foote, R. H. 1972. Aging of spermatozoa during storage in liquid nitrogen. Proc. IV Tech. Conf. Artif. Insemination Reprod., Chicago, pp. 28-30.
57. Foote, R. H. 1970. Fertility of bull semen at high extension rates in tris-buffered extenders. J. Dairy Sci. *53* :1475.
58. Foote, R. H. 1964. Influence of pH on survival and fertility of bull sperm. J. Dairy Sci. *47* :807.
59. Foote, R. H. 1969. *Physiological Aspects of A. I. Reproduction in Domestic Animals*. Academic Press, New York.
60. Foote, R. H. 1969. Organic buffers for preserving semen. J. Dairy Sci. *52:* 921.
61. Foote, R. H., Bratton, R. W., and Henderson, C. R. 1962. Survival of bovine spermatozoa at room temperature in citrate and CUE and TRIS extender containing whole and fractionated coconut milk. J. Dairy Sci. *45(11)*:1383-1391.
62. Foote, R. H., Gray, L. C., Young, D. C., and Dunn, H. O. 1960. Fertility of bull semen stored up to four days at 5°C in 20% egg yolk extenders. J. Dairy Sci. *43:* 1330.
63. Forgason, J. L., Berry, W. T., and Goodwin, D. E. 1961. Freezing bull semen in liquid nitrogen vapor without instrumentation. J. Anim. Sci. *20*:970-971.
64. Fowler, A. K., Pickett, B. W., Gosslee, D. G., and Cowan, W. A. 1961. Effects of -196°C and -79°C storage on the resistance of bull sperm to three

repeated freeze-thaw treatments. J. Dairy Sci. *44(4)*:715-720.

65. Gibson, C. D., and Graham, E. F. 1969. The relationship between fertility and post-freeze motility of bull spermatozoa (by pellet freezing) without glycerol. J. Reprod. Fertil. *20*:155.
66. Goffaux, M. 1968. Fertility of bull semen frozen according to 2 different techniques, one involving pellets, the other straws. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris.
67. Graham, E. 1974. Frozen sperm loses much of its fertility after long-term storage, study indicates. Wall St. J., Feb. 13, 1974
68. Graham, E. F., and Crabo, B. G. 1972. Some factors influencing the freezing of boar spermatozoa. VII Int. Congr. Anim. Reprod. Artif. Insemination, Munich *11*:1627-1632.
69. Graham, E. F., Crabo, B. G., and Brown, K. I. 1972. Effect of some Switterion buffers on the freezing and storage of spermatozoa I. Bull. J. Dairy Sci. *55*:372.
70. Graham, E. F., and Pace, M. M. 1967. Some biochemical changes in spermatozoa due to freezing. Cryobiology. *4*:75.
71. Graham, E. F., Rajamannan, A. H. J., Schmehl, M. K. L., Maki-Laurila, M., and Bower, R. E. 1971. Fertility studies with frozen boar spermatozoa. Artif. Insemination Dig. *19*:6.
72. Graham, E. F., Rajamannan, A. H. J., Schmehl, M. K. L., Maki-Laurila, M., and Bower, R. E. 1971. Preliminary report on procedure and rationale for freezing boar semen. Artif. Insemination Dig. *19*:12.
73. Graham, E. F., Schmehl, M. K. L., Maki-Laurila, M. 1970. Some physical and chemical methods of evaluating semen. Proc. III Tech. Conf. Artif. Insemination. Reprod. NAAB.
74. Graham, E. F., Vazquez, A. I., Schmehl, M. K. L., and Evensen, B. K. An assay of semen quality by use of sephadex filtration. Submitted to VIII Int. Congr. Anim. Reprod. Artif. Insemination, Cracow.
75. Graham, E. F., Vogt, P. W., and Fisher, G. R. 1958. Effect of method of glycerol addition on the fertility of frozen bovine spermatozoa. J. Dairy Sci. *41*:1553.
76. Griffin, J. F. T., Nunn, W. R., and Hartigan, P. J. 1971. An immune response to egg yolk semen diluent in dairy cows. J. Reprod. Fertil. *25*:193.

77. Good, N. E. 1962. Uncoupling of the Hill reaction from photophosphorylation by anions. Arch. Biochem. Biophys. *96*:653.

78. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, E., and Singh, T. M. M. 1966. Hydrogen ion buffers for biological research. Biochemistry. *5*:467.

79. Hafez, E. S. E. 1974. *Reproduction in Farm Animals*. 3d ed. Lea and Febiger, Philadelphia.

80. Hafs, H. D., Boyd, L. J., Cameron, S., Johnson, W. L., and Hunter, A. G. 1971. Fertility of bull semen with added Beta-Glucoronidase. J. Dairy Sci. *54*:420-422.

81. Hafs, H. D. and Elliott, F. I. 1954. Effect of thawing temperature and extender composition on the fertility of frozen bull semen. J. Anim. Sci. *13*:958.

82. Hahn, R. 1966. On the dilution of bull semen deep frozen in liquid nitrogen. Tieraerztl. Umsch. *21*:164.

83. Hamdy, A. H. 1972. Linco-Spectin sterile solution use as an antimicrobial agent for the control of mycoplasmas and Vibrio in processed bovine semen. The Upjohn Company, 7 pp.

84. Uaq, I., and Mullen, J. E. C. 1949. Phosphomono-esterases in bull semen. Vet. Rec. *61(12)*:145.

85. Hatziolos, B. 1937. Biochemistry of semen and of the male reproductive tract. Z. Zucht. B, *38*:199.

86. Hunter, A. G. 1972. Immunological aspects of reproduction associated with repeat breedings. Proc. IV Tech. Conf. Artif. Insemination Reprod. NAAB.

87. Hunter, A. G. 1972. Immunological aspects of reproduction associated with repeat breedings. Proc. IV Tech. Conf. Artif. Insemination Reprod. NAAB.

88. Ilda, I. 1966. Effects of various rapid freezing rates on survival of bovine spermatozoa. Bull. Fac. Agric. Shizuoka Univ. No. *16*:95-98.

89. Izumi, M. 1954. N-bis-(β-chlorethyl) amino acid and their effect upon the Yoshida sarcoma. Pharmacedical Bull. (Japan), *2*:275.

90. Inskeep, E. K., Stevens, J. T., Peters, J. B., and Kauf, L. 1974. A.I. in sheep. W. Va. Univ. Agric. Exp. Stn. Bull. 629, March, 39 pp.

91. Jaskowski, L.,and Koryckil, S. 1965. Investigations on the freezing of bull semen. II. The effect of rate of cooling to -79°C on the quality of semen

after thawing. Pol. Arch. Weter. 8(Rocz. Nauk Roln. E, 71):571-589.

92. Johnston, J. E., Branton, C., and Hathorn, F. 1952. Semen evaluation techniques and fertility of dairy bulls. J. Anim. Sci. *11*:740.
93. Jondet, R. 1973. Effect of artificial insemination with small numbers of frozen sperm on fertility and sperm transport in cattle. Insemination *26*:357.
94. Jondet, R. 1964. The rapid freezing of bull semen conditioned in straws. V Int. Congr. Anim. Reprod. Artif. Insemination, Trento.
95. Josey, M. J. 1969. Motility and fertility of bovine semen stored at -79°C and -196°C. Queens 1. J. Anim. Sci. *26(4)*:651-657.
96. Kampschmidt, R. F., Mayer, D. T., Herman, H. A., and Dickerson, C. E. 1951. Viability of bull sperm as influenced by electrolyte concentration, buffer efficiency and added glucose in storage media. J. Dairy Sci. *34*:45.
97. Kareta, W., Wiezbowski, S., and Osikiwski, M. 1969. The value of basical estimations of semen characteristics in ram and the results of A.I. Zesz. Probl. Postepow Nauk Roln. *1969*:95.
98. Kennelly, J. J., Hoyt, R. S., Foote, R. H., Bratton, R. W. 1960. Survival rates of rapidly frozen bovine spermatozoa. J. Dairy Sci. *43*:1140-1146.
99. Kerreru, E. E., and Leon, F. 1974. Effect of different solid metals and metallic pairs on human sperm motility. J. Fertil. *19(2)*:81-84.
100. Kirton, K. T., Hafs, H. D., and Hunter, A. G. 1964. Levels of some normal constituents of bull semen during repetitive ejaculation. J. Reprod. Fertil. *8*:157.
101. Kutscher, W., and Wolbergs, H. 1935. Prostophosphatase. Hoppe-Seyler's Z. 236.
102. Landsteiner, K. 1899. Zur Kennetinis der Spezlfisch auf Blutkorperchen wirkenden sera. Zentrabl. Bakteriol. Parasitenkd.
103. Lardy, H. A., and Phillips, P. H. 1943. Effect of pH and certain electrolytes on the metabolism of ejaculated spermatozoa. Am. J. Physiol. *138*:741.
104. Larson, B. L., and Salisbury, G. W. 1953. The proteins of bovine seminal plasma. I. Preliminary and electrophoretic studies. J. Biol. Chem. *206*:741.
105. Larson, G. L., and Graham, E. F. 1958. Effects of rapid freezing on progressive motility of semen. Artif. Insemination Dig. *7*:6-7.

106. Lasley, J. F., Easley, G. T., and Bogart, R. 1942. Some factors influencing the resistance of bull sperm to unfavorable environmental conditions. J. Anim. Sci. *1*:79 (Abstr).
107. Lasley, J. F., and Mayer, D. T. 1944. A variable physiological factor necessary for the survival of bull spermatozoa. J. Anim. Sci. *3*:129.
108. Lightfoot, R. J., and Salamon, S. 1970. Fertility of deep frozen ram spermatozoa. J. Reprod. Fertil. *21*:366.
109. Lightfoot, R. J., and Salamon, S. 1970. Fertility of ram semen frozen by the pellet method. I. Transport and viability of spermatozoa within the genital tract of the ewe. J. Reprod. Fertil. *22*:385.
110. Lightfoot, R. J., and Salamon, S. 1970. Fertility of ram spermatozoa frozen by the pellet method. II. The effects of method of insemination on fertilization and embryonic mortality. J. Reprod. Fertil. *22*:399-408.
111. Lindstrom, U. 1973. Pellets: Experiences and results of storage over 3 years. VII Int. Congr. Anim. Reprod. Artif. Insemination, Munich *11*:1427.
112. Luyet, E., and Keane, J. Jr. 1955. A critical temperature range apparently characterized by sensitivity of bull semen to high freezing velocity. Biodynamica *7(152)*:281-292.
113. MacPherson, J. W. 1955. Long term dry ice storage of bovine semen. Can. J. Comp. Med. *19(9)*:287-289.
114. MacPherson, J. W. 1955. Fertile bovine semen after three years storage. J. Am. Vet. Med. Assoc. *129(9)*:416.
115. MacPherson, J. W. 1968. Semen placement of fertility in bovines. J. Dairy Sci. *51(5)*:807-808.
116. MacPherson, J. W., Chatterjee, S., and King, G. J. 1968. The effects of freezing method on sperm frozen in plastic straws. VI Int. Congr. Anim. Reprod. Artif. Insemination *11*:1085.
117. MacPherson, J. W., Chattejee, S., and Friars, G. W. 1969. Frozen turkey semen. Can. J. Comp. Med. *22*:37.
118. Malkiel, S., and Mason, J. P. 1943. Reactions of 4-(2-chlorethyl) morpholine and rates of reaction of 2-(2-chlorethyl) morpholine and other halides with sodium propoxide. J. Org. Chem. *8*:199.
119. Mann, T. 1945. Studies on the metabolism of semen. I. General aspects, occurrences and

distribution of cytochrome, certain enzymes and co-enzymes. Biochem. J. *39*:451.

120. Mann, T. 1946. Studies on the metabolism of semen. III. Fructose as a normal constituent of seminal plasma. Site of formation and function of fructose in semen. Biochem. J. *40*:481.

121. Mann, T. 1964. *The Biochemistry of Semen and of the Male Reproductive Tract.* Barnes and Noble, Inc., New York.

122. Martig, R. C., and Almquist, J. O. 1966. Effect of glycerol equilibration time on fertility of bull spermatozoa frozen in homogenized milk-fructose diluent. Artif. Insemination Dig. *14*:8.

123. Martin, I. C. A. 1965. Influence of equilibration freezing rate, method of dilution and diluent on the survival of deep-frozen bull spermatozoa. Aust. J. Biol. Sci. *18*:901-915.

124. Martin, I. C. A., and Emmers, C. W. 1961. Effects of time of equilibration and addition of fructose on survival and fertility of bull spermatozoa deep-frozen to -79°C. J. Reprod. Fertil. *2*:404.

125. Masom, N. and Barnabe, R. C. 1965. Vegetable diluents for the semen of domestic livestock. III. Infusion of dried leaves of alfalfa and dried guandu leaves as diluents for bull semen. Rev. Fac. Med. Vet. Univ. Sao Paulo *7*:401-407.

126. Mattner, P. E., and Voglmayr, J. K. 1962. A comparison of ram semen collected by the artificial vagina and electro-ejaculation. Aust. J. Exp. Agric. Anim. Husb. *2*:78.

127. McKenzie, F. F., and Berliner, V. 1937. Res. Bull. Mo. Agric. Exp. Stn. No. 265.

128. Meding, J. H. 1970. Breeding efficiency of bull semen frozen in straws and in pellets. Ann. Rep. R. Vet. Agric. Coll., Copenhagen, pp. 249-258.

129. Milk Marketing Board. 1973-1974. Long-term storage of semen. Report of the Breeding and Production Organization, pp. 68-69.

130. Mixner, J. P., and Wiggin, S. H. 1957. Fertility results with frozen semen stored up to one year. J. Dairy Sci. *40(5)*:537-540.

131. Mixner, J. P., and Wiggin, S. H. 1957. Fertility results with frozen semen stored for two years. J. Dairy Sci. *40(12)*:1650.

132. Mixner, J. P., and Wiggin, S. H. 1960. Stored frozen semen fertility. Artif. Insemination Dig. *8(7)*:19.

133. Mortimer, R. G., Berndtson, W. E., and Pickett, B. W. 1975. Fertility of bull semen frozen in straws or ampules. J. Anim. Sci. *41*:370. (Abstr).
134. Nagase, H. 1966. Artificial insemination. Deep freezing of bull, goat and stallion semen in concentrated pellet form. Jap. Agric. Res. *1(2)*.
135. Nagase, H.,and Graham, E. F. 1964. Pelleted semen: Comparison of different extenders and processes on fertility of bovine spermatozoa. V Int. Congr. Anim. Reprod. Artif. Insemination, Trento *4*:387-391.
136. Horman, C., Johnson, C. E., Porterfield, I. D., and Dunbar, R. C. Jr. 1958. Effect of pH on the life span and metabolism of bovine sperm kept at room temperature. J. Dairy Sci. *41*:1803.
137. O'Conner, M. L., Robbins, R. K., Vinson, W. E., and Saacke, R. G. 1975. Response of sperm to anti-egg yolk diluent sera. J. Anim. Sci. *39*:220.
138. O'Dell, W. T., and Almquist, J. O. 1954. Techniques for freezing bull spermatozoa in heated milk and preliminary breeding results. J. Dairy Sci. *37*:652.
139. O'Dell, W. T., Almquist, J. O., and Marsh, L. A. 1958. Freezing bovine semen. III. Effect of freezing rate on bovine spermatozoa frozen and stored at -79°C. J. Dairy Sci.*41(1)* :79-89.
140. Olar, T. T., Becker, W. C., and Senger, P. L. 1975. Effect of thaw rate on bovine semen stored in ampules. J. Anim. Sci. *41*:372-373. (Abstr).
141. Onuma, H. 1963. Studies on the acrosomic system of spermatozoa of domestic animals. VI. The acrosomic system of spermatozoa in fresh semen of bulls and boars and its morphological changes due to the storage or the temperature shock. Bull. Nat. Inst. Anim. Ind. *3*:105.
142. O'Shea, T. 1969. Metabolism of semen after freezing. I. Effects of washing, skim milk solids, and K and Mg ions on the metabolism of thawed ram and bull spermatozoa. Aust. J. Biol. Sci. *22*:721-732.
143. Pace, M. M., and Graham, E. F. 1970. The release of glutamic oxaloacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. Biol. Reprod. *3(2)*:140-146.
144. Pace, M. M., and Graham, E. F. 1974. Components in egg yolk which protect bovine spermatozoa during freezing. J. Anim. Sci. *39(6)*:114-149.
145. Parez, M. 1965. New method of preserving semen by freezing. I. Freezing in the concentrated form

and redilution for preserving 24-48 hours at 5°C. II. Freezing of concentrated semen, redilution at time of use. Elevage Insem. *88*:3.

146. Pavelko, M. K., and Crabo, B. G. 1976. Possible importance of some sperm coating proteins and their behavior during preservation of boar spermatozoa. Submitted to VIII Int. Congr. Anim. Reprod. Artif. Insemination, Cracow.
147. Phillips, P. H. 1939. The preservation of bull semen. J. Biol. Chem. *130*:415.
148. Phillips, P. H., and Lardy, H. A. 1940. A yolk-buffer pabulum for the preservation of bull semen. J. Dairy Sci. *23*:399.
149. Pickett, B. W. 1966. Field techniques and semen handling. Proc. I Tech. Conf. Artif. Insemination Reprod., Chicago.
150. Pickett, B. W., and Back, D. G. 1973. Procedures for preparation, collection, evaluation and insemination of stallion semen. Colorado State University, General Series 935.
151. Pickett, B. W., and Berndtson, W. E. 1974. Preservation of bovine spermatozoa by freezing in straws: A review. J. Dairy Sci. *57*:1287-1301.
152. Pickett, B. W., Fowler, A. K., Gossler, D. G., and Cowan, W. A. 1960. Fertility of frozen bovine spermatozoa stored at -79 and -196 degrees centigrade. Univ. Conn. Coll. Agric. Exp. Stn. Bull. 361.
153. Pickett, B. W., Hall, R. E., Lucas, J. J., and Gibson, E. W. 1965. Investigations on thawing frozen bovine spermatozoa. Fertil. Steril. *16*:642-651.
154. Pickett, B. W., Jones, R. A., Hiller, P., Cowan, W. A., and Gossler, D. G. 1959. Semen stored in liquid nitrogen. Artif. Insemination Dig. *7(4)*:6.
155. Pickett, B. W., MacDonald, W. A., Gossler, D. G., and Cowan, W. A. 1961. Correlation between certain laboratory stress tests and fertility of frozen bovine spermatozoa. J. Dairy Sci. *44(6)*:1134-1140.
156. Pickett, B. W., Martig, R. E., and Cowan, A. W. 1961. Preservation of bovine spermatozoa at -79 and -196°C. J. Dairy Sci. *44*:2089.
157. Pilch, J. and Wiezbowski, S. 1969. Nitrogen vapor freezing of semen in polyethylene ampules. Med. Weter. *25*:221.
158. Polge, C. 1951. Functional survival of fowl

spermatozoa after freezing at -79°C. Nature (London) *167*:949.

159. Polge, C. 1953. The storage of bull semen at low temperatures. Vet. Rec. *65*:557-559.

160. Polge, C. 1956. Artificial insemination in pigs. Vet. Rec. *68*:62.

161. Polge, C. 1957. Low temperature storage of mammalian spermatozoa. Proc. R. Soc. (London) B *147*:498.

162. Polge, C., and Lovelock, J. E. 1952. Preservation of bull semen at -79°C. Vet. Rec. *64*:396-397.

163. Polge, C., Smith, A. U., and Parkes, A. S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature *164*:666.

164. Proline, M. C., McCabe, W. J., and Shannon, P. 1958. Selection of bulls for artificial breeding on the basis of semen storage characteristics. J. Agric. Sci. *50*:260.

165. Pursel, V. G., and Johnson, L. A. 1971. Procedure for the preservation of boar spermatozoa by freezing. USDA, ARS 44-227.

166. Pursel, V. G., and Johnson, L. A. 1975. Freezing of boar sperm: Fertilizing capacity with concentrated semen and a new thawing procedure. J. Anim. Sci. *40*:99.

167. Pursel, V. G., Johnson, L. A., and Rampacek, G. B. 1972. Acrosome morphology of boar spermatozoa incubated before cold shock. J. Anim. Sci. *34*:278.

168. Pursel, V. G., Johnson, L. A., and Schulman, L. L. 1972. Interaction of extender composition and incubation period on cold shock susceptibility of boar spermatozoa. J. Anim. Sci. *35*:580.

169. Rajamannan, A. H. J. 1969. A new method--straw freezing of semen. 9th Annu. Symp. Fertil. Cattle. The Workham Foundation, Cypress, Tex.

170. Rajamannan, A. H. J. 1970. A new method of packaging semen--the straw. Proc. III Tech. Conf. Artif. Insemination Reprod. NAAB.

171. Rajamannan, A. H. J., Graham, E. F., and Schmehl, M. K. L. 1971. Effects of holding on bovine semen. Artif. Insemination Dig. *19*:6.

172. Rashwas, M. E. 1953. Semen criteria evaluations and their relation to fertility in dairy bulls. M.S. thesis, Cornell University.

173. Reid, J. T., Ward, B. M., and Salisbury, G. W. 1948. Acid and alkaline phosphatase levels in consecutive semen ejaculates from bulls. Am. J. Physiol. *153*:235.

174. Robbins, R. K., Gerber, L. E., and Saake, R. G. 1972. Influence of thaw rate on maintenance of the acrosomal cap. J. Anim. Sci. *35*:253. (Abstr).

175. Robbins, R. K., Saacke, R. G., and Chandler, P. T. 1976. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa in straws. J. Anim. Sci. *41*:129-136.

176. Rothschild, Lord, and Barnes, H. 1954. Constituents of bull seminal plasma. J. Exp. Biol. *31*:561.

177. Rodriguez, O. L., Berndtson, W. E., Enned, B. D., and Pickett, B. W. 1975. Effects of rates of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws. J. Anim. Sci. *41*:129-136.

178. Roussel, J. D., Kellgren, H. C., and Patrick, T. E. 1964. Bovine semen frozen in liquid nitrogen vapor. J. Dairy Sci. *47(12)*:1403-1406.

179. Roussel, J. D., and Stallcup, O. T. 1966. Parallelism between semen characteristics and bovine blood serum phosphatases and transaminases. J. Reprod. Fertil. *17*:483.

180. Saacke, R. G., and White, J. M. 1972. Semen quality tests and their relationship to fertility. Proc. IV Tech. Conf. Artif. Insemination Reprod., Chicago.

181. Salamon, S. 1973. Deep freezing of boar semen. III. Effects of centrifugation, diluent and dilution rate, pellet volume and method of thawing of survival of spermatozoa. Aust. J. Biol. Sci. *26*:239.

182. Salamon, S., and Morrant, A. J. 1963. A comparison of two methods of artificial breeding in sheep. Aust. J. Agric. Anim. Husb. *3*:72.

183. Salamon, S., and Visser, D. 1974. Fertility of ram spermatozoa frozen-stored for 5 years. J. Reprod. Fertil. *37*:433-435.

184. Salisbury, G. W. 1976. Aging phenomena in spermatozoa. III. Effect of season and storage at -79 to -88°C on fertility and pre-natal losses. J. Dairy Sci. *50*:1683.

185. Salisbury, G. W. 1968. Fertilizing ability and biological aspects of sperm storage in vitro. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris *2*:1189-1204.

186. Salisbury, G. W., and Flerchinger, F. H. 1967.

Aging phenomena in spermatozoa. I. Fertility and prenatal losses with use of liquid semen. J. Dairy Sci. *50*:1675-1678.

187. Salisbury, G. W., Fuller, H. K., and Willett, E. B. 1941. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. J. Dairy Sci. *24*:905.
188. Salisbury, G. W., and Kinney, W. C. Jr. 1957. Factors influencing metabolic activity of bull spermatozoa. III. pH. J. Dairy Sci. *40*:1343.
189. Salisbury, G. W., and VanDemark, N. L. 1961. *Physiology of Reproduction and Artificial Insemination of Cattle.* W. H. Freeman and Co., San Francisco
190. Salisbury, G. W., VanDemark, N. L., Lodge, J. R., and Cragle, R. G. 1960. Inhibition of spermatozoan metabolism by PCO_2, pH, K ion and antibacterial compounds. Am. J. Physiol. *198*:659.
191. Schindler, H., and Amir, D. 1973. The conception rate in ewes in relation to sperm dose and time of insemination. J. Reprod. Fertil. *34*:191-196.
192. Shaffner, C. S. 1964. Observations on freezing chicken semen. V Int. Congr. Anim. Reprod. Artif. Insemination, Trento *4*:Sec. *3*:426.
193. Sherman, J. K. 1955. Temperature shock in human spermatozoa. Proc. Soc. Exp. Biol. Med. *88*:6.
194. Sherman, J. K. 1964. Research on frozen human semen. Past, present and future. Fertil. Steril. *15(5)*:485-499.
195. Sherman, J. K.,and Florence, C. 1974. Stability of Y chromosome fluorescence during freeze-thawing and frozen storage of human spermatozoa. Fertil. Steril. *25(4)*:311-314.
196. Singleton, W. L., and Shelly, D. R. 1972. Variations among boars in semen characteristics and fertility. J. Anim. Sci. *24*:762.
197. Smith, A. U., and Polge, C. 1950. Storage of bull spermatozoa at low temperatures. Vet. Rec. *62*:115-116.
198. Smith, J. E.,and Graham, E. F. 1976. High resolution size distribution of spermatozoa. Submitted to VIII Int. Congr. Anim. Reprod. Artif. Insemination, Cracow.
199. Sorenson, E., and Anderson, F. 1956. The influence of sodium and potassium ions upon the motility of sperm cells. III Int. Congr. Anim. Reprod. Artif. Insemination *1*:45-57.
200. Stallcup, O. T. 1965. Acid and alkaline phosphatase

activity in bovine semen as related to fertility. J. Dairy Sci. *48*:752.

201. Steinback, J., and Foote, R. H. 1967. Osmotic pressure and pH effects on survival of frozen bovine spermatozoa. J. Dairy Sci. *50*:205.
202. Stewart, D. L. 1964. Observations on the fertility of frozen semen stored at -79°C and -196°C. VI Int. Congr. Anim. Reprod. Artif. Insemination *3;99*:617-623.
203. Strom, B. 1968. Influence upon fertility of bull semen of storage time in liquid nitrogen. VI Int. Congr. Anim. Reprod. Artif. Insemination *2;*1171-1174.
204. Sullivan, J. J., Bartlett, D. E., Elliott, F. I., Brouwer, J. R., and Kloch, F. B. 1972. A comparison of recto-vaginal, vaginal and speculum approaches for insemination of cows and heifers. Artif. Insemination Dig. *20(1)*.
205. Sullivan, J. J., Parker, W. G., Larson, L. L., and Turner, P. C. 1972. Some aspects of reproduction in the horse and present status of artificial insemination with frozen stallion semen. Symp. Reprod. Cattle Horses. Michigan State University, November 6-7.
206. Swanson, L. V., and Hunter, A. G. 1969. Egg-yolk antigens and their effect on fertility in rabbits. Biol. Reprod. *1*:324.
207. Swanney, J. M. 1953. The storage of bull semen at low temperature. Vet. Rec. *65*:562-563.
208. Tampion, D., and Gibbons, R. A. 1963. Effect of pH on the swimming rate of bull spermatozoa. J. Reprod. Fertil. *5;* 249.
209. Tesron, F., Amir, D., and Schindler, H. 1974. Storage of ram semen under freezing conditions. In; *Scientific Activities 1973-1974* Agric. Res. Org., Israel Inst. Anim. Sci., Div. Anim. Reprod. Bet Dagon.
210. Tessier, H., and Rose, D. 1956. Composition of the liquid portion of frozen milk. Can. J. Technol. *34*:211.
211. Turanyu, J., and Nagy, G. 1968. Freezing, storage and use of pelleted bull semen. Aelattenyesztes *17*:149.
212. VanDemark, N. R., Miller, W. J., Kinney, W. C. Jr., Rodriguez, C., and Friedman, M. E. 1957. Preservation of bull semen at sub-zero temperatures. Ill. Agric. Exp. Stn. Bull. 621.
213. VanDemark, N. L., and Sharma, U. D. 1957. Prelimi-

nary fertility results from the preservation of bovine semen at room temperatures. J. Dairy Sci. *40*:438.

214. Van den Berg, L. 1959. The effect of addition of sodium and potassium chloride to the reciprocal system: KH_2PO_4- Na_2HPO_4-H_2O on pH and composition during freezing. Arch. Biochem. Biophys. *84*:305.
215. Van den Berg, L. 1960. pH measurement at low temperature using modified calomel and glass electrodes. Anal. Chem. *32*:628.
216. Van den Berg, L. 1966. pH changes in buffers and foods during freezing and subsequent storage. Cryobiology *3*:236.
217. Van den Berg, L., and Rose, D. 1959. Effect of freezing on the pH and composition of sodium and potassium phosphate solutions: The reciprocal system KH_2PO_4-Na_2HPO_4-H_2O. Arch. Biochem. Biophys. *81*:319.
218. Van Duijn, C., Jr. 1968. Seminal characteristics and fertility of stallions. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris *2*:1565.
219. Van Duijn, C., Jr., and Rikmenspoel, R. 1960. The mean velocity and velocity distributions of normal spermatozoa at different hydrogen-ion concentrations derived from photo-electric measurement. J. Agric. Sci. *54*:300.
220. Van Duijn, C., Jr., and Verver, H. W., 1970. Sire specificity of movement characteristics and longevity of bull spermatozoa. Anim. Biol. Anim. Biochem. Biophys. *10*:51.
221. Visser, D., and Salamon, S. 1974. Fertility following insemination with frozen-thawed, reconcentrated and unconcentrated ram semen. Aust. J. Biol. Sci. *27*:423.
222. Waldron, R., and Read, S. W. 1954. Factors affecting the recovery rate of frozen bull semen. Am. Dairy Sci. Assoc., West. Div. Proc. 35th Annu. Meet., July.
223. Wales, R. G., Scott, T. W., and White, I. G. 1961. Biuret reactive materials in semen. Aust. J. Exp. Biol. Med. Sci. *39*:455.
224. Watanabe, M. 1967. Studies on deep freezing preservation of chicken semen. Jap. Fac. Fish. Anim. Husb., Hiroshima *7*:9.
225. Watanabe, M., Miura, M., and Moda, Y. 1970. Studies on deep freezing preservation of fowl semen. Jap. Poult. Sci. *7*:23.

226. Webster, W. M. 1932. Bovine sterility in New Zealand. Aust. Vet. J. *8*:199.

227. Weitze, K. F. 1972. Protective effects of glycerol and lactose in pellet freezing of bull semen in relation to dilution, equilibration and freezing velocity. VII Int. Congr. Anim. Reprod. Artif. Insemination, Munich *972*:263.

228. Wentworth, B. C., and Mellen, W. J. 1963. Effects of spermatozoal antibodies and methods of insemination of the fecundity of white Leghorn males. Poult. Sci. *42*:1317.

229. White, I. G. 1953. The effect of potassium on the washing and dilution of mammalian spermatozoa. Aust. J. Exp. Biol. Med. Sci. *31*:193.

230. Wiggin, H. B., and Almquist, J. O. 1974. Effect of glycerol equilibration time and thaw rate on acrosomal maintenance of bull sperm frozen in plastic straws. J. Dairy Sci. *57*:625. (Abstr).

231. Wiggin, H. B., and Almquist, J. O. 1975. Combinations of glycerol percent, glycerol equilibration time and thawing rate upon freezability of bull spermatozoa in plastic straws. J. Dairy Sci. *58*:416-419.

232. Wiggin, H. B., and Almquist, J. O. 1975. Effect of glycerol equilibration time and thawing rate upon acrosomal maintenance and motility of bull spermatozoa frozen in plastic straws. J. Anim. Sci. *40*:302-305.

233. Wiggins, E. L., Terrill, C. E., and Emik, L. O. 1953. Relationships between libido, semen characteristics and fertility in range rams. J. Anim. Sci. *12*:684.

234. Willet, E. L., and Ohms, J. I. 1958. Inactivation of spermatozoa by lactate and reactivation with alkali. J. Dairy Sci. *41*:275.

235. Yassen, A. M., and Foote, R. H. 1966. Sodium and potassium concentration of extended bull semen. J. Anim. Sci. *25*:975.

236. Yassen, A. M., and Foote, R. H. 1967. Freezability of bovine spermatozoa in tris-buffered yolk extenders containing different levels of tris, sodium, potassium, and calcium ions. J. Dairy Sci. *50*:887.

237. Young, L. G., and Nelson, L. 1974. Calcium ions and control of the motility of sea urchin spermatozoa. J. Reprod. Fertil. *41(2)*:371-378.

238. Zaugg, N. L., and Almquist, J. O. 1973. Motility

of spermatozoa and control of bacteria in bovine semen diluents containing penicillin and neomycin or epicillin. J. Dairy Sci. *56(2)*:202.

239. Zavos, P. 1976. The effects of the degree of supercooling on motility and fertility of frozen turkey spermatozoa. Sixty-fifth Annu. Meet. Poult. Sci., August, 1976, Manhattan, Kans.

SUMMARY OF RESEARCH AT THE PENNSYLVANIA STATE UNIVERSITY

J. O. Almquist

MAXIMIZING THE SPERM HARVEST FOR BANKING FROZEN BOVINE SEMEN

Semen Collection and Bull Management Producedures

Total numbers of sperm harvested when seven successive ejaculates were collected from 10 beef bulls did not differ significantly between collection by artificial vagina and by electroejaculation; mean of 21 x 10^9 sperm (Foster, Almquist, and Martig, J. Anim. Sci. *30:*245, 1970).

Freezability did not differ for sperm obtained from the same beef bulls by artificial vagina and by electroejaculation. Sexual preparation involving one false mount +5 min of restraint prior to each ejaculation did not improve freezability of sperm collected by artificial vagina (Martig, Almquist, and Foster, J. Anim. Sci. *30:*60, 1970).

Sexual preparation (prolonging the period of sexual stimulation beyond that adequate for mounting and ejaculation) by false mounting and restraint significantly increased the number of sperm harvested per ejaculation (Almquist, J. Anim. Sci. *36:*331, 1973).

Freezability of sperm from beef bulls increased significantly with bull age between puberty and 2 yr of age (Cunningham *et al.,* J. Anim. Sci. *26:*182, 1967). Fertility of beef bulls, using only ejaculates which froze satisfactorily (at least 10 x 10^6 motile sperm after freezing and storage in ampules for 3 wk), increased significantly between 1 and 2 yr of age (Martig and Almquist, J. Anim. Sci. *28:*375, 1969).

Frequency of Semen Collection

From a series of studies we concluded that mature bulls should ejaculate 40 to 60 x 10^9 spermatozoa weekly when collected frequently by artificial vagina. This can be accomplished by collection of two or three successive ejaculates every 3 or 4 days with intensive sexual preparation (three false mounts) before each ejaculation. This procedure is useful when it is desired to harvest most of the testicular production of sperm and to bank frozen semen as rapidly as possible. The prodigious numbers of sperm produced daily by the testes of bulls, which can be harvested by frequent semen collection, was confirmed by measuring the number of sperm recovered from a vas deferens cannula (Amann *et al.*, J. Dairy Sci. *57*:93, 1974).

Does use of a high ejaculation frequency over prolonged periods of time to increase the harvest of sperm for freezing have a harmful effect on spermatozoan freezability or fertility?

Young Bulls

There was no difference at 3.25 yr of age in freezability of sperm from six pairs of Holstein bulls that had been maintained on one (1X) or six ejaculates weekly (6X) after 1 yr of age (Almquist *et al.*, J. Dairy Sci. *46*:1176, 1963). Fertility of frozen semen from these 1X and 6X bulls at 4 yr of age also did not differ. Fertility, based on 60- to 90-day nonreturn rates for 3,238 first inseminations, averaged 75 percent for 1X and 76 percent for 6X bulls (Almquist, unpublished data, 1976).

Freezability of sperm and fertility were not affected significantly by ejaculation frequency when 17 Angus and Hereford bulls were collected at 1X, 3X, or 6X weekly between 1 and 2 yr of age (Cunningham *et al.*, J. Anim. Sci. *26*:182, 1967, Martig and Almquist, J. Anim. Sci. *28*:375, 1969).

Thus, prolonged collection of up to six ejaculates weekly from young bulls starting at 1 yr of age does not impair their subsequent reproductive usefulness. When ejaculated 6X weekly a mean of 3.5 times more motile sperm are harvested from a Holstein bull between puberty and 2 yr of age than when ejaculated only 1X weekly (Almquist and Amann, J. Dairy Sci., in press, 1976). Based on 20 x 10^6 motile sperm per insemination unit prefreeze, a bank of 33,500 insemination units could be achieved by

2 yr of age collecting semen 6X weekly, compared to only 9,500 units on 1X weekly.

Mature Bulls

Freezability of sperm and fertility of frozen semen was not significantly affected by continuous ejaculation of five pairs of Holstein bulls for 7 yr, i.e., from 1 to 8 yr of age at 1X or 6X weekly (Almquist, unpublished data, 1976).

Spermatozoan freezability and fertility did not differ among seven successively collected ejaculates from 10 Angus bulls. However, collection of more than three or four successive ejaculates is not an efficient procedure because of the higher incidence of ejaculates that do not meet minimum standards for freezing, i.e., at least 40 percent progressively motile sperm and 840×10^6 total motile sperm (Martig *et al.*, J. Anim. Sci. *30:*60, 1970).

Freezability of sperm collected from dairy bulls in a series of depletion trials (6 to 37 successive ejaculates per trial without sexual preparation) showed that freezability of acceptable ejaculates (at least 30 percent progressively motile sperm and 150×10^6 motile sperm) did not differ among the first 10 ejaculates. Pooling of acceptable ejaculates resulted in freezability comparable to freezing ejaculates separately. Based on 20×10^6 motile sperm per insemination unit prefreeze, 924 insemination units could be frozen from 10 consecutive ejaculates per week, or 2.5 times the 368 units that could be frozen by collecting two ejaculates a week (O'Dell *et al.*, J. Dairy Sci. *42:*1209, 1959).

Sexual Rest

First ejaculates collected from six 4- and 5-yr-old Holstein bulls after prolonged sexual rest froze satisfactorily; freezability did not differ from that of six bulls ejaculated 1X weekly and six bulls ejaculated 6X weekly continuously from 1 to 5 yr of age. Each bull was given three 44-wk periods of sexual rest between 2 and 5 yr of age; at 3, 4, and 5 yr of age they were ejaculated at high frequency for 8 wk and freezability of sperm determined (Martig *et al.*, J. Anim. Sci. *25:*927, 1966).

Estimated Number of Insemination Units per Bull per Year

An average of about 80,000 insemination units should be yielded from a mature bull per year; the majority of mature bulls should yield between 60,000 and 100,000 units yearly. The average is based on a sperm harvest of 50×10^9 weekly, 68 percent initial progressive spermatozoan motility, 20×10^6 motile sperm per insemination unit prefreeze, and a loss of 8 percent of the potential number of insemination units.

Collecting semen from dairy bulls 6X weekly yields about 3.2 times more insemination units per year than collecting semen 1X weekly.

CONTROL OF BACTERIAL GROWTH IN BULL SEMEN

Combinations of penicillin (units) and neomycin (micrograms) of 0, 500, 1,000, and 1,500 of each per milliliter of semen diluent did not reduce the ability of sperm to remain motile at 5°C over a 20-day storage period. Each penicillin-neomycin combination effectively controlled bacterial growth during a 16-day storage (Figure 1). Epicillin alone at levels up to 1,200 µg/ml did not significantly affect sperm motility; however, bacterial growth was not efficiently retarded (Figure 2) at 1,200 µg/ml diluent (Zaugg and Almquist, J. Dairy Sci. *56:*202, 1973).

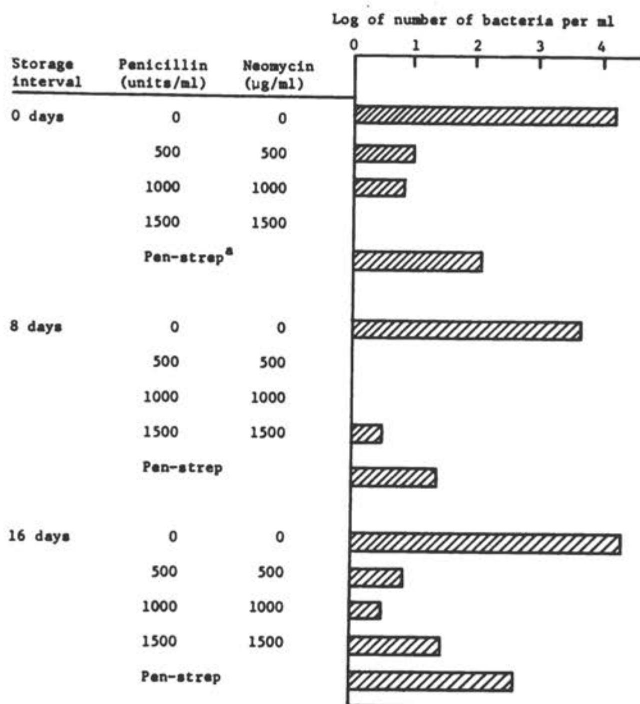
A fertility trial with frozen semen involving 2,679 first service cows showed that combinations of 1,000 units penicillin and 1,000 µg neomycin or 150 µg lincomycin and 300 µg spectinomycin per ml diluted semen are satisfactory substitutes for 1,000 units penicillin and 1,000 µg streptomycin/ml commonly used to treat semen in commercial artificial insemination (Almquist and Zaugg, J. Dairy Sci. *57:*1211, 1974).

COOLING RATE FROM 35°C TO 5°C

Slow (4 h) cooling of bull spermatozoa diluted in heated milk gave a higher ($P < 0.05$) post-thaw motility than rapid (1.5 h) cooling, but fertility of semen frozen in ampules did not differ (Almquist, unpublished data, 1976).

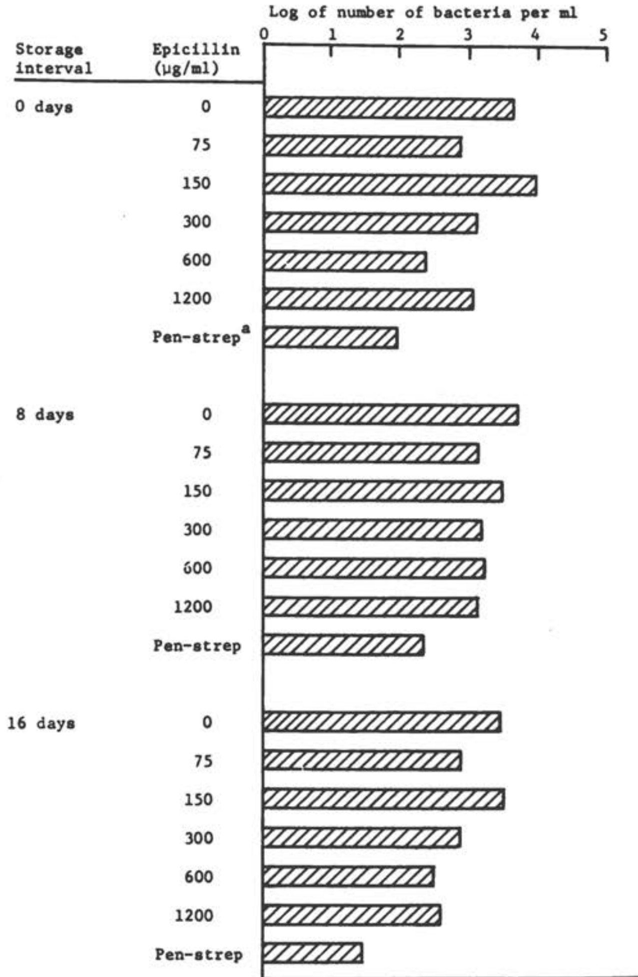
Rate from 35°C to 5°C	Post-Thaw Motility, %	No. First Services	Nonreturn Rate, % (60 to 90 days)
Fast, 1.5 h	21	719	66.5
Slow, 4.0 h	25	680	69.8

Similarly, there was no significant difference between fertility of semen frozen in plastic straws after cooling from 35°C to 5°C in 1.5 or 3 h. Average 66-day nonreturn rate for 1,403 first service cows was 74.7 percent for diluted semen cooled from 35°C to 5°C in 1.5 h and 74.2 percent for that cooled in 3 h (Zaugg and Weidler, Artif. Insemination Dig. *23*(8):9, 1975).



[a]Combination of 1000 units/ml of penicillin and 1000 µg/ml of dihydrostreptomycin.

FIGURE 1 Effect of penicillin-neomycin combinations upon bacterial populations in diluted semen stored for 0, 8 and 16 days at 5°C (mean of nine ejaculates).



[a]Combination of 1000 units/ml of penicillin and 1000 µg/ml of streptomycin.

FIGURE 2 Effect of epicillin upon bacterial populations in diluted semen stored for 0, 8 and 16 days at 5°C (mean of six ejaculates).

FERTILITY COMPARISON OF PACKAGING BULL SEMEN IN STRAWS OR AMPULES

In a fertility experiment involving 2,855 first-service inseminations, mean 66-day nonreturn rate of 70.7 percent for straws did not differ significantly from the 72.6 percent nonreturn rate for ampules. This represented our first attempt in Pennsylvania to use plastic straws for freezing bull spermatozoa in commercial artificial insemination (Almquist, Artif. Insemination Dig. *23*(7):7, 1975)

THAWING RATES FOR PLASTIC STRAWS

Rapid thawing in warm water at 35°C or above of bull semen frozen in plastic straws was beneficial for post-thaw percentage of sperm motility and significantly superior to thawing straws slowly in 5°C iced water (Almquist and Wiggin, Artif. Insemination Dig. *21*(9):10, 1973).

There was a significant increase in the mean post-thaw percentage of intact acrosomes and motile sperm for each increase in thawing rate when straws were thawed in water at 35°C for 15 s, 55°C for 12 s, 75°C for 9 s or 95°C for 7 s. Thawed semen samples were incubated at 37°C and evaluated for presence of the apical ridge and sperm motility at 0, 3, 6, and 9 h of incubation. Results of regression analysis revealed that for each 20°C rise in thawing temperature, intact acrosomes increased a mean of 5.5 percentage units while sperm motility increased a mean of 2.0 percentage units (Wiggin and Almquist, J. Anim. Sci. *40*:302, 1975).

Cold shock injury to bull spermatozoa in straws exposed to cold air or cold inseminating equipment after thawing can be avoided by limiting the thawing time at 35°C to 10 s. Cold shock injury, based on acrosomal retention and sperm motility, was significant only when straws thawed at 35°C for more than 10 s (20 or 80 s) were exposed to a 5°C iced water bath long enough to cool the semen to 5°C (Almquist, Artif. Insemination Dig. *24*(3):17, 1976).

SPERM NUMBERS AND FERTILITY OF FROZEN BULL SPERMATOZOA

Fertility, based on 60- to 90-day nonreturn rates for 4,603 first-service inseminations, was significantly lower for inseminating doses of 10 and 15 x 10^6 than

20×10^6 motile sperm (61, 62, and 70 percent). The 1-ml units of skim milk diluted semen were frozen in glass ampules. The post-thaw number of motile sperm after freezing and storage for 3 wk in liquid nitrogen averaged 5.5, 8.4, and 12.4×10^6 for corresponding pre-freeze numbers of 10, 15, and 20×10^6 motile sperm per ml and ranged from 4.0 to 15.7×10^6/ml. Frozen semen packaged in ampules should contain more than 8.4×10^6 motile cells post-thaw per ampule (Almquist, J. Dairy Sci. *58:*420, 1975).

TECHNIQUES FOR THE CRYOPRESERVATION AND FIELD HANDLING OF BOVINE SPERMATOZOA*

W. E. Berndtson and B. W. Pickett

INTRODUCTION

Smith and Polge[87] first reported that bovine spermatozoa were capable of withstanding freezing in the presence of glycerol in 1950. Since then the art and science of the cryopreservation of bovine spermatozoa has continued to develop, and approximately 9 million cattle are now inseminated with frozen semen annually in the United States. The application of techniques for the cryopreservation of bovine spermatozoa to spermatozoa of other mammals has produced variable and often disappointing results. Nonetheless, the wealth of research and field experience with frozen bovine semen has led to the identification of numerous essential factors and potential pitfalls associated with the use of frozen semen. The technical information that follows is based primarily upon research with bovine spermatozoa. Although specific procedures for the freezing and handling of spermatozoa from other species, including the human, are likely to differ, many of these same factors may be equally important. Thus, this discussion is intended primarily to point out the many potentially important factors that warrant critical, systematic evaluation during the development of successful techniques for the cryopreservation of human spermatozoa. Many of these factors are discussed elsewhere in greater detail.[14,70]

*Portions of this manuscript have been taken from "Techniques for Processing and Packaging Bovine Semen" by B. W. Pickett, W. E. Berndtson, and J. J. Sullivan[70] and "Procedures for Field Handling of Bovine Semen in Plastic Straws" by W. E. Berndtson, B. W. Pickett, and C. D. Rugg.[14]

TECHNIQUES FOR MEASURING FERTILITY

Much of the progress in improving the survival and fertility of frozen bovine spermatozoa resulted from laboratory studies in which post-thaw motility was used to assess treatment effects. Although motility is positively correlated with the fertility of frozen bovine spermatozoa, spermatozoa from several species, such as the dog and stallion, frequently exhibit very high motility after freezing, yet are infertile. Even for the bull, there is no completely reliable laboratory criterion for predicting fertility, and several different laboratory tests are often applied. However, each factor under study must ultimately be evaluated by actual fertility testing.

Reliable fertility data are difficult to obtain even with livestock animals. For example, one of the most widely adopted criteria for measuring fertility is the nonreturn rate, i.e., the percentage of cows inseminated that was not reported for an additional insemination in a specific period. However, such data include many inaccuracies. For example, many cows that fail to become pregnant are not reported for an additional insemination because they are sold or die, are bred by natural mating, or are artificially inseminated with semen supplied by other artificial insemination (AI) organizations. Thus, nonreturn rates frequently are much greater than actual fertility. Problems of this nature must be considered when evaluating fertility data, and efforts should be made to control those factors that can influence the reliability of the data.

PACKAGING SYSTEMS

A variety of methods for packaging bovine semen for freezing have been developed. The primary considerations in the selection of a system for packaging semen are fertility, inseminator preference, ease of handling, ease of identification, freedom from contamination, economics of storage, and the proportion of ejaculates that can be frozen. Of these the most important objective should be to obtain *maximum reproductive efficiency.* Since the various packaging systems were developed under different conditions, philosophies, areas of the world, etc., direct comparisons are difficult. In fact, it must be understood that systems of packaging, rather than packages *per se,* were being compared in most studies.

Fertility

Pickett *et al.*[70] recently summarized the results of 17 studies in which the fertility of semen frozen in ampules or straws was compared. The results are presented in Table 1. Studies shown above the broken line are ones in which fertility was greater for semen frozen in ampules. In most studies, differences in fertility due to method of packaging were small. Furthermore, the observation that neither method of packaging was consistently superior to the other is consistent with the hypothesis that some factor(s) other than method of packaging *per se* also contributed to the differences in fertility observed.

Sire *x* packaging-method interactions have also been observed. The results of one study are presented in Table 2. In Herd 1, fertility averaged 70.1 vs. 49.5 percent for semen frozen in straws vs. ampules, respec-

TABLE 1 Effect of Packaging Bovine Semen in Straws or Ampules on Fertility

Packaging System			
Straws	Ampules	Difference	Reference
73.2	54.3	+18.9	61
63.7	57.9	+ 5.8	60
72.0	67.8	+ 4.2	2
71.0	67.0	+ 4.0	76
67.2	64.3	+ 2.9	5
70.4	67.7	+ 2.7	48
74.9	72.8	+ 2.1	42
71.1	69.5	+ 1.6	3
69.0	67.5	+ 1.5	46
71.5	70.3	+ 1.2	76
74.3	73.2	+ 1.1	76
61.1	65.5	- 4.4	38
70.7	73.8	- 3.1	4
70.7	72.6	- 1.9	8
67.7	69.1	- 1.4	30
73.0	73.9	- 0.9	1
67.5	67.6	- 0.1	38

SOURCE: Adapted from Pickett *et al.*[70]

TABLE 2 Percent Pregnant[a] After a 38-(Herd 1) and a 44-(Herd 2) Day Breeding Season

	Packaging System			
	Straws		Ampules	
Bull	No. Bred	Pregnant[b] (%)	No. Bred	Pregnant[b] (%)
Herd 1				
A	75	76.7	72	72.3
B	75	63.3	70	47.9
C	72	61.3	65	12.6
D	72	79.2	73	65.2
Mean	294	70.1	280	49.5
Herd 2				
C	101	82.6	103	66.8

SOURCE: Adapted from Mortimer.[61]
[a]Least squares mean.
[b]Determined by rectal palpation.

tively. However, the actual differences in pregnancy rates for the four sires used were 4.4, 15.4, 48.7, and 14.0 percentage points. Thus, method of packaging was relatively unimportant for semen of some bulls, but resulted in a marked difference in fertility for others. Methods of packaging should probably be selected on an individual-bull basis to ensure optimal fertility.

The largest difference between the two methods of packaging in Herd 1 was for Bull C (48.7 percentage points). The corresponding difference for the same bull used in Herd 2 was only 15.8 percentage points. Since different ejaculates were used in each herd, and because the cattle were maintained under different levels of management, a direct comparison of the fertility results

would not be appropriate. However, all ampules and straws used in Herd 1 contained 20×10^6 motile spermatozoa prior to freezing, while 30×10^6 motile spermatozoa were provided prior to freezing of semen used in Herd 2. We suspect that differences in fertility due to packaging method would not have been as great in Herd 1 if more spermatozoa were included per inseminate. Regardless of the reason(s) for the difference in fertility of semen from Bull C in the two herds, it is obvious that factors other than method of packaging *per se* can have marked effects on fertility.

Other techniques have been utilized for packaging and/or freezing of bovine semen. Some investigators have frozen semen in pellets by placing small drops of extended semen into small depressions on a block of dry ice, or in steel plates suspended in liquid nitrogen (LN) vapor. The comparative fertility of semen frozen in pellets, ampules, or straws in some studies in shown in Table 3. Again, the influence of packaging methods on fertility have been inconsistent.

Risks of Contamination

Pellets have not gained acceptance in the United States and Canada because of severe limitations in handling and distribution. Although pellets can be labeled,[80] permanent identification has presented some difficulties and the semen is still exposed to LN. Therefore, pieces of the pellets can be transferred to other pellets while in LN storage, creating a potential for mixing semen from different bulls.[47,56] Stewart[89] conducted an extensive series of experiments related to storage of pellets and concluded:

1. Pellets can become contaminated with organisms present in LN.
2. Pellets can "pick up" spermatozoa in LN. The occurrence was rare and the spermatozoa "picked up" were nonmotile.
3. Organisms from contaminated pellets are shed into LN and can contaminate pellets in a different canister.
4. Spermatozoa became attached to forceps during handling of pellets.
5. Straws stored in the same LN with pellets became contaminated externally.

Thus, the potential for spread of disease is excel-

TABLE 3 Fertility of Spermatozoa Frozen in Pellets, Ampules, or Straws

Pellets		Ampules		Straws		
% NR	Inseminations	% NR	Inseminations	% NR	Inseminations	References
68.3	467	56.9	545	--	--	62
70.4	440	65.4	1,216	--	--	82
59.0	1,872	--	--	68.5	1,993	7
69.7	--	57.9	--	63.7	--	60
66.5	5,515	--	--	66.7	5,481	34
53.1	160	56.6	198	--	--	86
39.7	253	41.9	281	--	--	94
66.1	9,035	--	--	66.4	8,804	35
61.4	--	71.5	--	--	--	32
65.7	2,839	--	--	66.1	2,937	55
55.1	--	59.2	--	--	--	43
55.7	--	68.5	--	--	--	93

SOURCE: Adapted from Pickett *et al.*[70]

lent and a pregnancy from the wrong bull possible.

A technique for freezing pellets in gelatin capsules was developed[47] to eliminate the possibility of contamination. However, the open end of the capsule was sealed with a drop of extender, which is exposed to LN and represents a potential source of contamination.

Straws can be sterilized externally[6] but contamination of French straws and the powder used for sealing has been reported.[49] When properly sterilized and sealed, glass ampules are the most suitable package with respect to potential contamination of semen.

Contamination of ampules and straws can occur during thawing, primarily due to faulty seals. Improperly sealed ampules generally explode, particularly if they are stored under LN; while plugs generally pop from straws.[90] Ampules that have exploded may have been contaminated, but contamination is of little consequence, since the semen is unusable. When a plug pops from a straw, the semen should not be used, because the semen may have become contaminated. It is suspected that LN enters the straws during freezing or storage, and the seals are expelled due to vaporization during thawing. If nitrogen can enter the straw, other contaminants may also enter. Since expulsion of "plugs" is an occasional phenomenon, it is not known whether nitrogen entry is occasional or occurs routinely.

Influence of Package Size

The volume per inseminate does not appear to be an important factor in fertility of cattle, at least in the range currently utilized for AI. For example, American Breeders Service[12] reported 60- to 90-day nonreturns of 77.3 vs. 76.2 percent with 0.5- and 1.0-ml ampules, respectively, when the same total number of spermatozoa was included per inseminate. Hafs *et al.*[37] also reported similar 60- to 90-day nonreturn rates of 73.2 vs. 70.5 percent for insemination volumes of 0.9 vs. 0.5 ml, when all inseminates were frozen in 1.0-ml glass ampules and contained the same total number of spermatozoa.

Pickett *et al.*[70] reviewed results of fertility trials involving straws containing different inseminate volumes. Fertility was generally greater when semen was frozen in 0.5-ml as compared to 1.0-ml straws. However, many of the studies were confounded by the use of a different number of spermatozoa within each volume. Differences in fertility for 0.25- and 0.5-ml straws ranged from 0.6 percentage

points lower to 1.2 percentage points greater with the smaller straw. It would appear that where differences in fertility have been associated with inseminate volume, these have probably been due to concomitant changes in freezing rate, thawing rate, or some other aspect of seminal processing and/or handling.

SEMINAL PROCESSING PROCEDURES

Incubation of Raw Semen

A limited number of studies have been conducted to evaluate the influence of incubation of raw semen (herein designated as holding) prior to initial dilution and cooling on the survival and fertility of bovine spermatozoa. Rajamannan *et al.*[77] reported a gradual increase in post-thaw motility as holding time at 26°C was increased for up to 60 min. Furthermore, the fertility of semen from two low fertility bulls was increased when semen was held for 20 min at 26°C compared to that of semen not held. In contrast, Jondet[40] obtained similar fertility (69.6 vs. 68.5 percent nonreturn) when semen was diluted and cooled immediately vs. held for 15 min at 32°C. In a preliminary study at our laboratory, a total of 14 of 26 heifers (54 percent) conceived when bred with unincubated semen compared to 12 of 26 (46 percent) inseminated with semen held for 20 min at 37°C. In this study, each inseminate contained only 10×10^6 motile spermatozoa prior to freezing. Elliott *et al.*[25] and Ennen *et al.*[28] both found no advantage to holding raw semen on post-thaw motility. In fact, post-thaw motility tended to decrease as holding time increased. Until additional investigations are completed, holding of raw semen from fertile bulls, prior to the initiation of cooling, is not recommended.

Initial Dilution

Bovine spermatozoa may be damaged by cold shock if they are cooled rapidly at temperatures above freezing. The consequences of cold shock include coiling of the tails with an accompanying irreversible loss of motility, an increase in the number of spermatozoa that are permeable to vital stains, a reduction in respiratory activity and glycolysis, loss of lipid, an influx of enzymes into the seminal plasma, an uptake of sodium and calcium, and a loss of potassium and magnesium by the cells.[16,19,21,53,71,74,75,84,97]

Cold shock can be prevented by slow cooling in the presence of protective agents. Most extenders for bovine semen contain milk or egg yolk. The former contains casein, while the latter contains lecithin, lipoproteins, and phospholipids, which provide protection against cold shock.[15,21,33,41,44,51,65] The concentration of protective substances present during the cooling of semen will depend upon the dilution ratio of semen to extender prior to cooling (initial dilution) and the concentration of these substances in the extender. Benson *et al.*[13] obtained decreased post-thaw motility as initial seminal dilution was increased for semen subsequently frozen in ampules. Post-thaw motility averaged 36, 37, 34, and 32 percent when semen contained 1,000, 250, 40, or 20 x 10^6 spermatozoa/ml during cooling to 5°C. The extender used for seminal dilution in this study contained a fixed level of egg yolk. Thus, higher rates of dilution were also associated with higher egg yolk concentrations during cooling. Zarazúa *et al.*[101] studied the influence of initial dilution ratio on the post-thaw motility of spermatozoa frozen in plastic straws. In this study, the level of egg yolk in the extenders was adjusted so that 4, 8, 12, and 16 percent egg yolk were present within each dilution ratio. The results are given in Table 4. In this study, motility increased as the dilution ratio increased, regardless of the level of egg

TABLE 4 Influence of Initial Dilution Ratio and Egg Yolk Level during Cooling from 37 to 5°C on Motility of Bovine Spermatozoa Subsequently Frozen in 0.25-ML Continental Straws

Dilution Ratio	Egg Yolk (%)[a]				
	4	8	12	16	Mean
1:0.5	19.7	17.6	17.2	17.2	18.0
1:1	21.1	20.7	19.2	19.0	20.0
1:2	20.7	21.5	19.9	20.9	20.7
1:4	22.0	20.9	20.6	20.9	21.1
Mean	20.9	20.2	19.2	19.5	--

SOURCE: Adapted from Zarazúa *et al.*[101]
[a]All samples were adjusted to a final concentration of 16 percent egg yolk and 7 percent glycerol for freezing.

yolk. It was recommended that semen should be diluted with at least an equal volume of extender prior to cooling.

Cooling Time

The rate at which semen is cooled to about 5°C can have a significant effect on fertility. Erickson[29] compared the fertility of split samples cooled from 32.2°C to 3.3°C in 110 min vs. 4 h and used without freezing. The 30- to 40-day nonreturn rates were 74.5 and 80.6 percent respectively. Slower cooling has also been associated with greater post-thaw motility for spermatozoa frozen either in ampules or straws.[13,28,52,54] In contrast, Weidler and Zaugg[98] found no difference in fertility for semen cooled from 35°C to 5°C in 1.5 or 3.0 h, and Jondet[40] found no difference in fertility of semen cooled from 30°C to 5°C in 0.5, 1, or 2 h. In view of the results of laboratory studies, we suspected that bull fertility and/or the number of spermatozoa per inseminate were sufficiently high to mask any effect of rate of cooling on fertility in these studies. Although there are some contradictory data, we must recommend cooling times of 2 h or more. The National Association of Animal Breeders[63] conducted a survey among semen processors and found that 15 percent cooled semen in less than 1 h, 52 percent in 1.0 to 1.5 h, 10 percent in 1.5 to 2.0 h, and 23 percent in more than 2 h. From these results, we suspect that the desire to complete seminal processing and freezing on the same day that semen is collected has resulted in a cooling rate of convenience rather than on based upon sound experimental evidence.

Glycerolization

Once extended semen has been cooled to 5°C, it is customary to further dilute the semen so that the spermatozoal concentration is twice as great as that to be present in the final product. Final dilution is then accomplished by adding a volume of glycerol-containing extender equal to that of the extended semen. Glycerol is added to semen as a cryoprotective agent. The optimal concentration of glycerol depends upon several factors, including the type of extender and freezing rate. Generally, levels of approximately 7 percent glycerol are optimal for citrate-yolk and tris-yolk extenders, and levels of 11 to 13 percent for fresh and reconstituted skim-milk extenders.[69] Although

there are some contradictory data, many investigators have reported damage to spermatozoa as the temperature at which glycerolization occurred was increased.[13,17,22,23,24,58,73] Thus, extended semen is generally cooled to about 5°C before glycerolization. A variety of procedures for the addition of glycerol-containing extender to the extended semen have also been evaluated. These include glycerolization in a single addition, stepwise addition at intervals of several minutes, or by allowing the glycerol extender to drip slowly into the extended semen.[22,58,64,99] The influence of glycerolization method on post-thaw motility in these studies was inconsistent. Following a review of the available data, we[69] concluded that under certain conditions the stepwise addition of glycerol to bull semen may enable greater post-thaw motility. However, when present, this advantage has generally been small, and may not be reflected in increased fertility.[31,39]

The glycerolization procedure is used to provide both the proper level of glycerol for cryoprotection of the spermatozoa and the desired concentration of spermatozoa in the extended semen. Ejaculates with a low spermatozoal concentration have posed a distinct problem when semen was to be frozen in 0.25-ml straws, since the use of a 1:1 initial dilution and an additional 1:1 dilution at glycerolization can result in too few spermatozoa per inseminate. Therefore, Zarazúa *et al.*[101] studied the effect of glycerolization by dilution at ratios of 1:1, 2:1, 3:1, or 4:1 (extended semen:glycerol-containing extender) with extenders containing 14, 21, 28, and 35 percent glycerol, respectively. These dilution ratios resulted in a final level of 7 percent glycerol for all samples. No difference in post-thaw motility was observed due to glycerolization procedure. Thus, it would appear that lower dilution ratios (i.e., volumes) may be used for glycerolization, provided the final product contains the proper level of glycerol for optimal cryoprotection.

Number of Spermatozoa per Insemination

The influence of the number of spermatozoa per inseminate has been discussed elsewhere in considerable detail.[68,70] It appeared that the number of live spermatozoa per insemination for optimal fertility was similar for semen frozen in straws or ampules.[68] In only one of seven studies with semen in ampules was fertility significantly greater with the highest number of spermatozoa studied.[70] However, in

all but one of the seven studies, actual mean fertility was higher with the highest spermatozoal number. The same trend was generally evident in eight studies with semen frozen in plastic straws.[70] When all of the studies on number of spermatozoa per inseminate were considered, there appeared to be a trend towards the use of fewer and fewer spermatozoa. If this trend continues using nonsignificant data, it is certainly possible, if not inevitable, that overall fertility will be reduced.

The optimal number of spermatozoa per insemination depends, in part, upon the fertility level of the bull. Hafs *et al.*[37] reported no influence of number of spermatozoa per inseminate, over the range studied, on the fertility of highly fertile bulls, while fertility of bulls of lower fertility decreased when spermatozoal numbers were reduced. Although this trend was not significant, it was similar to that reported by Sullivan,[92] as depicted in Figure 1. From these results, it appears that for optimum fertility more spermatozoa are required per insemination when bulls are of low fertility. Thus, the fertility level of each bull must be considered in establishing the number of spermatozoa per insemination. Unfortunately, the established optimum is only valid for that bull under those circumstances.

Equilibration Time

Numerous investigators[18,26,27,36,50] have established that

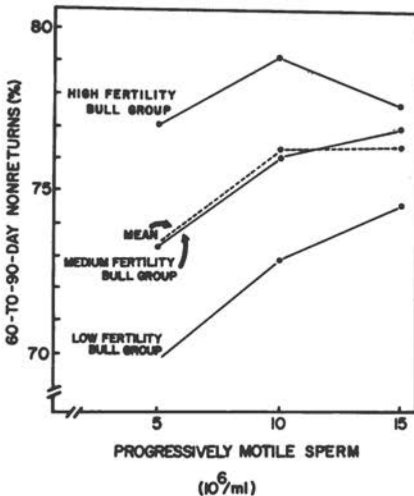


FIGURE 1 The effect of number of spermatozoa on nonreturn rate of Holstein bulls.[92]

the fertility of spermatozoa frozen in ampules was enhanced when semen was stored with (equilibrated) or without (aged) glycerol for several hours after cooling prior to freezing. The optimal length of this period has varied, but generally is between 6 and 18 h.[69] Although it was previously thought that the presence of glycerol during equilibration was important, it was subsequently demonstrated that the period of storage or aging was the important factor, and that glycerol could be added at any time after cooling and before freezing, without influencing fertility.[31]

Laboratory investigation of optimal equilibration time is probably not fruitful, because it is suspected that the industry will adopt an aging or equilibration time of convenience rather than one based upon research results. Fortunately, it appears that extended equilibration or aging is not necessary for semen to be frozen in straws.

Freezing Rate

Some of the basic causes of cell death during freezing and thawing and methods for their prevention have been presented elsewhere.[69] While many of the principles of cryopreservation of living cells are well established, most procedures for the freezing of bovine spermatozoa have been developed empirically. Unfortunately, no fertility studies have been conducted to establish optimal rates of freezing for bovine spermatozoa. Rate of freezing can exert marked effects on sperm survival.[70] However, the optimal rate(s) of freezing is influenced by a variety of factors, including methods of seminal packaging, glycerol level, and subsequent thawing rates. From the available evidence it is obvious that spermatozoa are capable of withstanding a wide range of freezing rates. Nonetheless, procedures should be developed and utilized that minimize variation. This is important, as rate of freezing interacts with many other factors, and each of these factors must be controlled to obtain a uniformly high-quality product.

Storage

The key factor enabling the long-term preservation of bovine spermatozoa is low temperature. At one time, most frozen semen was stored in a mixture of dry ice and alcohol at -79°C. When stored at this temperature, the fertility of the semen ultimately decreased.[59,83,88] However, Ström[91]

found no evidence of reduced fertility when approximately 60,000 inseminations were performed with semen in pellets after storage in liquid nitrogen (LN) at -195°C for approximately 1 to 1.5 yr. Cassou[20] reported no difference in fertility after 285,551 inseminations with semen in straws stored in LN up to 54 mo. If fertility of semen stored in LN is reduced with time, regardless of packaging technique, some factor other than storage is responsible.

PROCEDURES FOR THE FIELD HANDLING OF FROZEN SEMEN

One of the major causes of low fertility associated with the use of AI is mishandling of semen by the inseminator. This fact has been confirmed by numerous studies conducted with full-time, professional inseminators closely supervised by AI organizations.[66,67,72] Although data are lacking, mishandling of semen is probably more extensive today than at any time in the past, due to direct sale of semen to ranchers and dairymen who inseminate their own cattle, often with little training or supervision.

Principles for the field handling of frozen semen and specific recommendations for the field handling of bovine semen frozen in ampules or straws have been presented elsewhere in detail.[14,67] Therefore, the following discussion will focus primarily on specific aspects of field handling that have, or are likely to have, profound influences upon fertility.

From the available evidence, it appears that seminal temperature should be maintained at -130°C or lower at all times.[45,57,72,88,96] Although LN has a temperature of about -195°C, there are many opportunities for semen stored in LN to be exposed to damaging temperatures from the time it is shipped from the AI organization until it is thawed for use. One such opportunity is during transfer from the unit in which it was shipped into the field storage unit. Seminal temperature can increase rapidly during exposure to ambient conditions. The rate of warming depends, in part, upon the method of packaging. This is illustrated in Figure 2. Seminal temperature rose much more rapidly within straws than within glass ampules, because the straws contained a smaller volume of extended semen and because they had a much greater surface area-to-volume ratio. The greater increases in temperature were also associated with greater reductions in post-thaw motility (Figure 3). Straws placed in plastic goblets for field distribution are much less vulnerable to changes

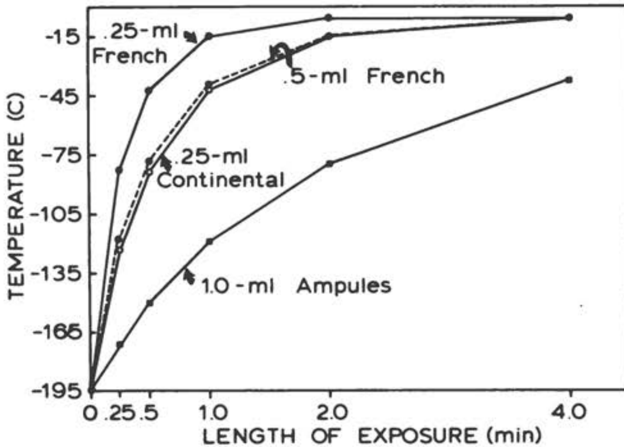


FIGURE 2 Temperature of semen within individual straws held by forceps or ampules clipped to metal racks (canes) during exposure to ambient (20 ± 0.6°C) conditions. (Each curve represents the mean of five replicates. The curve for ampules is a mean of the temperature of semen within the top and bottom ampule on the same rack.[14]

in temperature during exposure.[14] Thus, we have recommended that AI organizations package straws in plastic goblets or other suitable protective devices for distribution and field storage. Rates of warming during exposure to ambient temperatures may also be influenced by factors such as wind velocity and the intensity of solar radiation. Thus, it is impossible to establish a safe interval for the transfer of semen from one unit to another. The most reasonable approach is to transfer the semen as quickly as possible, out of the wind and sunlight.

Semen may also be exposed to damaging temperatures each time it is raised to the neck of a field unit to permit removal of an individual straw for thawing. The temperature within the neck of a typical field unit is much higher towards the top of the tank (Figure 4). Since spermatozoa will be damaged at these temperatures, the canister containing the semen should be raised no higher than absolutely necessary, and the individual straw removed for thawing as quickly as possible.

One need not be nearly as concerned about the tempe-

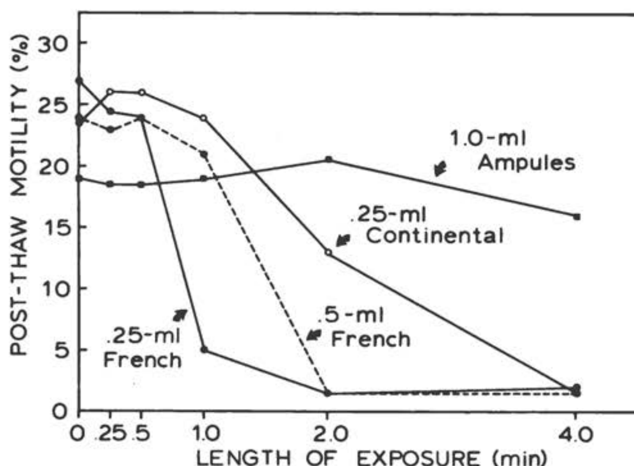


FIGURE 3 Influence of exposure to ambient (20 ± 0.6°C) conditions on the post-thaw motility of spermatozoa within individual straws or ampules on racks. (Semen in straws was thawed in water at 37°C, while ampules were thawed in iced water. Each value represents the mean of duplicate samples from each of vice bulls.[14]

rature of semen within the straw being removed as with the other semen in the same canister or goblet. This semen is exposed to elevated temperatures each time another straw is removed for thawing. The damage from exposure to elevated temperatures is additive. In addition, unless sufficient time is allowed between exposures to permit the semen to cool completely, higher temperatures will be reached during subsequent exposures.

It is obvious that the temperature reached by semen during exposure to the neck of a field storage unit depends upon the height to which it is raised, length of exposure, level of LN in the tank, and the intervals between exposures. Therefore, semen should be raised no higher than necessary to facilitate removal of a straw for thawing, and removal should be executed as quickly as possible. Since, under normal circumstances it is impossible to maintain a full LN level at all times, inseminators must be instructed to allow more time between exposures when the LN level is low.

Procedures for thawing semen can exert marked influ-

ences upon fertility. After reviewing the available evidence, Pickett[67] concluded that semen in glass ampules should be thawed in ice water. In contrast, the quality of semen frozen in plastic straws has consistently been enhanced in laboratory studies by rapid thawing in warm water.[3,10,11,28,78,79,100] On the basis of fertility trials conducted at our laboratory, we currently recommend that semen in plastic straws be thawed in water at 35°C.[14]

Thawing in warm water is not difficult, but several precautions should be exercised. Spermatozoa are damaged by excessive heating. In one study, spermatozoa in 0.25-ml Continental straws were removed from LN and plunged into water at 35 to 60°C for 5 min, and evaluated immediately. Motility declined precipitously at temperatures above 45°C. Temperatures below 45°C may also be detrimental. For example, high temperatures can stimulate motility briefly, but are then followed by rapid cell death. Also, it has been shown that rabbit spermatozoa incubated for 2 h at 38 or 40°C were equally capable of fertilization, but fertilization by sperm incubated at 40°C was followed by high rates of embryonic mortality.[95] Thus, there may be considerable damage to spermatozoa warmed above body temperature.

Damage to spermatozoa thawed in water at above body temperature can be prevented by timing the thawing process very carefully. At the same time, it would be futile to recommend a thawing method that was so difficult to employ that it would greatly increase the opportunity for mishandling during and after thawing. Under such circumstances, fertility might be lower than with a method that was less than ideal but more easily accomplished. In view

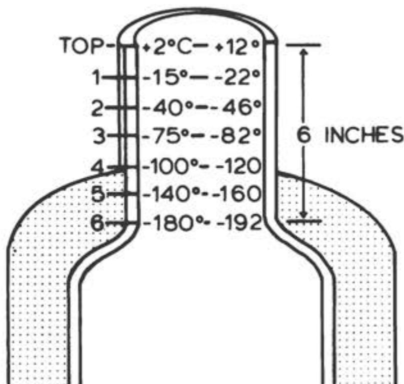


FIGURE 4 Typical temperature range in the neck of an LN field storage unit.[81]

of the extremely detrimental effects of excessive heating, and the fact that post-thaw motility has generally been only slightly enhanced at temperatures above 35 to 37°C, we currently recommend that semen in straws be thawed in water at 35°C.

Spermatozoa warmed to higher temperatures (~35°C) after thawing are susceptible to cold shock.[9,85] Therefore, we[14] recommended that thawing in warm water be carefully timed to permit thawing without causing further elevation in seminal temperature.

REFERENCES

1. Aamdal, J. 1967. A new insemination appliance. Anim. Breed. Abstr. *35:*246.
2. Aamdal, J., and Andersen, K. 1968a. Fast thawing of semen frozen in straws. Zuchthygiene *3:*22.
3. Aamdal, J., and Andersen, K. 1968b. Fast thawing of bull semen frozen in straws. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*973.
4. Aamdal, J., Lyngset, L., and Filseth, O. 1969. Insemination experiment with a new type of insemination equipment for straws. Anim. Breed. Abstr. *37:*56.
5. Adler, H. C. 1962. Storage and transport of semen in cellophane straws, single dosage glass vials, and larger glass vials. Anim. Breed. Abstr. *30:*191.
6. Adler, H. C., and Andersen, J. B. 1970. Method for the inactivation of virus on semen straws. Acta Vet. Scand. *11:*484.
7. Adler, H. C., Jespersen, C., Meding, J. H., and Rasbech, N. O. 1968. Fertilizing efficiency of bull semen frozen in straws or in pellet form. Anim. Breed. Abstr. *36:*229.
8. Almquist, J. O. 1975. Fertility results with straws favorable. Artif. Insemination Dig. *23*(7):7.
9. Almquist, J. O. 1976. Effect of cold shock after thawing on acrosomal maintenance and motility of bovine spermatozoa frozen in plastic straws. J. Dairy sci. *59:*1825.
10. Almquist, J. O., and Wiggin, H. B. 1973a. Survival of bull spermatozoa frozen and thawed by different methods in plastic straws. Artif. Insemination Dig. *21*(7):12.
11. Almquist, J. O., and Wiggin, H. B. 1973b. Effect of different combinations of freezing and thawing rates

upon survival of bull spermatozoa in U.S. plastic straws. Artif. Insemination Dig. *21*(9):10.

12. American Breeders Service. 1975. Personal communication. DeForest, WI 53232.
13. Benson, R. W., Sexton, T. J., Pickett, B. W., Lucas, J. J., and Gebauer, M. R. 1968. Influence of processing techniques and dilution rates on survival of frozen bovine spermatozoa. Univ. Conn. (Storrs) Agric. Exp. Stn. Res. Rep. No. 28.
14. Berndtson, W. E., Pickett, B. W., and Rugg, C. D. 1976. Procedures for field handling of bovine semen in plastic straws. Proc. VI Tech. Conf. Artif. Insemination Reprod. NAAB, p. 51.
15. Bialy, G., Ludwick, T. M., Hess, E. A., and Ely, F. 1957. Influence of lipoprotein on the freezing of bovine spermatozoa. J. Dairy Sci. *40:*1189.
16. Blackshaw, A. W. 1954. The prevention of temperature shock of bull and ram semen. Aust. J. Biol. Sci. *7:*573.
17. Blackshaw, A. W. 1955. Factors affecting the revival of bull and ram spermatozoa after freezing to -79°C. Aust. Vet. J. *31:*238.
18. Blackshaw, A. W., Emmens, C. W., Martin, I., and Heyting, J. 1957. The preparation of deep frozen semen. Artif. Insemination Dig. *5*(1):6.
19. Blackshaw, A. W., and Salisbury, G. W. 1957. Factors influencing metabolic activity of bull spermatozoa. II. Cold-shock and its prevention. J. Dairy Sci. *40:*1099.
20. Cassou, R. 1972. A technique for semen preservation and its application to breed improvement programmes. Artif. Insemination Co-op Centre, 61-L'Aigle, France. Personal publication.
21. Choong, C. H., and Wales, R. G. 1962. The effect of cold shock on spermatozoa. Aust. J. Biol. Sci. *15:*543.
22. Choong, C. H., and Wales, R. G. 1964. The effects of glycerol addition and equilibration on the revival of bull spermatozoa frozen in reconstituted skim milk. Res. Vet. Sci. *5:*228.
23. Clegg, E. D., Komarek, R. J., and Pickett, B. W. 1965. Preliminary investigations on glycerol entry into bovine spermatozoa. J. Dairy Sci. *48:*1709.
24. Dunn, H. O., and Hafs, H. D. 1953. Extenders and techniques for freezing bovine spermatozoa. J. Dairy Sci. *36:*577.
25. Elliott, F. I., Murphy, D. M., Bartlett, D. E., and

Kubista, R. A. 1962. The use of polymyxin B sulfate with dihydrostreptomycin and penicillin for control of "Vibrio fetus" in a frozen semen process. Artif. Insemination Dig. *10*(2):10.

26. Emmens, C. W., and Martin, I. 1956. The controlled introduction of deep-freeze techniques into artificial insemination. III Int. Congr. Anim. Reprod. Artif. Insemination *3*:18.
27. Emmens, C. W., and Martin, I. C. A. 1961. The effects of equilibration period and sugar content of the diluent on the survival and fertility of bull spermatozoa deep-frozen to -79°C. IV Int. Congr. Anim. Reprod. Artif. Insemination *4*:964.
28. Ennen, B. D., Berndtson, W. E., Mortimer, R. G., and Pickett, B. W. 1976. Effect of processing procedures on motility of bovine spermatozoa frozen in .25-ml straws. J. Anim. Sci. *43*:651.
29. Erickson, W. E. 1958. East Central Breeders Assoc. Coop., P. O. Box 191, Waupun, WI 53963.
30. Filseth, O. 1970. Conception rates using the French straw. Anim. Breed. Abstr. *38*:252.
31. Foote, R. H. 1970. Influence of extender, extension rate, and glycerolating technique on fertility of frozen bull semen. J. Dairy Sci. *53*:1478.
32. Gavrikov, A. M. 1971. Some improvement in the techniques for freezing bull semen and inseminating cows. Anim. Breed. Abstr. *39*:694.
33. Gebauer, M. R., Pickett, B. W., Komarek, R. J., and Gaunya, W. S. 1970. Motility of bovine spermatozoa extended in "defined" diluents. J. Dairy Sci. *53*:817.
34. Goffaux, M. 1968. Pouvoir fecondant du sperme de taureau traite selon deux techniques de congelation, l'une en pellets, l'autre en paillettes. VI Int. Congr. Anim. Reprod. Artif. Insemination. *2*:1041.
35. Goffaux, M., Tourneur, J. C., and Baudet, J. 1969. Fertilizing ability of bull semen treated by two freezing techniques, one involving pellets and the other straws. Final results. Anim. Breed. Abstr. *37*:439.
36. Graham, E. F., Erickson, W. E., and Bayley, N. D. 1957. Effect of glycerol equilibration on frozen bovine spermatozoa. J. Dairy Sci. *40*:510.
37. Hafs, H. D., Boyd, L. J., and Cameron, S. 1970. Fertility of cattle inseminated with 12, 24 or 35 million sperm in 0.5 or 0.9 milliliter. J. Dairy Sci. *53*:1693.
38. Hahn, R., Cassou, B., and Eibl, K. 1969. Comparative

studies of semen freezing techniques using French straws and ampules (Summary). Dtsch. Tierarztl. Wochensch. *76:*346.

39. Hunter, W. K. 1968. Glycerolisation and freezing techniques with bull semen. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1053.
40. Jondet, R. 1972. Etudes sur de pouvoir fecondant du sperme de taureau. Societa Italiana per il progresso della Zootecnica. p. 137.
41. Kampschmidt, R. F., Mayer, D. T., and Herman, H. A. 1953. Lipid and lipoprotein constituents of egg yolk in the resistance and storage of bull spermatozoa. J. Dairy Sci. *36:*733.
42. Kellgren, H. C. 1973. Personal communication. Select Sires, Inc., Route 3, Box 126, Plain City, OH 43064.
43. Kim, J. K., Oh, Y. K., Suh, G. S., and Lee, Y. B. 1973. Methods of glycerol addition and equilibration time and its effect on semen quality and fertility of pelleted bull semen. Anim. Breed. Abstr. *41:*605.
44. Lanz, R. N., Pickett, B. W., and Komarek, R. J. 1965. Effect of lipid additives on pre- and post-freeze survival of bovine spermatozoa. J. Dairy Sci. *48:*1692.
45. Larson, G. L., and Graham, E. F. 1958. Effects of low temperatures in storage of bovine semen. Artif. Insemination Dig. *6*(12):6.
46. Linares, I. A. 1970. A comparison of the methods of freezing semen in straws and in ampoules for artificial insemination. Anim. Breed. Abstr. *38:*84.
47. Lorrmann, W. 1969. On the possibility of mixing semen by using Nagase's pellet technique. Anim. Breed. Abstr. *37:*63.
48. Macpherson, J. W., and King, G. J. 1966. Plastic straws for frozen semen (A field evaluation). Can. J. Comp. Med. Vet. Sci. *30:*109.
49. Marinov, P., and Bohnel, H. 1974. Hygienic conditions of materials for freezing bovine semen. J. Dairy Sci. *57:*707.
50. Martig, R. C., and Almquist, J. O. 1966. Effect of glycerol equilibration time on fertility of bull spermatozoa frozen in homogenized milk-fructose diluent. Artif. Insemination Dig. *14*(5):8.
51. Martin, I. C. A. 1963. Effects of lecithin, egg yolk, fructose and period of storage at 5°C on bull spermatozoa deep-frozen to -79°C. J. Reprod. Fertil. *6:*441.
52. Martin, I. C. A. 1965. Effects of rate of cooling to

5°C storage at 5°C glycerol concentration, sodium chloride, fructose and glycine on the revival of deep-frozen bull spermatozoa. J. Agric. Sci. *64:*425.

53. Mayer, D. T. 1955. The chemistry and certain aspects of the metabolic activities of mammalian spermatozoa. Mich. State Univ. Cent. Symp. Rep. Reprod. Infertil. p. 45.
54. McFee, A. F., and Swanson, E. W. 1960. The influence of cooling rates and storage temperatures on sperm activity. Artif. Insemination Dig. *8*(10):12.
55. Meding, J. H. 1970. Breeding efficiency of bull semen frozen in straws and in pellets (Summary). Arsberetning, Den kgl. Vet.-og Landbohøjskole Sterilitetsforskn. p. 249.
56. Merkt, H., Weitze, K. F., and Lorrmann, W. 1968. Semen pellets sealed in a capsule, a means of improving the pellet method in the deep-frozen storage of bull semen. Anim. Breed. Abstr. *36:*596.
57. Meryman, H. T. 1956. Mechanics of freezing in living cells and tissues. Science *124:*515.
58. Miller, W. J., and VanDemark, N. L. 1954. The influence of glycerol level, various temperature aspects and certain other factors on the survival of bull spermatozoa at sub-zero temperatures. J. Dairy Sci. *37:*45.
59. Mixner, J. P. 1968. Fertility of bull semen frozen for twelve years. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1095.
60. Morales, J. R., and Duverger, O. 1970. Three different techniques for the freezing of cattle semen and their conception results. Anim. Breed. Abstr. *38:*422.
61. Mortimer, R. G. 1976. Frozen bovine spermatozoa; U.S. straws vs ampules. M.S. Thesis, Colorado State University, Fort Collins.
62. Nagase, H., and Graham, E. F. 1964. Pelleted semen: Comparison of different extenders and processes on fertility of bovine spermatozoa. V Int. Congr. Anim. Reprod. Artif. Insemination *4:*387.
63. National Association of Animal Breeders. 1958. Report of the Semen Preservation Committee. Proc. XI Annu. Conv. NAAB. p. 91.
64. O'Dell, W. T., and Almquist, J. O. 1954. Techniques for freezing bull spermatozoa in heated milk and preliminary breeding results. J. Dairy Sci. *37:*652.
65. O'Shea, T., and Wales, R. G. 1966. Effect of casein, lecithin, glycerol, and storage at 5°C on diluted ram and bull semen. Aust. J. Biol. Sci. *19:*871.

66. Pickett, B. W. 1966. Field techniques and semen handling. Proc. I Tech. Conf. Artif. Insemination Bovine Reprod. p. 64.
67. Pickett, B. W. 1971. Factors affecting the utilization of frozen bovine semen for maximum reproductive efficiency. Artif. Insemination Dig. *19*(2):8.
68. Pickett, B. W., and Berndtson, W. E. 1974. Preservation of bovine spermatozoa by freezing in straws: A review. J. Dairy Sci. *57:*1287.
69. Pickett, B. W., and Berndtson, W. E. 1977. Principles and techniques of spermatozoan preservation by freezing. In: *Physiology of Reproduction and Artificial Insemination of Cattle*. 2d ed. G. W. Salisbury, N. L. VanDemark, and J. R. Lodge, Eds. W. H. Freeman & Co.
70. Pickett, B. W., Berndtson, W. E., and Sullivan, J. J. 1976. Techniques for processing and packaging bovine semen. Proc. VI Tech. Conf. Artif. Insemination Reprod. NAAB. p. 34.
71. Pickett, B. W., and Komarek, R. J. 1967. Effect of cold shock and freezing on loss of lipid from spermatozoa. J. Dairy Sci. *50:*753.
72. Pickett, B. W., Martig, R. C., and Cowan, W. A. 1961. Preservation of bovine spermatozoa at -79 and -196°C. J. Dairy Sci. *44:*2089.
73. Polge, C. 1953. The storage of bull semen at low temperatures. Vet. Rec. *65:*557.
74. Quinn, P. J., Salamon, S., and White, I. G. 1968. Effect of osmotic pressure and temperature gradients on cold shock in ram and bull spermatozoa. Aust. J. Biol. Sci. *21:*133.
75. Quinn, P. J., and White, I. G. 1966. The effect of cold shock and deep-freezing on the concentration of major cations in spermatozoa. J. Reprod. Fertil. *12:*263.
76. Rajamannan, A. H. J. 1970. A method of packaging semen--the straw. Proc. III Tech. Conf. Artif. Insemination Reprod. p. 49.
77. Rajamannan, A. H. J., Graham, E. F., and Schmehl, M. K. L. 1971. Effects of holding on bovine semen. Artif. Insemination Dig. *19*(4):6.
78. Robbins, R. K., Gerber, L. E., and Saacke, R. G. 1972. Influence of thaw rate on maintenance of the acrosomal cap. J. Anim. Sci. (Abstr.) *35:*253.
79. Rodriguez, O. L., Berndtson, W. E., Ennen, B. D., and Pickett, B. W. 1975. Effect of rates of freezing, thawing and level of glycerol on the survival of bovine spermatozoa in straws. J. Anim. Sci. *38:*129.

80. Roldán, R. R. 1970. A means of individual identification of semen frozen in pellets. Anim. Breed. Abstr. *38:*423.
81. Saacke, R. G. 1974. Concepts in semen packaging and use. Proc. VIII Conf. Artif. Insemination Beef Cattle. p. 11.
82. Sabbione, J. C. 1968. Semi-rapid freezing of pelleted semen. The intensive use in the field and a comparison of its efficiency with semen frozen in ampoules and refrigerated semen. Anim. Breed. Abstr. *36:*432.
83. Salisbury, G. W. 1967. Aging phenomena in spermatozoa. III. Effect of season and storage at -79 to -88°C on fertility and prenatal losses. J. Dairy Sci. *50:*1683.
84. Salisbury, G. W., Willett, E. L., and Seligman, J. 1942. The effect of the method of making semen smears upon the number of morphologically abnormal spermatozoa. J. Anim. Sci. *1:*206.
85. Senger, P. L., Becker, W. C., and Hillers, J. K. 1976. Effect of thawing rate and post-thaw temperature on motility and acrosomal maintenance in bovine semen frozen in plastic straws. J. Anim. Sci. *42:*932.
86. Sic, R., and Vergles, V. 1968. Bull semen, pelleted and deep frozen. Anim. Breed. Abstr. *36:*432.
87. Smith, A. U., and Polge, C. 1950. Storage of bull spermatozoa at low temperatures. Vet. Rec. *62:*115.
88. Stewart, D. L. 1964. Observations on the fertility of frozen semen stored at -79°C and -196°C. V Int. Congr. Anim. Reprod. Artif. Insemination *4:*617.
89. Stewart, D. L. 1968. Ministry of Agriculture, Fisheries and Food, Agricultural Development and Advisory Service, Reading Cattle Breeding Centre. Annual Report.
90. Stewart, D. L. 1972. Ministry of Agriculture, Fisheries and Food, Agricultural Development and Advisory Service, Reading Cattle Breeding Centre. Annual Report.
91. Ström, B. 1968. Influence upon fertility of bull semen of storage time in liquid nitrogen. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1171.
92. Sullivan, J. J. 1970. Sperm numbers required for optimum breeding efficiency in cattle. Proc. III Tech. Conf. Artif. Insemination Reprod. p. 36.
93. Tjupina, K. F. 1971. Comparison of different methods of freezing bull semen. Anim. Breed. Abstr. *39:*698.
94. Turányi, J., and Nagy, G. 1969. Freezing, storage and use of pelleted bull semen. Anim. Breed. Abstr. *37:*68.

95. Ulberg, L. C., and Burfening, P. J. 1967. Embryo death resulting from adverse environment on spermatozoa or ova. J. Anim. Sci. *26:*571.

96. VanDemark, N. L., Miller, W. J., Kinney, W. C. Jr., Rodriguez, C., and Friedman, M. E. 1957. Preservation of bull semen at sub-zero temperatures. Ill. Agric. Exp. Stn. Bull. 621.

97. Wales, R. G., and White, I. G. 1959. The susceptibility of spermatozoa to temperature shock. J. Endocrinol. *19:*211.

98. Weidler, J. F., and Zaugg, N. L. 1975. Fertility of semen in straws unaffected by shortened cooling time to 5°C. Artif. Insemination Dig. *23*(8):9.

99. Wickersham, E. W., and Almquist, J. O. 1959. Use of egg yolk in skim milk-glycerol diluents to improve bovine sperm livability and simplify glycerolation. J. Dairy Sci. *42:*931.

100. Wiggin, H. B., and Almquist, J. O. 1975. Effect of glycerol equilibration time and thawing rate upon acrosomal maintenance and motility of bull spermatozoa frozen in plastic straws. J. Anim. Sci. *40:*302.

101. Zarazúa, M. V., Berndtson, W. E., and Pickett, B. W. 1977. Unpublished data. Colorado State University, Fort Collins.

BANKS FOR FROZEN HUMAN SEMEN: CURRENT STATUS AND PROSPECTS

J. K. Sherman

INTRODUCTION

A synopsis of the art of human semen cryobanking was published in 1973.[1] It included the history of cryobiology of human spermatozoa from 1776 to 1964 and research highlights and clinical status of semen cryopreservation from 1964 to 1973. Data were presented on about 600 births, with fewer abnormalities and spontaneous abortions than in the normal population, as well as on normal births from semen inseminated after over 10 yr of frozen storage. In addition, support was given for the efficacy of frozen human semen banks relative to acceptable, safe, and valuable methods and applications. Finally, commercial banking with regulated, high standards of operation and a clarified "no guarantee of fertility" policy for frozen-stored spermatozoa was endorsed as essential to the requisite large-scale application critical to the future of research and development of frozen human semen. Most of what was reported and discussed in the synopsis is still relevant and valid today. Continuous updating of the data, with reemphasis on applications analysis of accomplishments and perspectives of the field, is essential for proper assessment of the value and future of frozen human semen. The purpose of this overview is to further update and modify the presentation made in 1975.[2]

APPLICATIONS OF BANKS FOR HUMAN SEMEN

The demand for therapeutic (artificial) donor insemination (AID) by childless couples with an infertile male partner will continue to be stimulated greatly by the abortion laws, which severely reduce the opportunity for adoption.

Donor semen with particular and uncommon genetic characteristics is not always available to the physician for AID. Such a logistical problem is resolved readily through the use of a bank for frozen-stored semen. Other clinical applications of frozen-stored human semen, in both infertility and population control, as proposed in 1964,[3] are still valid and have been implemented to some degree.

Many ejaculates from oligospermic husbands (H) can be collected and stored over a period of several months, then thawed, pooled, concentrated by centrifugation, and inseminated (AIH) at spermatozoal concentrations manyfold greater than the original single ejaculates. This technique has proven successful, but only on occasion, when a reduced number of motile spermatozoa was the sole cause of male infertility. It is probable, however, that factors of infertility other than reduced numbers of spermatozoa also are involved in oligospermia, and that successful application of freezing methods in its "treatment" is quite limited. Oligospermic samples often show a poor revival after freezing and thawing, further restricting this application. Sixteen of the normal births reported with frozen-stored semen (Table 1), however, were achieved by AIH from oligospermic husbands.

Stored semen can be used to deliver by insemination large numbers of motile spermatozoa at times most likely to coincide with ovulation. A procedure of consecutive multiple inseminations improves chances of successful fertilization by husband or donor, especially in the female with irregular cycles or subfertile behavior.

Donor semen banks permit storage of sperm cells bearing the genetic characteristics for desired unusual blood types and the like. This facilitates ready availability for satisfying specific needs and increased demands of AID. A system of centralized banks supplying physicians throughout each state, and between states and countries, is quite feasible. We have sent frozen-stored semen overseas on several occasions, with the most recent success after shipment reported from the Virgin Islands.

By storing his ejaculates, man can induce conception in absence of testes, after exposure to sterilizing or possible mutagenic effects of radiotherapy or chemotherapy, in old age, and long after his death. Also, several children in one family can be fathered from the stored semen of the same desirable donor independent of his availability or viability. The reproductive effectiveness of husband or donor, therefore, can be greatly extended.

TABLE 1 Births from Frozen-Stored Human Semen[a]

No. of normal children born	1,464
No. of abnormal children born	11
No. of spontaneous abortions	113
No. of pregnancies still unreported as births	71
Longest period of cryopreservation of fertility	
Asia (Iizuka, Japan)	13 mo
Europe (Lebech, Denmark)	5 yr
United States (Sherman)	10¼ yr
Before international shipment (Sherman United States)	6 yr.

[a]Pregnancies for births reported followed, on the average, a protocol of two inseminations of 0.5 to 1 ml of semen in each of four cycles, aside from results of one clinician, who routinely inseminates 14 times per cycle.

Frozen human semen banks may provide a means of control of population in planned parenthood. Population control means not only the reduction in the number of births in our world's population explosion, but also the genetic improvement of the population. Selection of donor semen bearing the characteristics of mind and body deemed most suitable by the recipient couple is facilitated through semen banks. The concept of germinal choice or parental selection may extend beyond that couple, with its infertile male member, to fertile couples wishing to improve upon the genetic constitution of their offspring. Frozen human semen banks can offer a wide range of genetic material for selection at any time. Also, the ultimate in donor evaluation is made feasible through a program of progeny testing with donor semen. Frozen semen banks offer scientists the unique opportunity to evaluate genetics in man on an experimentally controlled basis from generation to generation.

The promise of future fertility in frozen storage, to a minor degree, has encouraged voluntary submission to vasectomy in attempts to restrict family size. Its prime effect for those already committed to vasectomy, however, has been to offer a greater chance for fertility maintenance

than attempts at surgical reversal. Also, banks storing semen from those outstanding vasectomized potential fathers who, by choice or death, leave their genetic legacy to others, may in turn be used within the framework of a program of germinal choice. In spite of relatively reduced demand, prevasectomy storage is still the main function of one or two commercial cryobanks.

Banks of frozen sperm cells protected from radiation may minimize or obviate the potential genetic danger of man's exposure to radiation on earth and in space and help to control such mutations in the population.

Carrying this further into theoretical considerations, which are of questionable advocation, it may become possible to preserve spermatogonia and oogonia by freezing or freeze-drying, to cultivate these gonia on demand, to select sex and other genetic characteristics, and finally to realize *in vitro* fertilization and development. Early (preimplantation) embryos of mice and rabbits already have been preserved at -196°C, subsequently demonstrating normal development and reproductive capacity.[4,5]

Whether or not we favor each of the above applications, as scientists we must appreciate that their use may be implemented. The need for proper evaluation of some of these applications still is apparent. There is little doubt that some degree of control of heredity is on the horizon in man's biologic destiny.[6] The realization of the necessary manipulation of the coding elements to achieve alterations in mind and body, however, is really a projected dream into at least the next century. In the interim, those who feel that programs of genetic improvement through germinal choice should be pursued may use frozen human semen banks for this purpose.

CURRENT RESULTS

Thousands of therapeutic, or artificial, donor inseminations (AID) with fresh semen are performed each year as an endorsed and accepted medical practice in the treatment of childless couples with infertile or relatively infertile husbands. Estimates of recent annual births through AID can be projected to range from 10,000 to 100,000.[7,8] It is foolhardy, however, even to suggest the authoritative nature of such figures in this area of human reproductive biology. The most frustrating part in the preparation of this overview was the search for vital statistics in critical and obvious areas of inquiry. Figures on the results of

insemination with frozen-thawed human semen were obtained in part through review of the literature, but primarily via questionnaires and personal contacts. Such an inquiry is limited because some sources do not respond, some are unknown, and many responses are incomplete. The results shown in Table 1, however, are representative and authoritative, indicating minimal but documented numbers. They strongly suggest, within the limitation of relatively small numbers, as compared with millions in animal breeding, that frozen storage of human spermatozoa is compatible with fertilization and the subsequent embryonic development that results in normal progeny. As was true of the 1973 figures, the number of abnormalities and spontaneous abortions are below that seen in the normal population. The observation has been made that fewer abnormalities and spontaneous abortions also result from AID with fresh (unfrozen) semen.[8] The explanation for better reproductive performance after therapeutic (artificial) insemination, with either fresh or frozen-stored semen, probably is that the procedure demands a more careful control of the timing of ovulation. This minimizes possible prefertilization aging-induced effects on ovulated egg cells that are considered a basis for early embryonic developmental errors subsequent to their fertilization.

Results of a survey of the physicians and banking facilities known to use frozen-stored semen were reported 3 yr ago.[1] The figures in Table 1 are based upon updated data obtained from the sources listed in the 1973 report, as well as from the following additional participants: J. Barkay and H. Zuckerman, Department of Obstetrics and Gynecology, Central Emek Hospital, Afula, Israel; D. Cottinet, Laboratoire d'Histologie et Embryologie, 8 Avenue Rockefeller 69, Lyon 8°, France; G. David, Hospital de Bicetre, 94270 Kremlin--Bicetre, France; S. Evans, 609-925 West Georgia Street, Vancouver, B.C., Canada; J. deMaria, Department of Obstetrics and Gynecology, University of Oregon Medical School, Portland, Oreg.; J. Feldschuh, Idant Corporation, 645 Madison Avenue, New York, N.Y.; M. Jondet, 35 Rue Mathurin Rignier, Paris, France; S. Leto, Washington Fertility Study Center, 2600 Virginia Avenue, N.W., Washington, D.C.; R. R. Quinlan, Chartered International Cryobank, 1801 Bush Street, San Francisco, Calif.; J. H. Ravina, 148 Avenue Wagram, 75017 Paris, France; J. C. Rioux, Department of Gynecology, Le Centre Hospitalier de l'Universite Laval, Quebec, P.Q., Canada; R. Schoysman, Avenue R. Comhaire, 69-1080 Brussels, Belgium; G. Sillo-Seidl, Schwanthaler Strasse 65, Frankfurt am Main, Germany.

There are undoubtedly many more births from cryopreserved spermatozoa than the number reported in our survey (Table 1), but, even if the figure was tripled, results still would indicate only token use of frozen-stored semen in comparison with fresh (unfrozen) samples. The prediction of rapid growth for commercial banks, which was made in 1973,[9] has not been realized. To the contrary, there have been cancellations of planned openings of new branches and closing of some existing branches in what was expected to become a thriving industry. The explanation given by some commercial banks is that the expected accelerated activity in prevasectomy storage of semen never has materialized. But, this observer sees more than this simple miscalculation as the cause of the disappointing rate of development in semen banking.

BASIS FOR SLOW PROGRESS

The benefits of cryobanking human semen for use in both infertility and population control have yet to be realized to any appreciable degree. The chief reason still[1] appears to be the reluctance of the lay and medical community to accept, as proven, the safety and efficacy of frozen-stored human spermatozoa. There has been apprehension, therefore, in the support and use of cryobanking in appropriate therapeutic and planned parenthood applications. Public acceptance and demand are determined, to a large degree, by both medical practice and information that appears in newspapers and lay medical publications. The hesitancy to accept cryobanking of human semen is quite understandable in the face of warnings and doubts raised by certain medical groups. Public statements in 1972 by a task force committee of the American Public Health Association and the Planned Parenthood-World Population, as cautions or expressed fears, have insinuated questionable genetic adequacy and raised without basis and thorough review[1] the awesome possibility of birth defects after frozen storage.

Recently, discouragement from using cryopreserved human semen has been reenforced and extended by emphasis on possible dangers of *in vitro* aging of animal spermatozoa. There have been controversial reports that reduced fertility and an increase in spontaneous abortions have been demonstrated as a consequence of aging during frozen storage of bull and ram spermatozoa.[10] There is no evidence, however, that *in vitro* aging of frozen mammalian spermatozoa

causes birth defects, changes in sex ratio, or any other manifestations of genetic instability or inadequacy in progeny. This is a critical point for those who cite *in vitro* aging effects as a basis for concern for the danger of increase in birth defects through the use of frozen-stored semen. In addition, more recent and extensive data, involving up to 100,000 inseminations of bull semen stored for as long as 5 yr, reveal no decline in breeding efficiency (fertility) or any alteration in the rate of spontaneous abortions.[11,12]

Millions of dairy calves are born annually through the use of frozen-stored bull spermatozoa. The authoritative general opinion of artificial breeders is that frozen storage is efficient, genetically safe, and of great importance in the improvement of the quality of farm animals and their products. Frozen-storage has proven itself in the breeding industry for the past two decades. One would think that contraindications for its widespread national and international use, if real, would have been detected by now.

TECHNIQUES FOR CRYOPRESERVATION

The basic, relatively simple, liquid-nitrogen-vapor freezing technique that was introduced in 1963 has been proven successful in cryobanking.[1] It compares favorably with newer, more complicated, stepwise and electronically controlled freezing systems. There appears to be a wide spectrum of rates of freezing and thawing in techniques that result in satisfactory cryosurvival of human spermatozoa, rather than a limited defined set of conditions. Some methods are claimed to give superior cryosurvival than others within a milieu of sometimes contradictory findings in the literature, but there has been no evidence of real differences on the basis of fertility and progeny production. Actually, properly controlled evaluation remains to be pursued on this and other aspects of semen cryobanking.

Current techniques include the use of paillettes or plastic straws in general preference to glass ampules and the dilution of semen with an egg yolk citrate extender with antibiotics, as well as the use of raw or undiluted semen. All methods employ liquid nitrogen for freezing and storage, with glycerol as the cryoprotectant. The author's method of cryopreservation involves undiluted semen with 7.5 to 10 percent glycerol by volume, freezing primarily in heat-sealed plastic straws either in air at -85°C or in liquid nitrogen vapor (-190 to -196°C), and

frozen storage in liquid nitrogen. Preliminary comparison in our laboratory suggests that the egg yolk extender does improve cryosurvival. This is based on results of motility observations on split samples of raw and diluted semen in parallel freezing and thawing experiments. I am still reluctant to use or advocate the clinical use of this extender, which has proved so successful in the animal-breeding industry. There are reservations concerning the wisdom of: (1) diluting an already sparse population of spermatozoa with possible reduction of its effectiveness, (2) the introduction of additional nonphysiological substances of unknown effects in human subjects into the female, and (3) the potential danager of the provocation of a drug sensitivity reaction in the recipient. These reservations probably will be proved invalid, aside from the reservation concerning the use of penicillin. Until then it seems medically prudent to avoid such possible limitations, especially since the use of raw semen has been so successful in realizing most of the births reported to date with frozen-stored semen. This conservative approach, however, may change with subsequent evidence that egg yolk or other extenders or conditions of cryopreservation are both superior and safe for cryobanking of human cells. Evaluation of egg yolk extenders, cryoprotectants, rates of freezing and thawing, and other factors continue to be evaluated in our laboratory.[14] Recently, pregnancies have been reported in Israel by Barkay *et al.* using a pellet method of freezing and storage, the same used by some for bull and boar semen.[14]

Our present level of technology is adequate to establish cryobanks of human semen on a large scale. It is the standards concerning use or abuse of the techniques that should be the matter for concern. "Fly-by-night" unscientific and irresponsible commercial enterprises may not employ care in genetic selection of donors, screening of semen, testing for venereal disease, and the like. Regulations in terms of acceptable scientific and ethical practices seem warranted in cryobanking of human semen.

RESEARCH AND OTHER NEEDS

The author's views on the subject of cryobanking human spermatozoa have not changed[1] in considering the practice as efficacious and safe. There is neither a clinical indication for increased spontaneous abortions or birth defects, nor is there test-tube evidence for DNA alterations or

chromosomal aberrations, from evaluative studies on frozen-stored human spermatozoa.[1,13] Genetic stability during frozen storage has been demonstrated also with hundreds of other cell types and strains that are routinely preserved by freezing.[1] It is unfortunate, however, that the overwhelming majority of the approximately 1,500 normal human births reported to date (Table 1) have resulted from insemination of semen stored for less than a year. Chances are that, as with bull spermatozoa, long-term storage of human spermatozoa also is genetically safe, but this has not been established. There is, therefore, a serious deficiency in the critical evaluation of genetic adequacy or stability of human spermatozoa during long-term storage. This deficiency must be remedied if cryobanking of human spermatozoa is to fulfill its valuable potential and if there is to be authoritative data for standards in the control of methods and practices of commercial and clinical cryobanking. This is essential for the assurance of protection of participants in donor inseminations (AID), prevasectomy storage, and for other applications, such as the use of the same donor semen for several births in the same family, which will involve the use of semen stored for periods exceeding a year.

A program is needed to evaluate the genetic stability of human spermatozoa stored for periods of 5 to 10 yr, especially in terms of birth defects, in a significant population of progeny resulting from AID. Decreased fertility potential (percent fertility) and an increased rate of abortion (early embryonic mortality), if induced by *in vitro* aging during long-term frozen storage of human spermatozoa, certainly are undesirable and traumatic events in a woman's reproductive cycle. They become relatively minor calculated risks, however, in attempts at realizing progeny in cases where cryopreserved semen is particularly advantageous or essential to achieving fertility. Induction of birth defects, to the contrary, is a far more serious possible consequence of prolonged cryobanking, one that should be avoided at all costs. The critical deleterious end point in evaluating possible effects of aging in storage, therefore, should be an increased incidence of birth defects. Information on fertility and abortion rates can be collected at the same time, of course, in investigations of such possible phenomena. Unless the research is undertaken now, it may never be initiated and completed. The past relative inactivity of investigators in serious programs of related research efforts in semen cryobanking attest to this probability.[1,3]

Frozen-stored spermatozoa will be used and should be used in clinical applications, in spite of the unresolved question of genetic stability in long-term storage. Certainly there should no longer be inhibitory concern about the safety of semen used after storage periods not exceeding 1 yr. An additional advantage of semen cryobanking, which may have an important bearing on increased use of frozen-semen banks, is that it permits semen to be stored for future use during the time (2 to 3 days) in which it can be screened, by culture methods, for gonococci.[15] This has particular importance because of recent research that uncovered a high incidence of asymptomatic males whose fresh or frozen-stored semen could transmit gonorrhea via AID. The use of penicillin in semen, as the alternative to such an application of frozen storage, is of questionable clinical prudence because of possible allergic reactions to the drug.[15] Anticipated clinical use of frozen-stored human spermatozoa, therefore, makes research on evaluation of its safety after prolonged storage of more than academic importance. Parallel studies on more basic aspects of pretreatment, and the like, also should be undertaken. For example, no study has been made on the evaluation of possible deleterious effects of pretreatment with a cryoprotectant (glycerol), of freezing and thawing, and of aging during long-term frozen storage, in terms of motility, viability (membrane integrity), and ultrastructure of spermatozoa (especially the membrane and acrosome) from the same ejaculate. Such an investigation on a number of semen samples should provide information on critical cryoprotection and cryoinjury of human spermatozoa. These data also could be correlated with the demonstrated fertility potential of split-ejaculate semen samples from the same donors, especially if pursued in parallel with the proposed long-term (5 and 10 yr) storage evaluation.

SUGGESTED PROGRAMS

In addition to the long-term research evaluation proposed above, there are other requisites that should be satisfied in the interim, to provide a more stable foundation for the field. These include:

1. A directory of the group of experts and practitioners in frozen-semen research and therapeutic insemination should be compiled and distributed.
2. Standards should be established by the group as

to donor procurement and screening for communicable and genetic diseases, as well as the handling, analysis, processing, storage, and clinical use of frozen-stored semen.

3. These standards eventually should be incorporated into a system of appropriate guidelines and regulations, to insure the highest level of technical and business practices in cryobanking. It is strongly recommended that state or federal regulations be developed cautiously and deliberately, in stepwise fashion, guided by the standards and suggestions of the cryobanking group (2 above). Guidelines rather than regulations gradually should be formulated for the technology, as premature interference would stifle development of the field. Aspects of screening for health hazards, such as venereal disease,[9] however, should be implemented and regulated as soon as feasible.

4. A central clearing house should be established for periodic filing of data from the cryobanking group and the compilation and dissemination, as an authoritative source, of such data as: (a) improved methods of aspects of processing and clinical use of frozen semen; (b) pertinent basic and applied research findings; (c) team approach to the counseling of patients by experts in infertility, psychology, and family planning, prior to AID; (d) number of patients involved in therapeutic AID and AIH; (e) number of normal children born; (f) number of spontaneous abortions; (g) number and kind of malformations (birth defects); (h) number and volume of insemination units per cycle; (i) number of cycles per pregnancy; (j) methods for timing ovulation; (k) methods for insemination; (l) percent fertility; (m) follow-up examinations on the early and later development, as well as the reproductive history of progeny, if at all possible; and (n) comparison of fresh and frozen semen on the basis of above data.

The principle of an identified and organized group of persons for exchange and dissemination of research and clinical findings also is applicable to other workers. I recommended it for the area of cryobanking of eggs, embryos, and other cells and tissue transplants used in human medicine. The practicality of implementing the format described above, however, will depend upon the enlightened attitude of active investigators and practitioners. Such an attitude can develop through educational forums and healthy communication and dialogues, such as this one.

SUMMARY

An overview of the clinical use of frozen human semen was presented especially in respect to: (1) reemphasis of the actual and potential clinical applications of semen banking, as well as the reason for the slow progress in their realization; (2) current results of an ongoing survey of the clinical use of frozen-stored human spermatozoa, which reveals about 1,500 births with fewer abnormalities and spontaneous abortions than in the normal population; (3) the evaluation of possible *in vitro* aging effects on genetic stability during the long-term storage of human spermatozoa, which is essential to the realization of its potential in widespread use, or its abandonment in clinical medicine; and (4) the need for the establishment and regulation of standards in both processing and use of frozen-stored semen, to provide maximum efficiency and safety.

DISCUSSION

ROBERT H. FOOTE: Your method of freezing without diluents is a good one. However, if additional cryoprotectants and stabilizers prove to be beneficial, a reduction of sperm concentration by adding diluting media need not be inimical to their use. One might first concentrate the specimen and then dilute or extend the spermatozoa. For example, research by Salamon in Australia has demonstrated that concentration of ram spermatozoa following extension and storage improves fertility, apparently because a small volume with a high concentration of cells at the cervix is desirable. Concentration of human spermatozoa before dilution and freezing might be desirable.

J. K. SHERMAN: I agree that additives to fresh human semen probably will prove beneficial to cryosurvival and will be used in clinical applications. Aside from the temporary reluctance of some to use nonphysiological or untested additives other than glycerol, the fear of penicillin use as an additive seems warranted. Sensitivity reactions to it in clinical inseminations already have been reported. Concentration and subsequent dilution with additives, or the reverse, certainly is feasible, if necessary, but we do not have much information on motility and even less on fertility concerning this procedure with human se-

men. Centrifugation for concentration of human spermatozoa from fresh untreated semen, followed by dilution with seminal plasm, is compatible with maintenance of fertility, of course, but the degree (percent) of fertility has not been assessed.

ROBERTA WHITE: I agree in your request for more followup on AID and numbers of patients treated. I have treated about 100 patients, only one with frozen semen, but if you would like exact figures I would be glad to help. I would also like to point out that this is a nursing procedure at the Yale New Haven Hospital and should be elsewhere, mainly because of the availability of the RN.

REFERENCES

1. Sherman, J. K. 1973. Synopsis of the use of frozen semen since 1964: State of the art of human semen banking. Fertil. Steril. *24:*397.
2. Sherman, J. K. 1976. Clinical use of frozen human semen. Transplantation Proc. *8*(2) (Suppl. No. 1): 165-170.
3. Sherman, J. K. 1964. Research on frozen human semen. Past, present, and future. Fertil. Steril. *15;*485.
4. Whittingham, D. G., Leibo, S. P., and Mazur, P. 1972. Survival of mouse embryos frozen to -196° and 269°C. Science *178;*411.
5. Bank, H., and Maurer, R. R. 1974. Survival of frozen rabbit embryos. Exp. Cell. Res. *89;*188.
6. Frankel, M. S. 1973. Genetic technology; promises and problems. Geo. Wash. U. Policy Studies Mono. 15.
7. Behrman, S. J. 1975. Techniques of artificial insemination. In: S. J. Behrman and R. W. Kistner, eds: *Progress in Infertility*. Little, Brown, Boston. p. 765.
8. Joel, C. A. 1971. Therapeutic insemination. In: C. A. Joel, ed. *Fertility Disturbances in Men and Women.* S. Karger, Basel. p. 580.
9. Frankel, M. S. 1973. The public policy dimension of artificial insemination and human-semen cryobanking. Geo. Wash. U. Policy Studies Mono. 18.
10. Salisbury, G. W., and Hart, R. G. 1975. Functional integrity of spermatozoa after storage. BioScience *25:*159.
11. Anderson, J. B. 1975. Personal communication.

12. Roettger, L. W., Salisbury, G. W., Lee, A. J., Boyd, L. J., and Ingalls, W. 1975. In vitro aging of frozen bull semen. J. Dairy Sci. *58:*767.
13. Sherman, J. K., and Char, F. 1974. Stability of y chromosome fluorescence during freeze-thawing and frozen storage of human spermatozoa. Fertil. Steril. *25:*311.
14. Sherman, J. K. Freezing human semen. In: E. S. Hafez, ed. *Techniques of Human Andrology.* Unpublished.
15. Sherman, J. K., and Rosenfeld, J. J. 1975. Importance of frozen-stored human semen in the spread of gonorrhea. Fertil. Steril. *26:*1043-1047.

GAMETIC INTEGRITY DURING STORAGE AT LOW TEMPERATURES--EVIDENCE FOR AN AGING EFFECT

G. W. Salisbury and J. R. Lodge

INTRODUCTION

Our first experience with the manifestations of the effect of the passage of time on fully formed mammalian spermatozoa were experiments on rabbits conducted by the senior author during the 1930's at Cornell with his mentor, S. A. Asdell.[2] That experiment was in fact a continuation experiment, after a break of several years, of a classic in this field conducted at the University of Cambridge in the middle 1920's by John Hammond and Asdell.[28] Those men had shown that the fully formed spermatozoa of the rabbit isolated in the sectioned epididymis confined in the scrotum achieved their optimum fertility in the second 10-day time period of testing of fertility after the section. The fertility tailed off gradually after that and lasted for a maximum of 38 days. (Fertility by 10-day periods: first = 89 percent; second = 100 percent; third = 36 percent; and fourth = 20 percent.) Our experiment[2] with rabbits having the ligated epididymides and testes anchored in the abdomen showed that the duration of fertile life of spermatozoa was 8 days. Fertility did not tail off, but ended with dramatic suddenness. Their motility lasted for 14 days. Temperature, the abdomen being 5° to 7°C or so higher than the scrotum, clearly can influence the time sequence.

Others earlier had demonstrated that spermatozoa of the marine worm, *Nereis limbata,* when used after 24 h storage at room temperature or after several days at refrigerator temperature,[20] were depressed in fertility. Also, when they were successful in penetrating the fresh ova used, sometimes conjunction of the male and female pronucleii did not occur, so no cleavage resulted; or there were various and profound changes in the subsequent development if it did.

In a series of papers from 1911 to 1916 G., O., and P. Hertwig[31,32,33,34,35] showed that, in worms, frogs, and fish, radium Xrays of unknown dosage directed on spermatozoa caused profound changes in their fertility and in the well-being of whatever progeny they produced. With frog spermatozoa, overdoses caused complete destruction of the male genome, but normal progeny were produced by parthenogenesis, as the spermatozoan capacity to penetrate eggs and to activate them to mitoses and subsequent sequential embryonic development was not so destroyed.

This series of researches, beginning with worms and extending through mammals and covering a period from 1911 through 1926, set the parameters of our present knowledge about the separate functional performances of spermatozoa and the order of their loss when subjected to environmental insult. These functions and the order of their loss are:

1. Integrity of the genetic message transmitted;
2. capacity for pronuclear formation and syngamy;
3. ovum penetrance and activation of the egg; and
4. independent motility.

These experiments also identified time and temperature as parts of the spermatozoan environment to which they react.

In a series of published papers beginning in 1931, Young and his students and colleagues extended such studies to other mammals, the guinea pig and the rat, and included the question of the age of ova as influencing embryonic development.[12,13,63,64,66] In 1943 Nalbandov and Card[44] published their data on the drop with time after mating in the fertility of chicken eggs and the increase in embryo death or developmental abnormalities. They believed the nucleus of the stale spermatozoa to have changed during their sojourn in the female reproductive tract. A decade earlier plant geneticists had proved nuclear involvement in aging by the increased mutations exhibited by long-stored seed.[18,45]

SPERMATOZOAN AGING

Data from Commercial AI of Cattle

Experience with commercial AI of a larger mammal began in 1938. In the period prior to the establishment of the system for artificial insemination of domestic animals in

large numbers, the few university physiologists working with male gametes in an endeavor to undergird the process with sound science were hard put to get much data on the fertility of the spermatozoan samples with which they worked. The criteria used first for assay of the efficacy of preserving spermatozoan life were estimates of the proportion of the spermatozoa in treated samples capable of motion and the level of vigor of that display. The techniques utilized for these purposes included not only visual microscopic observation of activity, but also a broad array of physical and biochemical techniques, including analyses of the energy utilizing chemical transformations necessary for the movement of the spermatozoan tail.[47,59]

After some of us in academe working closely with commercial AI designed situations where we had control of the males producing the semen and how it was processed and used for AI of cattle, the fertility results became fascinating objects of study. No one in prior history had had such voluminous data for study.

A major new experience was the necessity of generating a statistical concept of fertility of males. One of the first things noticed about such data was the question of the relative validity of an estimate of male fertility based on the number of inseminations providing it. The basic assumptions for adequate tests of male fertility were that the females inseminated by a semen sample were numerous and were in fact a random sample of females in the population. With too few females inseminated per test semen sample assayed, the normal binomial distribution characteristic of such all-or-none phenomena contributes so much variance to the outcome when expressed as a ratio as to obscure the significance of any other items under assay.[19] Elaborate, detailed, and continuing studies were necessary to prove these points.[61]

Another problem was the question of how long after insemination one had to study an inseminated cow to determine whether or not it was in calf. Unfortunately, in cattle no quick mobilization of this or that hormone in the pregnant animal and its extrusion in the urine afforded a reliable early diagnosis of pregnancy. We relied on what cattlemen had for all of history--the absence of recurring estrus as evidence of pregnancy and its recurrence after an insemination as a sign of failure. However, the sequential events of pregnancy from fertilization to parturition run their hazards through the 280-285-day period, and the difference in apparent success at any one early stage in gestation to a later one is an important estimate of the

cumulative effectiveness of any pregnancy. These concepts are demonstrated in Figure 1.[49]

The technique finally adopted for estimation of the relative fertility of AI practice was designated as the non-return-to-service estimate, which has provided the research data for most of the progress recorded over time in the commercial practice of artificial insemination of cattle. The technique has its basic and well-recognized errors,[61] none of which need to contribute bias in properly conducted experiments with sufficient number of inseminated cows randomly distributed within the test population of females.

An estimate of the number of non-return-to-service cows made relatively soon after insemination, within a 30-60-day interval and after a mean period, including about two normal estrus periods of 21 days each, is considered as evidence for the fertilization of the egg. Those cows found on return to service, to be in estrus

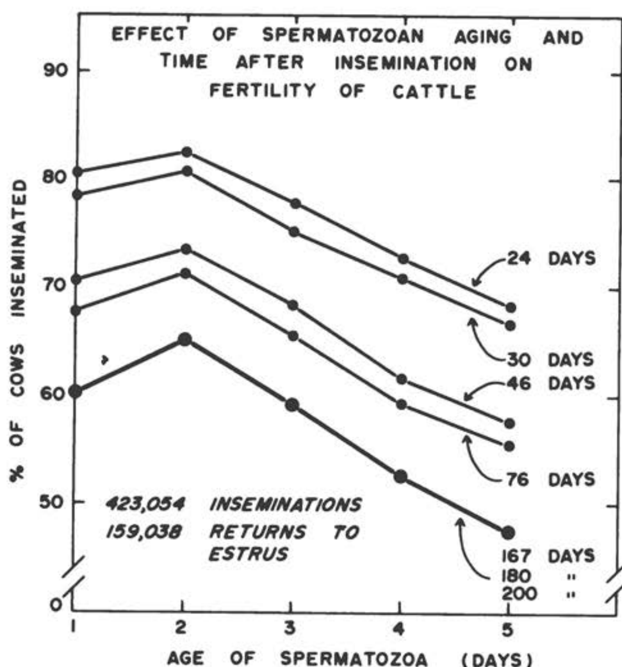


FIGURE 1 The percentage of nonreturns at varying times between 24 and 200 days after insemination for semen stored at 5°C.[49]

after that period, are considered as evidence for faults in embryogenesis resulting in the late returns (Figure 2). The technique has its adherents and its critics, but it has no other satisfactory alternative in the breeding of large numbers of cows. It has become standard practice in the industry. In our statistical studies of commercial AI data, great care has been taken to use data that permitted imposition of an experimental design in their analyses.

With these concepts in mind, the results from the first study on spermatozoan aging involving a small number of cows are presented in Table 1.[54] These results show that, as semen was stored for 4 days at 5°C, there was an initial increase in fertility after 1 day of storage, which was followed by a decline in fertility with each subsequent day of storage. These changes in fertility were observed for both the early estimate (1 mo NR) and the late estimate (5 mo NR). If we now examine the difference between the early and late estimates of fertility, we see that the difference becomes larger with each day of storage, and

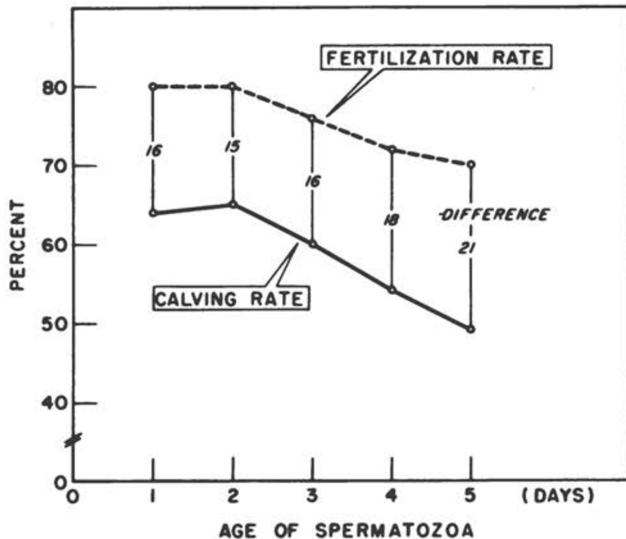


FIGURE 2 Fertility of a population of 306,513 cows determined 30 days (fertilization rate) and 180 days after insemination (calving rate) when inseminated with semen aged for 5 days or less at 5°C.

TABLE 1 The Effect of the Length of Time of Storage at 5°C of Extended Semen on Its Fertility Level and the Difference Between 1-Mo and 5-Mo Nonreturns

	Age of Extended Semen When Inseminated in Relation to Day of Collection				
	Same Day	2d Day	3d Day	4th Day	5th Day[a]
No. of inseminations	12	726	756	970	56
1-mo nonreturns (%)	58.3	67.0	62.8	54.3	57.2
5-mo nonreturns (%)	50.0	57.0	50.7	41.5	39.3
Difference (%)	8.3	10.0	12.1	12.8	17.9

SOURCE: Salisbury *et al.*[54]
[a] 5th day or more.

thus a higher percentage of cows was presented for a second service at a later time as the semen aged during storage. This increasing difference reflects an increasing prenatal loss during this time period.

A similar study was designed in cooperation with the Southern Illinois Breeding Cooperative and involved more than 400,000 breedings.[55] The data in Table 2 show the results from the same semen samples used for 4 days. The initial increase in fertility, followed by the decline, and the inverse relationship with the difference can be seen. It was possible also from these data to examine the influence of season and individual sire effects in relation to time of semen storage. It was the warm months particularly that depressed fertility and increased prenatal losses. The fertility and embryonic loss curves of stored semen were found to be characteristic of the bull producing the semen.[49] The bulls of the highest fertility tended to show the least increase in fertility during the initial period of storage and the least decline with continued storage.

It was at about the time these studies were being concluded that the use of frozen semen came into wide-

TABLE 2 Fertility of Semen Samples Used on Day 4 and for Samples of the Same Semen Each Day of Prior Use

	Age of Diluted Semen at Time of Insemination (days)[a]				
	1	2	3	4	Total
No. inseminations	3,081	26,558	13,273	5,300	46,212
Fertility					
At 30 days (%)	78.6	80.7	76.6	71.6	78.7
At 180 days (%)	62.8	66.2	61.5	54.4	63.8
Difference	15.8	14.5	15.1	17.2	14.9

SOURCE: Salisbury and Flerchinger[55]
[a]Day 1 = day of collection.

spread practice, and it was assumed by many that storage in the frozen condition would overcome the aging effects seen with liquid semen storage. Thus experiments were designed in cooperation with the Central Ohio Breeding Association to determine if the results found for liquid semen storage occurred with semen frozen and stored in dry ice-alcohol (-79°C) and in liquid nitrogen (-196°C).

Results from the first experiment with dry ice-alcohol, involving nearly 200,000 inseminations (Table 3), revealed that the identical phenomena occurred in populations of cows bred by frozen semen, as had been the cases with those bred with liquid extended semen. The fertility increased and embryonic mortality declined from the first to the second month of storage. Fertility was optimum in the second month, remained so for about 3 mo, and then declined. By 6 mo the fertility had dropped about 13 percentage units, and embryonic losses were increasing.[48] The study was designed so that overall seasonal trends could be separated into male (based on month of semen collection; 176,432 inseminations) or female (based on month of insemination; 174,307 inseminations) effects. The results (Table 4) clearly show the seasonal effect. The effect under the environmental conditions of Ohio was due primarily to the male.[48]

During the time span of the study, the conversion from dry ice-alcohol to liquid nitrogen freezing and storage was made. An experiment was designed using the split ejaculate technique to study the effect of storage on fertility and embryonic loss at these two temperatures. The results (Figure 3) show a similar effect for the two temperatures during a 6-mo period of storage with a slight advantage for the colder temperature.[49]

An experiment to study the use of liquid nitrogen freezing and storage at -196°C for a period of 2 yr is presented in Figure 4. The distribution of numbers of inseminations shows that the largest number of inseminations were performed during the first year of storage with markedly fewer numbers of inseminations during the second year. The data from 196,488 inseminations show that optimum fertility was achieved after storage for about 4 mo. This optimum fertility and the minimum embryonic loss was maintained for another 5 or 6 mo when the inevitable decline in fertility began.[49] These results for storage at -196°C, along with the effect of storage on fertility of spermatozoa when stored at 4°C and -79°C, are presented for comparative purposes in Figure 5. The shapes of the curves are similar. The effect on the cow popula-

TABLE 3 Effect of Age of Bovine Semen During Storage at About -79°C on Fertility and on Embryonic Mortality as Estimated by the 46-167-Day Nonreturn Difference

Age of Semen	No. Inseminations	Fertility Level at 167 Days, %	46-167-Day Nonreturn Difference, %
< 1 mo	8,543	66.4	13.4
1-2 mo	50,235	68.0	10.0
2-3 mo	40,767	67.8	9.3
3-4 mo	23,648	67.8	9.0
4-5 mo	14,120	66.7	9.1
5-6 mo	9,088	66.4	9.2
> 6 mo	30,031	54.7	13.1
TOTAL	176,432	65.4	10.3

SOURCE: Salisbury[48]

TABLE 4 The Decrease in Fertility (Mean Difference in 167-Day Nonreturns Between 1-2-Mo Storage and >6-Mo Storage) on Aging of Semen at About -79°C for the Months in the Year of Insemination (Cow Response) and of Semen Collection (Bull Response)

Month	Cow Response Decrease on > 6-Mo Storage, %	Bull Response Decrease on > 6-Mo Storage, %
January	13.8	7.0
February	13.8	11.8
March	16.6	8.4
April	13.5	11.5
May	12.8	16.8[a]
June	13.2	16.7[a]
July	11.3	20.9[a]
August	14.4	14.2[a]
September	7.3	21.4[a]
October	13.1	15.7[a]
November	14.6	12.8
December	14.7	8.9
TOTAL	174,307	176,432
MEAN DECREASE	13.3	13.3

SOURCE: Salisbury[48]
[a] P = 0.01 that difference from mean is not due to chance.

tion was identical. The effect of lowering the temperature of spermatozoan storage only changes the time scale at which the effects occur. It must be remembered that an inverse relationship between fertility level and prenatal loss was demonstrated in all of the studies at the varying temperatures of sperm storage. The higher the initial fertilization rate, the lower the level of embryo loss.

More recent results obtained in cooperation with Michigan State University and the Michigan Artificial Breeding Cooperative are presented in Figures 6 and 7 for 555,449 inseminations. It can be seen that the distribution of numbers of inseminations (Figure 6) was similar

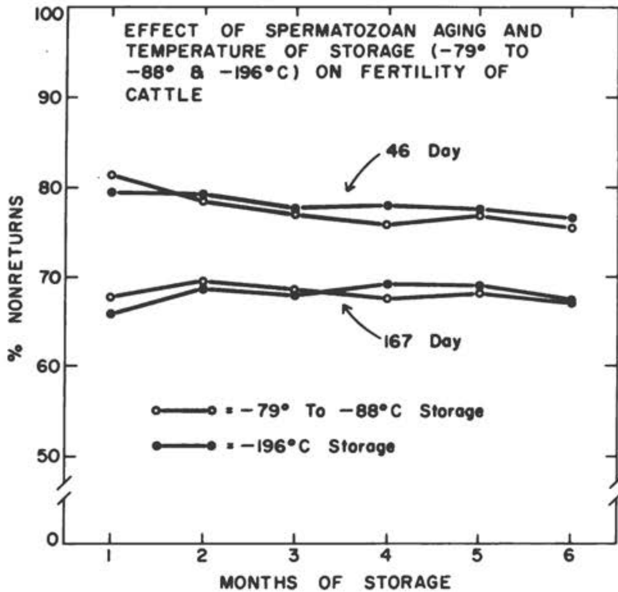


FIGURE 3 A comparison with split ejaculates of the effect of temperature of storage (-79° to -88° and -196°C) on fertility of cattle during 6 mo of storage.[49]

to that found in the earlier study in Ohio. Semen-use practices are similar among AI centers and do not appear to have changed with time. The regression curves of the nonreturn data are presented in Figure 7. These curves show again the initial increase in fertility during the first few months of storage and only a slight decline from optimum fertility during a storage period up to 4-5 yr. The short curve is for sets of semen samples used for 1 yr or less. The longer one is for sets used throughout the 5-yr period.

These results, along with others presented in this volume, are encouraging for extending the optimum fertility longer than had been shown previously. They undoubtedly reflect the improvements that have been made through the years in semen processing and handling techniques. However, from such data we should not generate the view that now spermatozoa can be stored forever without detrimental effects to their ability to fertilize eggs and produce viable offspring.

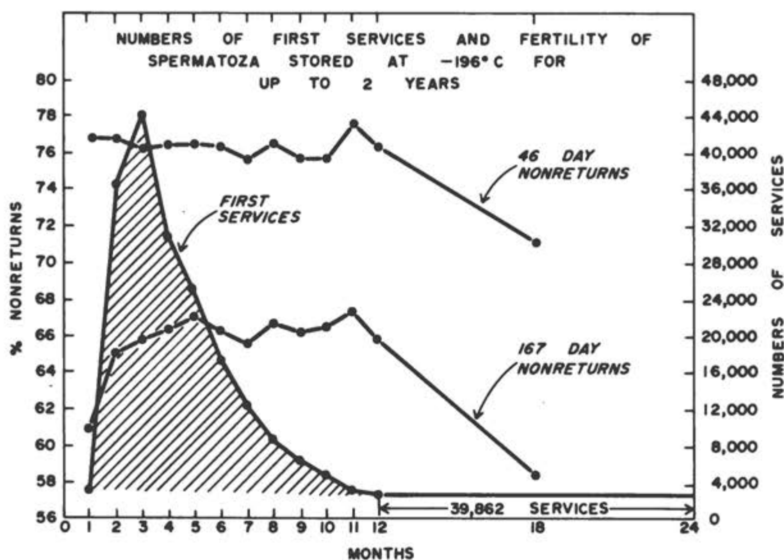


FIGURE 4 The distribution of numbers of inseminations and the effect of -196°C storage over 2 yr on fertility of cattle.[49]

Data from Other Species

The data using frozen semen of other species with sufficient numbers of inseminations over a long enough period of storage are too limited to determine if they support or refute the findings with cattle. However, data obtained from a number of other species, by us and others, with spermatozoa stored in varying *in vivo* and *in vitro* environments all at above freezing temperatures, confirm by direct observation of fertilized eggs, developing embryos, or fetuses the hypothesis generated by the cattle data. Many of these studies and others have been reviewed by various authors in a 1973 symposium.[1]

Studies of the effects of *in utero* sperm aging on fertility and embryonic development have been recently reviewed by Salisbury and Hart[56] and Shaver and Martin-DeLeon.[62] It is obvious from these reviews that, for a large number of species, spermatozoa that reside in the female reproductive tract beyond the optimum time for maximum fertility first cause an increase in prenatal loss due to developmental anomalies of one kind or another followed by a complete loss in fertilizing ability. An

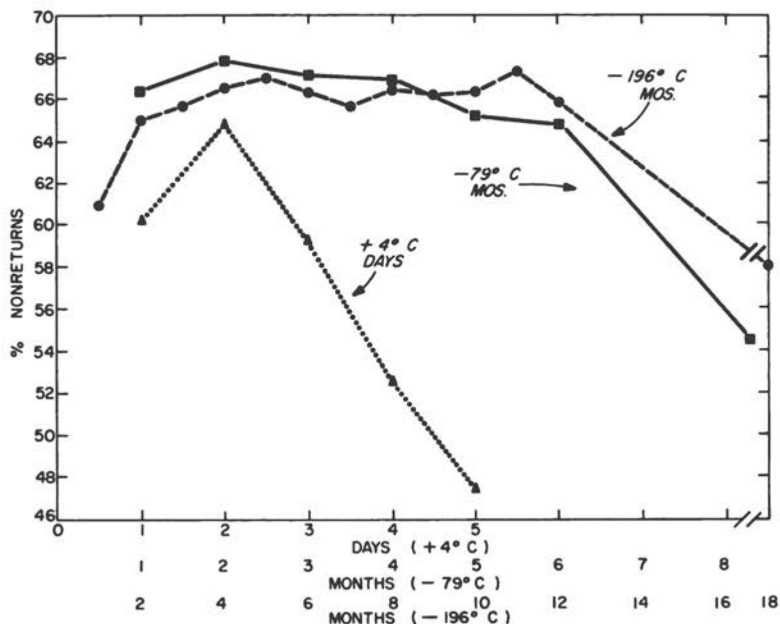


FIGURE 5 The effect of length of storage and of temperature of storage on fertility of bull spermatozoa. The duration of storage was 5 days at 5°C (423,054 inseminations), 1 yr at -79 to -88°C (174,307 inseminations), and 2 yr at -196°C (196,448 inseminations). Fertility was assayed to 167 to 180 days after insemination.[56]

example of these effects for the chicken, an excellent species for this kind of study, is presented in Figure 8.[41] Chromosome anomalies in developing embryos resulting from fertilization by aged spermatozoa have been reported for the rabbit[62] and the chicken.[40] The types of anomalies found would undoubtedly result in prenatal death of the developing embryo.

The effect of *in vitro* storage of spermatozoa in the liquid state has been studied in swine,[21,23] rabbits,[37] mice,[7] and frogs.[30] The results from these studies all show that following maximum fertility there is increasing embryonic and early fetal loss with increasing time of storage of the spermatozoa used to fertilize the eggs. The data presented in Table 5 show a highly significant increase in the percentage of developing frog embryos

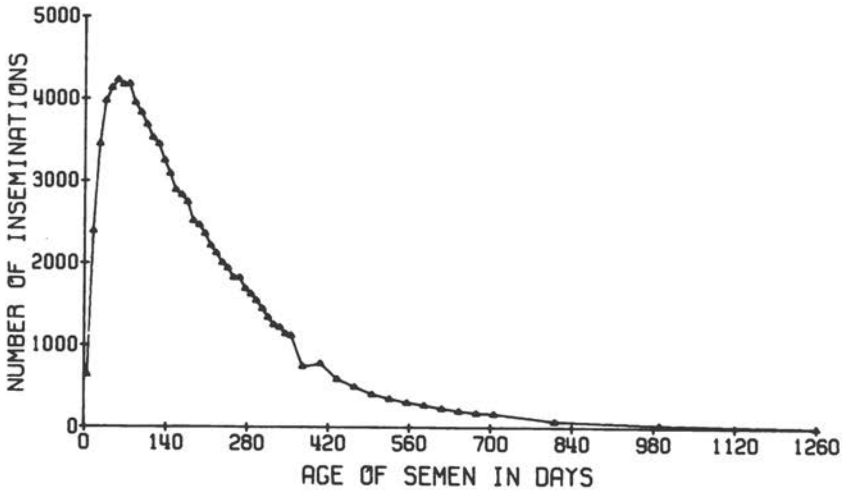


FIGURE 6 The distribution of the use of semen stored at -196°C for 4 to 5 yr.

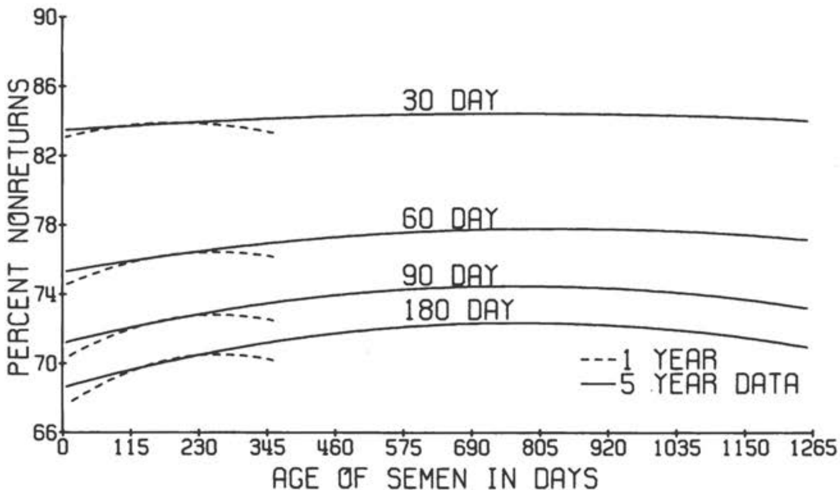


FIGURE 7 Regression curves for nonreturns from semen stored at -196°C and used during 1 yr or during 5 yr.

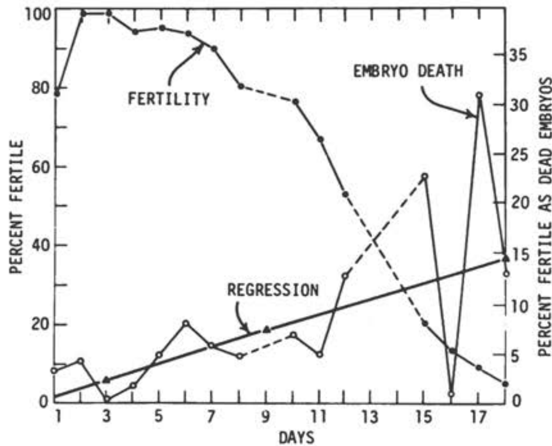


FIGURE 8 The effect of *in vivo* sperm age following a single insemination on fertility and embryonic death in the chicken. Approximately 140 eggs examined after 18 days incubation for each day.[41]

arrested at the gastrula stage of development when fresh eggs were fertilized by sperm, which had been stored for 24 h at 18°C.

From the results reviewed above for a number of species in varying environmental conditions, we conclude that with time ejaculated spermatozoa initially increase in fertility, they undergo a plateau period of optimum fertility, and then decline. There is an inverse relationship between fertility level and prenatal loss. The temperature of storage, the season of the year, the fertility level of the male, and the processing and handling procedures will all influence the time at which these inevitable effects occur. Our data with cattle clearly infer that such changes in effectiveness of the male gamete occurring with the passage of time occur at any temperature within the range of body temperature down to -196°C. Temperature influences the rate of change but does not postpone indefinitely the ultimate occurrence.

TABLE 5 Effect of Aging Spermatozoa at 18°C for 24 H on Embryonic Development in *Rana pipiens*--Approximately 19,000 Embryos Were Scored.

		Stage of Development at Embryonic Arrest, %				
Sperm	Unfertilized, %	Cleavage	Blastula	Gastrula	Neurula	Hatching, %
Fresh	4.3	0.7 ± 0.3	0.8 ± 0.2	1.4 ± 0.7	1.6 ± 0.5	91.8 ± 3.2
Aged	4.2	0.6 ± 0.2	1.0 ± 0.7	35.0 ± 4.4[a]	1.9 ± 0.9	57.3 ± 5.6[a]

SOURCE: Hart[29]

[a]$P < 0.01$.

CONCERN WITH GENETIC INFORMATION SYSTEM

The basic cause of the phenomena shown by the data presented here have preoccupied us for some time. In 1952, in a paper published in collaboration with some of those expressing other views today, the senior author wrote, while discussing the problem of embryonic mortality in cattle, that "end-products of spermatozoan metabolism resulting from handling and storage of semen may be at the foundation of the problem, since at least one of the possible end-products, H_2O_2, is known to produce mutations in bacteria and to retard cleavage of *Arbacia* eggs fertilized by spermatozoa exposed to H_2O_2."[53]

Since 1959 my colleagues and I have sought means of testing experimentally the hypothesis that the aging of male gametes ultimately affected adversely their transmission of genetic information. We were attracted first to the technique of microspectrophotometric quantitation of Feulgen staining intensity as reflecting the deoxyribonucleic acid (DNA) content of the individual sperm cell nucleus.[52] Today we recognize much better than formerly its limitations.[60] Nevertheless, such analysis of many individual spermatozoa of many bulls reveals two things about them that are of great interest.

First, the Feulgen positive material in the spermatozoa of semen of bulls varies within samples from the same bull, indicating that not all bull spermatozoa carry the same amount of chromatin or, as we tend to call it now, deoxyribonuclear-protein complex. Such data suggest that the sort of one or another of the pair of each set of homologous chromosomes at meiosis in spermatocytogenesis is not anywhere near 100 percent perfect and as precise as it is considered to be. There appear to be aneuploid spermatozoa containing more and fewer chromosomes produced in a normal distribution pattern about the exact haploid number,[3] and diploid sperm cells are sometimes found.[50]

Second, as shown in Figure 9, the Feulgen-positive staining reaction of stored spermatozoa changes during storage and apparently in about the same quantitative amounts for all spermatozoa, as the distribution surfaces show the same shape after storage as before it. We had for some time considered these nuclear absorbance measurements at 570 µ wavelength to reflect the relative content of DNA. Collaborative experiments using absorbance of ultra-violet light,[10,14,15] the fluorescence of acridine orange[10,43] for DNA measurements, and fast green-staining for nuclear histone content[10] convinced us that, except

for the Feulgen measurements, there was a constancy of means of measurements during storage. Such results suggested that, if changes in the DNA *per se* of spermatozoa occurred during storage, the techniques we were employing were not sharp and precise enough to reveal them. The shift in Feulgen stainability of aged spermatozoa is most likely due to a continuation throughout storage of the formation of protein-to-protein disulfide bonding, which occurs with the condensation of the cell nuclei during maturation in the male reproductive tract.[17] An increased uptake of Feulgen reagents occurs on treating ram spermatozoa with dithiothreitol breaking these disulfide bondings.[22]

Recall that the thalidomide controversy, reflecting an effect of an intake of the drug during pregnancy on the subsequent embryogenic development of humans, was raging at about this time and that our data on the metabolic processes for motility of bull spermatozoa showed that the

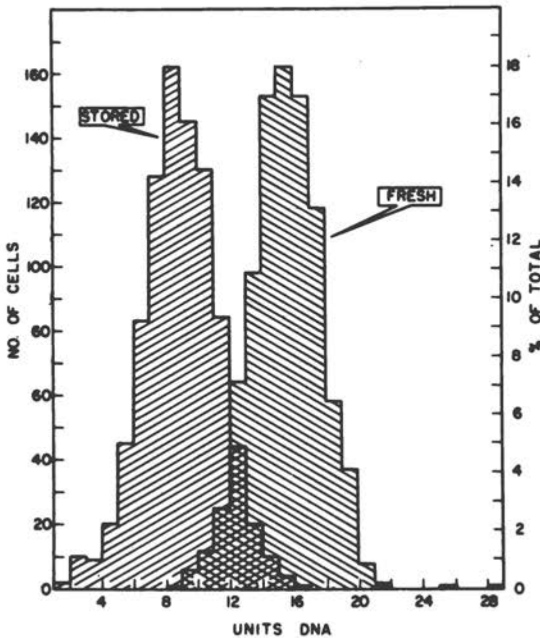


FIGURE 9 The Feulgen-DNA distributions among 900 spermatozoa with a single semen sample before and after 11 days storage at 5°C.[3]

teratogenic effect was unlikely to be due to a simple alteration in energy metabolism.[27] We were keen to evolve some sort of assay of spermatozoa influenced by time or chemicals, or radiation, or what not, reflecting other influences on the sperm cell contribution to the development of a healthy, normal embryo. We tried the effect of X-irradiation on bull spermatozoa to find that Feulgen density reflected a change, but only when the dose in rads was at lethal levels.[51]

A technique that seemed to offer promise for disclosure of change in the genetic information system of the sperm cell was that of determining the melting point of the DNA isolated from spermatozoa before and after experimental treatments of one or another kind.[8,9] The melting profile of isolated DNA was reflective of the proportion of paired purine (guanine and adenine) and pyrimidine (thymine, cytosine, and methylcytosine) bases found in DNA. The ratio of bases are a part of the genetic code peculiar to the species.

Thus, such thermal denaturation curves were determined for the DNA isolated from samples of semen containing 50×10^6 bull spermatozoa stored under a variety of conditions and treated in various ways including exposure to 120,000 roentgens by X-irradiation. The technique for isolation of the DNA involved trypsin digestion of the protein of chromatin leaving the double stranded helical coil to break apart the hydrogen bonding on heating and causing an increase in ultraviolet light absorption. The melting point of $86.4 \pm 0.25°C$ and the profiles of changing hyperchromicity at 258 μ did not change for the purified DNA isolated from those spermatozoa after storage or treatment.

In all these studies we had used a standard solvent for the DNA during heat denaturation studies of 0.15 *M* NaCl and 0.015 *M* sodium citrate. We had found that use of solutions of lower ionic strength had shifted the melting point downward from the almost constant melting temperature of $86.4 \pm 0.25°C$ for the more concentrated solvent used. We believed the change observed to be reflected exactly by the change in the logarithm of the salt concentration, but in fact tested that assumption only with the DNA of fresh spermatozoa. Later that assumption was reevaluated by Graves and his student, Beil,[4,5,6] but not before we had studied other approaches to this problem.

Immunoserological techniques seemed to offer some basis for expectation of differentiating spermatozoan chromatin before and after aging. The antigenic material

used for injection into rabbits was the DNA isolated from spermatozoa by the techniques mentioned above, including the treatment with trypsin for the separation of the DNA from protein.[65] In spite of this treatment, it was later found that the DNA contained about 7 to 8 percent protein. The results of these techniques showed that the DNA-protein complex isolated from aged spermatozoa was, in fact, different from that isolated from fresh spermatozoa.

Using the gel double-diffusion precipitation test disclosed that at least two proteins were involved with the DNA isolated from aged spermatozoa, while only one was found with the isolate from fresh spermatozoa. The antigens of aged spermatozoa when pretreated with ribonuclease and deoxyribonuclease were completely destroyed but not when they were treated with either enzyme alone. The antigenic property of the DNA-protein isolated from fresh spermatozoa was not influenced by either enzyme or the two combined. Immunoelectrophoresis techniques applied to the same materials confirmed the larger number of proteins for the aged spermatozoa.[65] These two studies led Salisbury and Hart[56] to the conclusion that the DNA-protein complex of spermatozoa did change on aging, even though the DNA *per se* could not be shown to have changed by the melting point technique employed earlier.[9]

Graves and his student, Beil, have continued examination of this discrepancy to find that the earlier conclusion by Berchtold *et al.*[9] to the effect that a reduction in the ionic strength of the solvent used for the determination of the temperature at DNA denaturation (Tm) resulted in a decreased temperature directly related to the logarithm of the salt concentration was not correct. By use of the same solvent, but a lower ionic strength of it, they have found differences between the Tm of DNA-protein isolated from fresh and aged spermatozoa. These differences have been reduced by drastic proteolysis with pronase[5] and by repetitive protein extraction with sodium dodecyl sulfate.[6] They strongly support the contention that the spermatozoan chromatin is altered during aging. The change they found is an increase in the intensity of sulfide binding in the chromatin.

Although the studies in our laboratory have not yet provided chemical data proving an alteration of the DNA *per se* during storage, we do have, we believe, irrefutable evidence that there is a potential for such alteration in the metabolic turnover of the DNA in the nucleus of living spermatozoa incubation at 37°C and 7-day storage at 5°C.[26] Spermatozoa have been so stored in the presence of ^{14}C-

labeled glycine with the results shown in Figure 10. Earlier the same authors[25] had studied the incorporation of ^{14}C from glycine labeled either in the 1-C or 2-C position. Most of the carbon labeled in the 1-C (carboxyl) position was found in the respired carbon dioxide, and a major part of that labeled in the 2-C position was found in the isolated thymine base as shown in Table 6.

The parallelism of the biological data on fertility and early embryonic losses in cattle from spermatozoa stored for varying limits of time at each of several temperatures varying through a range of 200°C indicate that the same kinds of biological events occur with the passage of time, temperatures in this wide range influencing only their rate of occurrence. Decreasing the temperature serves to delay senescence.[56]

An important question is whether or not it does so at a fixed and standard rate throughout the entire temperature range. We have earlier concluded that senescence in the cold occurs at rates "far higher than would be characteristic of a metabolic process having a temperature coefficient of 2" (Q_{10}, a doubling for each 10° upward increment in the range of 5° to 37°C), as is the case for energy

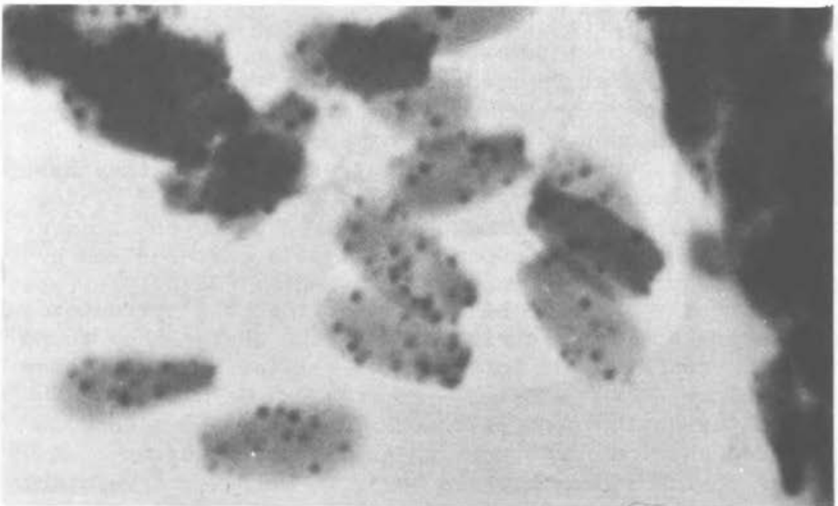


FIGURE 10 Radioautograph of bull spermatozoa incubated for 4 h at 37°C in a diluent containing radioactive glycine.[26]

TABLE 6 Incorporation of radioactivity from Glycine into the Bases of Bovine Sperm DNA After Spermatozoan Incubation for 4 H at 37°C

Base	Glycine 1-C^{14} (dpm)	Glycine 2-C^{14} (dpm)
Adenine	22	30
Cytosine	37	62
Quanine	36	74
Thymine	52	320

SOURCE: Graves and Salisbury[25]

exchange for spermatozoan motility at above freezing temperature.[11] A Q_{10} of 2, inverted for decreasing the temperatures, suggests that at -196°C events in maturation and senescence would occur only $(1/2)^{20}$ as fast as at 4°C. Optimum fertility occurring after 24 h or so of 4° to 5°C storage would thus occur 25 or so centuries later for semen storage at -196°C. The appearance of biological change as measured by fertility and embryonic loss seems not to take so long. Based on such calculations, however, it had been assumed that metabolic chemical changes occurring in semen during relatively short storage at -196°C would be too small to measure. Thus there are not much data on this issue. However, our colleague Graves[24] has data indicating that carbon turnover does occur in frozen bull semen held in a closed system of glass ampules and stored for 1 yr at -196°C. The results are shown in Table 7.

The bull spermatozoa were provided a substrate of randomly labeled fructose after slow cooling to 5°C and before the freezing in ampules to -196°C in N_2 vapor began. After freezing, aliquote numbers of ampules were dropped into a mortar containing the barium hydroxide-zinc sulfate solution used for precipitation of protein and the ampules quickly crushed by pestle to minimize change during such a thawing process. A similar process was used a year

TABLE 7 Organic Acids Produced by Bovine Spermatozoa During 12-Mo Storage at -196°C (n = 2)

Acid	Amount (μ eq/10^9)		Radioactivity (dpm/10^9)	
	Control	Stored	Control	Stored
Valeric	12.01	40.48	9,953	5,780
Butyric	3.69	18.72	6,274	6,436
Propionic	3.16	26.64	56,278	5,975
Acetic	0.35	31.34	43,423	133,589
Formic	32.20	69.33	169,250	97,689
Lactic	69.52	76.38	92,352	57,555
Succinic	116.13	84.59	37.942	51,418
Glycolic	155.44	59.88	81,305	41,245
Oxalic	11.23	55.33	9,522	48,720
Malic	219.45	312.79	36,397	83,332
Citric	5,114.28	906.16	19,489	67,827

SOURCE: Graves[24]

later in preparing samples for analysis.[24] Clearly turnover of the organic acids has occurred. Graves has other of these treated samples under continuous storage and intends to make similar analyses on them later.

After preparation of two recent reviews[57,58] on various aspects of spermatozoan life and aging, the present authors are inclined to the view that final resolution of the problem of the decrease in the fertility of spermatozoa and the increase in the proportion of embryonic and developmental anomalies that they produce in their progeny as they age has been narrowed to the veracity of the genetic message they transmit to their potential progeny.

Perhaps the best evidence demonstrating the effect of spermatozoan aging on the integrity of the genetic message they transmit are the data of R. G. Hart in treatment of the frog embryos produced by aged spermatozoa, a large proportion of which ceased embryonic development at the gastrula stage.[29] He isolated ribonucleic acid (RNA) from frog embryos at the gastrula stage of development from normal gastrulae produced by fresh spermatozoa and from those produced by aged spermatozoa, about 35 percent of which would be expected to cease development at that stage. Some of both RNA samples was subjected to RNase. He injected gastrulae produced by aged spermatozoa with RNA from normal gastrulae and shifted dramatically that portion that went on through development to hatching. The RNA from gastrulae produced by aged spermatozoa or that treated with RNase did not do so well in carrying the development beyond the gastrula stage. The data[29] are presented in Table 8.

Clearly, however, the genome of some of the arrested gastrulae had been affected by the aging of the male gametes that produce them. In spite of the fact that the RNA from embryos produced by fresh spermatozoa was able to assume completion of embryogenesis, many of those that hatched were anomalies of development (Figure 11), suggesting genetic alteration of a very serious nature. That these anomalies are in fact caused by mutations in the aged male genome is suggested by the data shown in Table 9 for mutations observed some years ago in that paragon for genetic study of mutations, *Drosophila melanogaster*.[16,36,46]

SUMMARY AND CONCLUSIONS

This paper has reviewed our research and that of others with a variety of species on the effect of time and the

TABLE 8 The Effect of Injecting Gastrula RNA into Frog Zygotes Arising from the Fertilization of Fresh Eggs by Fresh or Aged Spermatozoa. Gastrula Arrest Was Scored 72 H After Insemination. Percent Represents Mean ± Standard Deviation

Treatment	No. of Eggs Injected	No. Cleaved Normally	% Arrest at Gastrula
Control			
10% Holtfreter's	175	122	5.0 ± 4.1
RNA control gastrulae	100	73	5.6 ± 3.4
Aged			
10% Holtfreter's	250	207	30.9 ± 3.9
RNA control gastrulae	360	270	13.3 ± 3.2[a]
RNA arrested gastrulae	275	196	32.7 ± 3.7
RNA control gastrulae plus RNase	150	115	28.5 ± 2.6
RNA control gastrulae[b]	100	69	14.4 ± 2.7[a]

SOURCE: Hart[29]
[a] $P < 0.01$ that the mean deviations shown in comparison with eggs inseminated by aged sperm and injected with Holtfreter's are due to chance.
[b] RNA treated as described for RNase digested RNA, except RNase digestion was not carried out.

interaction with temperature on spermatozoan fertility and their ability to produce viable offspring. Data painstakingly obtained from planned experiments and from statistical studies of many artificial insemination rec-

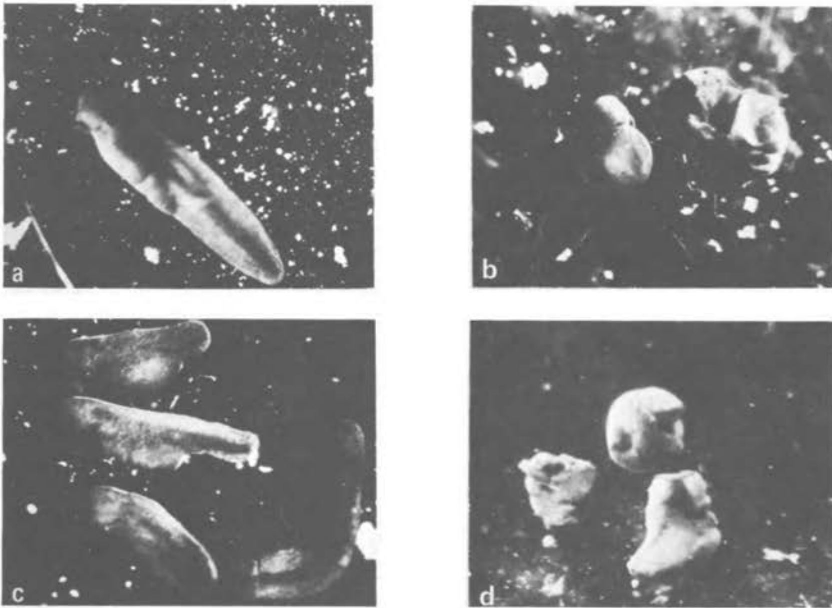


FIGURE 11 Photographs of frog embryos arising from the fertilization of fresh eggs by fresh spermatozoa (A) or by aged spermatozoa and subsequently injected with RNA extracted from normal control gastrula (B,C,D).[29]

ords of cattle indicate that, with the passage of time after the collection of spermatozoa before insemination and at rates dependent largely but not entirely on their handling and the temperature at which the spermatozoa are stored, a pattern of fertility increase, a plateau, and a decrease in fertility follows in sequence. Fertility is defined as the nearest possible estimate to calving rate for large numbers of cattle. Highly but inversely correlated with fertility is the proportion of embryonic and fetal losses exhibited in the population of cows inseminated. The higher the fertility of the bulls producing the spermatozoa, the lower is the proportion of eggs fertilized in the population that exhibit embryonic mortality.

Such patterns of biological behavior occurred for all temperatures of spermatozoan storage over a range of about 200°C, but at a later and later time as the temperature decreased. Evidence was presented indicating that spermatozoan metabolic processes occur during a 1-yr period of storage at the lowest temperature employed. Sequential

TABLE 9 Effect of Age of *Drosophila melanogastor* Spermatozoa on Spontaneous Mutation of Sex-Linked Lethals

Investigators	Sperm Cell Age	Number of Flies	Mutations, %
Rajewsky and Timofeef-Ressovsky[46]	Fresh	13,481	0.104 ± 0.028
	Aged (20 days at 22°C)	18,659	0.263 ± 0.038
Kaufmann[36]	1-2 days (22°C)	3,545	0.141 ± 0.063
	16-17 days (22°C)	3,471	0.317 ± 0.095
	32-33 days (22°C)	5,248	0.572 ± 0.104
Byers and Muller[16]	21 days at 7°C	8,312	0.168
	21 days at 27°C	3,490	0.401

events in manifestations of sperm senescence seem to occur more rapidly at the very low temperatures than would be expected from calculations of 10°-interval temperature coefficients for energy utilization at above freezing temperatures.

Direct observations of events in experiments on spermatozoan aging with laboratory animals confirm the biological inferences drawn from the cattle data. Available evidence points to nuclear rearrangements in spermatozoa during storage and aging. These spermatozoan nuclear changes include measurable changes in immunological reactions, changes in sulfide linkages between DNA and protein, and increased chromosome anomalies in developing embryos they produced. The RNA message transmittal systems of frog spermatozoa is altered by senescence.

DISCUSSION

WILLIAM N. FISHBEIN: It is essential to segregate the experiments presented according to storage temperature. Most describe deterioration of sperm stored at 0 to 37°C, which, of course, only illustrates the reason for freeze-storage. The evidence for deterioration at -196°C seems entirely based on a statistical inference of a moderate decrease in fertility in bovine field-trial AI, where many complicating factors are present. The data showing ^{14}C incorporation into sperm metabolites after storage at -196°C would be convincing only if the incorporation is shown *not* to occur in sperm frozen and thawed without storage, since concentration effects and membrane damage during freeze-thaw may be responsible, rather than storage.

J. K. SHERMAN: Latent cryoinjury, the accentuated loss in functional and structural integrity of cells with time after thawing, is an established phenomenon in cryobiology (Sherman, J. K., Low temperature research on spermatozoa and eggs, Cryobiology *1:*103, 1964). This should not be interpreted, however, as a contraindication for cell banking, especially if the functional activity for which the cells are preserved still is expressed normally after frozen-storage. Reported alterations in biochemical and physiological activities, as well as in ultrastructure of cryopreserved human spermatozoa, therefore, have proved

compatible with retention of their ability to fertilize and induce embryonic development (Sherman, J. K., Synopsis of the use of frozen human semen since 1964: State of the art of human semen banking, Fertil. Steril. *24:*397, 1973). It is possible that such latent cryoinjury may result in a decrease in percent fertility with fresh semen because of an accentuated fall off in motility, but proper timing of insemination relative to ovulation should obviate this relatively innocuous possibility.

BIBLIOGRAPHY AND REFERENCES

1. Blandau, R. J. Ed. 1975. *Aging Gametes, Their Biology and Pathology*. S. Karger, Basel.
2. Asdell, S. A., and Salisbury, G. W. 1941. Viability of spermatozoa in the abdominal epididymis and the failure of motile sperms to fertilize ova. Am. J. Physiol. *132:*791-795.
3. Baker, F. N., Salisbury, G. W., and Lodge, J. R. 1964. The variation of DNA (deoxyribonucleic acid) among spermatozoa within ejaculates of bull. Proc. V Int. Congr. Anim. Reprod. Artif. Insemination, Trento *2:*157-160.
4. Beil, Richard E. 1975. Alterations of chromatin during the *in vitro* storage of bovine spermatozoa. Unpublished Ph.D. Diss., University of Illinois at Urbana-Champaign.
5. Beil, R. E., and Graves, C. N. 1974. Alterations in sperm chromatin during *in vitro* storage. J. Anim. Sci. *39:*198.
6. Beil, Richard E., and Graves, C. N. 1976. Evidence for alterations in the chromatin of bovine spermatozoa during *in vitro* storage. In press.
7. Benitz, A. M., and Graves, C. N. 1972. In vitro aging of mouse spermatozoa. J. Anim. Sci. *35:*236-237.
8. Berchtold, M., and Salisbury, G. W. 1963. Determination of the melting profile of bull spermatozoa DNA. J. Dairy Sci. *46:*636. (Abstract)
9. Berchtold, M., Salisbury, G. W., and Graves, C. N. 1971. Aging phenomena in spermatozoa. V. Thermal denaturation characteristics of deoxyribonucleic acid isolated from fresh and aged bovine spermatozoa. J. Dairy Sci. *54:*1436-1540.
10. Blackshaw, A. W., and Salisbury, G. W. 1972. The

effect of storage *in vitro* on the DNA content of bull spermatozoa. Aust. J. Biol. Sci. *25:*175-184.

11. Blackshaw, A. W., Salisbury, G. W., and VanDemark, N. L. Factors influencing metabolic activity of bull spermatozoa. I. 37, 21 and 5 C. J. Dairy Sci. *40:*1093-1098.
12. Blandau, R. J., and Jordan, E. S. 1941. The effect of delayed fertilization on the development of the rat ovum. Am. J. Anat. *68:*275-291.
13. Blandau, R. J., and Young, W. C. 1939. The effects of delayed fertilization on the development of the guinea pig ovum. Am. J. Anat. *64:*303-329.
14. Bouters, R., Esnault, C., Ortavant, R., and Salisbury, G. W. 1967. Comparison of DNA revealed by Feulgen and by ultraviolet light in rabbit spermatozoa after storage in the male efferent ducts. Nature (London) *213:*181-182.
15. Bouters, R., Esnault, C., Salisbury, G. W., and Ortavant, R. 1967. Discrepancies in analyses of deoxyribonucleic acid in rabbit spermatozoa, involving Feulgen staining (Feulgen-DNA) and ultraviolet light absorption (UV-DNA) measurements. J. Reprod. Fertil. *14:*355-363.
16. Byers, Helen, and Muller, H. J. 1952. Influence of aging at two different temperatures on the mutation rate in mature spermatozoa of *Drosophila melanogaster*. Genetics *37:*570-571.
17. Calvin, H. I., and Bedford, J. M. 1971. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. J. Reprod. Fertil. *13*(Suppl. 1): 65-75.
18. Cartledge, S. L., and Blakeslee, A. F., 1934. Mutation rate increased by aging seeds as shown by pollen abortion. Proc. Natl. Acad. Sci. *20:*103-110.
19. Cochrane, W. G. 1943. Analysis of variance for percentages based on unequal numbers. J. Am. Stat. Assoc. *38:*287-301.
20. Dungay, N. S. 1913. A study of the effects of injury on the fertilizing power of sperm. Biol. Bull. *25:*213-260.
21. Dziuk, P. J., and Henshaw, G. 1958. Fertility of boar semen artificially inseminated following *in vitro* storage. J. Anim. Sci. *17:*554-558.
22. Esnault, C. 1973. Reactivation of the Feulgen reaction of ram spermatozoa by dithiothreitol. J. Reprod. Fertil. *32:*153-157.

23. First, N. L., Stratman, F. W., and Casida, L. E. 1963. Effect of sperm age on embryo survival in swine. J. Anim. Sci. *22:*135-138.
24. Graves, C. N. 1968. Metabolic activity of spermatozoa at -196°C. J. Anim. Sci. *27:*1191.
25. Graves, C. N., and Salisbury, G. W. 1963. Incorporation of radioactivity from glycine into the DNA of mature bovine spermatozoa. Fed. Proc. *22:*2.
26. Graves, C. N., and Salisbury, G. W. 1966. Spermatozoan DNA turnover during aging as measured by radioautography. Fed. Proc. *25:*315.
27. Graves, C. N., Salisbury, G. W., and Lodge, J. R. 1964. Effect of thalidomide on bovine spermatozoan metabolism. J. Anim. Sci. *23:*906.
28. Hammond, John, and Asdell, S. A. 1926. The vitality of the spermatozoa in the male and female reproductive tracts. Br. J. Exp. Biol. *4:*155-185.
29. Hart, R. G. 1967. The effect of spermatozoan senescence on embryonic mortality in *Rana pipiens* and *Arbacia punctulata*. Unpublished Ph.D. Diss., University of Illinois at Urbana-Champaign.
30. Hart, R. G., and Salisbury, G. W. 1967. The effect of sperm age on embryonic mortality in the frog. Fed. Proc. *26:*545.
31. Hertwig, G. 1913. Parthenogenesis bei Wierbeltieren hervorgerufen durch artsfremden radiumbestrahlten Samen. Arch. Mikrosk. Anat. *81:*87-218.
32. Hertwig, O. 1911. Die Radium Krankheit tiericher Keimzellen. Arch. Mikrosk. Anat. *77:*79-164.
33. Hertwig, P. 1911. Druch Radiumbestrahlung hervorgerufene Veranderungen in den Kernteilungsfiguren der Eier von Ascaris megalocephala. Arch. Mikrosk. Anat. *77:*301-312.
34. Hertwig, P. 1913. Das Verhalten des mit Radium bestrahlten Spermachromatins im Froschei. Arch. Mikrosk. Anat. *81:*173-182.
35. Hertwig, P. 1916. Durch Radiumbestrahlung verurschte Entwicklung von halbkernigen Triton und Fisch Embryonen. Arch. Mikrosk. Anat. *87:*63-122.
36. Kaufmann, B. P. 1947. Spontaneous mutation rate in *Drosophila*. Am. Nat. *81:*77-80.
37. Koefoed-Johnsen, H. H., Pavlock, A., and Fulka, J. 1971. The influence of the aging of rabbit spermatozoa *in vitro* on fertilizing capacity and embryonic mortality. J. Reprod. Fertil. *26:*351-356.
38. Laing, J. A. 1948. Infertility in cattle associated with death of ova at early stages after fertilization. J. Comp. Pathol. Ther. *59:*97-108.

39. Laing, J. A. 1952. Early embryonic mortality. Proc. II Int. Congr. Physiol. Pathol. Anim. Reprod. Artif. Insemination. Vol. II: Pathol. Reprod. pp. 17-34. Copenhagen.
40. Lodge, J. R., Ax, R. L., and Fechheimer, N. S. 1974. Chromosome aberrations in embryos from *in vivo* aged chicken sperm. Poult. Sci. *53:*1816-1819.
41. Lodge, J. R., Fechheimer, N. S., and Jaap, R. G. 1971. The relationship of *in vivo* sperm storage interval to fertility and embryonic survival in the chicken. Biol. Reprod. *5:*252-257.
42. Medes, Grace. 1917. A study of the causes and the extent of variations in the larvae of *Arbacia punctulata*. J. Morphol. *30:*317-432.
43. Miller, O. C., and Blackshaw, A. W. 1968. The DNA of rabbit spermatozoa *in vitro* and its relation to fertilization and embryo survival. Proc. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1921-1293, Paris.
44. Nalbandov, A., and Card, L. E. 1943. Effect of stale sperm on fertility and hatchability of chicken eggs. Poult. Sci. *22:*218-226.
45. Navaskin, M. 1933. Origin of spontaneous mutations. Nature (London) *131:*43.
46. Rejewsky, B. N., and Timofeeff-Ressovsky, N. W. 1939. Hohenstrahlung auf den mutationsrate von *Drosophila melanogaster*. Z. Indukt. Abstamm-Vererbungsl. *77:*488-500.
47. Salisbury, G. W. 1962. Ionic and osmotic conditions in relation to metabolic control. In: *Spermatozoan Motility,* pp. 59-87. AAAS, Washington, D. C.
48. Salisbury, G. W. 1967. Aging phenomena in spermatozoa. III. Effect of season and storage at -79° to -88°C on fertility and prenatal losses. J. Dairy Sci. *50:*1683-1689.
49. Salisbury, G. W. 1968. Fertilizing ability and biological aspects of sperm storage *in vitro*. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris. *2:*1189-1204.
50. Salisbury, G. W., and Baker, F. N. 1966. Frequency of occurrence of diploid bovine spermatozoa. J. Reprod. Fertil. *11:*477-480.
51. Salisbury, G. W., and Berner, Anna S. 1963. X-irradiation of Feulgen-DNA of sperm cells. J. Anim. Sci. *22:*865.
52. Salisbury, G. W., Birge, W. J., De LaTorre, L., and Lodge, J. R. 1961. Decrease in nuclear Feulgen-positive material (DNA) upon aging in *in vitro* sto-

rage of bovine spermatozoa. J. Biophys. Biochem. Cytol. *10:*353-359.

53. Salisbury, G. W., Bratton, R. W., and Foote, R. H. 1952. The bull as one cause of delayed returns to service in artificial insemination. J. Dairy Sci. *35:*250-255.
54. Salisbury, G. W., Bratton, R. W., and Foote, R. H. 1952. The effect of time and other factors on the nonreturn to service estimate of fertility in artificial insemination of cattle. J. Dairy Sci. *35:*256-260.
55. Salisbury, G. W., and Flerchinger, F. H. 1967. Aging phenomena in spermatozoa. I. Fertility and prenatal losses with use of liquid semen. J. Dairy Sci. *50:*1675-1678.
56. Salisbury, G. W., and Hart, R. H. 1970. Gamete aging and its consequences. Biol. Reprod. *2*(Suppl. 2):1-13.
57. Salisbury, G. W., and Hart, R. G. 1975. Functional integrity of spermatozoa after storage. BioScience *25:*159-165.
58. Salisbury, G. W., Hart, R. G., and Lodge, J. R. 1976. The fertile life of spermatozoa. Perspect. Biol. Med. *19:*213-230.
59. Salisbury, G. W., and Lodge, J. R. 1962. Metabolism of spermatozoa. In: F. F. Nord, ed. *Advances in Enzymology 24:*35-104. Interscience Publishers, Wiley & Sons, New York.
60. Salisbury, G. W., Lodge, J. R., and Baker, F. N. 1964. Effects of age of stain, hydrolysis time, and freezing of the cells on the Feulgen-DNA contents of bovine spermatozoa. J. Dairy Sci. *47:*165-168.
61. Salisbury, G. W., and VanDemark, N. L. 1961. *Physiology of Reproduction and Artificial Insemination of Cattle.* W. H. Freeman and Co., San Francisco and London.
62. Shaver, E. L., and Martin-DeLeon, P. A. 1975. Effects of aging of sperm in the female and male reproductive tracts before fertilization on the chromosome complement of the blastocysts. In: R. J. Blandau, ed., *Aging Gametes, Their Biology and Pathology.* S. Karger, Basel.
63. Soderwall, A. L., and Blandau, R. J. 1941. The duration of the fertilizing capacity of spermatozoa in the female genital tract of the rat. J. Exp. Zool. *88:*56-64.
64. Soderwall, A. L., and Young, W. C. 1940. The effect of aging in the female genital tract on the fertilizing capacity of guinea pig spermatozoa. Anat. Rec. *78:*19-29.

65. Todorovic, R. A., Graves, C. N., and Salisbury, G. W. 1969. Aging phenomena in spermatozoa. IV. Immunoserologic characterization of the deoxyribonucleic acid-protein extracted from fresh and aged bovine spermatozoa. J. Dairy Sci. *52:*1415-1421.

66. Young, W. C. 1931. A study of the epididymis. III. Functional changes undergone by spermatozoa during their passage through the epididymis and vas deferens. J. Exp. Biol. *8:*151-162.

MOLECULAR FACTORS AFFECTING GAMETIC INTEGRITY DURING STORAGE AT LOW TEMPERATURES

J. T. Mullhaupt

INTRODUCTION

Routine cryopreservation of mammalian spermatozoa is a practical reality. Enough is known of the relevant chemical and physical factors that sperm can be frozen, stored, and thawed without excessive damage. In spite of this, data presented in this volume provide evidence both for and against gametic injury in long-term cryostorage. These data express the problem in biological terms; in this paper, the problem will be examined on a molecular scale. The purpose here is (1) to identify additional chemical and physical factors that may affect cryopreservation and (2) to contribute to a better fundamental understanding of the events that occur and the empirically successful methods for controlling them. To achieve these ends, the processes that can occur at cryogenic conditions will be examined from two points of view: the energetics of such processes and the molecular mechanisms involved.

ENERGETICS OF PROCESSES AT CRYOGENIC CONDITIONS

Effect of Temperature on Rates

At the typical cryostorage temperature (77°K), the rates of biological processes are so slow that they are usually taken to be zero. These processes involve relatively complex series of chemical and physical processes, each of which will be slowed with decreasing temperatures. The rates of these individual molecular processes may not be negligible, however, The effect of temperature can be estimated by assuming that the rate constant is described by the Arrhenius law. In Table 1, the calculated ratios

TABLE 1 Effect of Temperature on Rate Constant

Activation Energy (kcal/mol.)	Rate Constant Ratio: k(T)/k(298°K)			
	277°K (4°C)	195°K (-78.5°C)	143°K (-130°C)	77°K (-196°C)
10	2.6×10^{-1}	8.4×10^{-5}	5.4×10^{-9}	--
5.0	4.9×10^{-1}	7.4×10^{-3}	5.1×10^{-5}	7.8×10^{-12}
2.0	7.2×10^{-1}	1.09×10^{-1}	1.25×10^{-2}	1.60×10^{-5}
1.0	8.2×10^{-1}	2.7×10^{-1}	7.7×10^{-2}	2.0×10^{-3}
0.5	8.8×10^{-1}	4.2×10^{-1}	1.92×10^{-1}	2.3×10^{-2}
0.2	9.1×10^{-1}	5.5×10^{-1}	3.3×10^{-1}	9.8×10^{-2}

Assumptions:

1. Arrhenius law describes rate constant.
2. Preexponential factor proportional to temperature.

of rate constants at various subambient temperatures to that at 298°K are presented for different values of activation energy. The assumptions used are indicated; these were chosen to provide better estimates over the large temperature range.[1] Values of activation energy were selected to represent both chemical and physical processes.

The rate constant ratios illustrate the expected decline in rate with decreasing temperature or increasing activation energy (ΔE). For ΔE = 2.0 kcal/mol, the rate constant ratio decreases slowly with temperature, then rapidly between 143° and 77°K to a value that could be measured over several months. Similarly, the rate constant ratio at 143°K, for example, decreases with increased ΔE, at first slowly, then rapidly for $\Delta E \geq 5.0$ kcal/mol.

To gauge what values of rate constant ratio are measurable and therefore are relevant to long-term storage, a ratio of reaction times can be estimated from the inverse of the rate constant ratio. Thus, a rate constant ratio of 5×10^{-5} corresponds to a time ratio of 2×10^{4}. If the characteristic reaction time at 298°K is 10 min, then the time to reach the same extent of reaction at the low temperature is about 5 mo. Thus, during months of storage, one can expect processes with $\Delta E \leq 2.0$ kcal/mol to occur to an appreciable extent at 77°K.

Equilibrium Distribution of Energy

Thermally activated reactions are driven by energy extracted from molecular collisions. To be effective, the available energy must exceed certain threshold values. In a gas at equilibrium under isothermal conditions, a relatively small fraction of the molecules have high energies. For a Boltzmann distribution of translational energy,[2,3] the fractions of molecules with energies in excess of selected values have been calculated at three temperatures. These data are shown in Table 2. For comparison, the energy values used were selected from the activation energy values in Table 1.

As illustrated in Table 2, the fraction of molecules with excess energy at each temperature decreases with increasing energy threshold, slowly at first, then rapidly. For a fixed energy, the fraction decreases with temperature in a similar manner. Some measure of what is a significant fraction of energetic molecules is given by the values for 5.0 kcal/mol at 298° and 195°K. Thus, at 77°K significant fractions obtain only below energy values of 2.0 kcal/mol,

TABLE 2 Effect of Temperature on Energy Distribution

Energy (kcal/mol)	Fraction of Molecules with Energy $\geq E$		
	298°K	195°K	77°K
5.0	4.2×10^{-4}	1.0×10^{-5}	4.2×10^{-14}
2.0	9.0×10^{-2}	2×10^{-2}	9×10^{-6}
1.0	3.4×10^{-1}	1.63×10^{-1}	5×10^{-3}
0.5	6.5×10^{-1}	4.9×10^{-1}	9.0×10^{-2}

Assumptions:

1. Boltzmann distribution.
2. Translational energy only.

and these fractions will be considerably reduced in condensed phases.

From energy considerations, one can see that molecular processes are slowed, but not stopped, at cryopreservation temperatures. Processes with relatively low activation energies can be driven thermally at 77°K, although the rates will be slow by conventional measures of reaction time. However, the increases with temperature in rate constant and in the fraction of energetic molecules illustrated in Tables 1 and 2 show why most chemical and physical processes are expected to occur on cooling and warming rather than during storage.

MOLECULAR PROCESSES AT CRYOGENIC CONDITIONS

Chemical Reactions

Although chemical reactions, at 195°K for example, are not unusual, reactions at or below 90°K are not normally considered in cryobiology. Examples of some of the reactions that do occur are outlined in Table 3 and will be described briefly in what follows. The purpose of this cursory review is to demonstrate that reactions involving covalent bonds

TABLE 3 Chemical Reactions at Cryogenic Temperatures

I. Radiation-Induced Reactions

A. Polymerization of solid CH_4 at 77°K by γ rays[4]

B. Addition of carbene to multiple bonds[5]

1. Photolysis of CH_2N_2 in solid matrices
2. Additions to:
    a. CO or C_2H_4 in N_2 at 20°K
    b. C_2H_2 in Ar at 4°K

C. Formation and trapping of radical ions in aqueous or organic glasses at 77°K; Xrays or γ rays[6]

II. Reactions of Atoms with Molecules

A. Dehydrogenation of C_2H_6 or C_2H_4 in liquid Ar at 87°K by O atoms generated photolytically *in situ*[7]

B. Oxidative addition of O atoms from molecular beam to *trans*-2-butene in solid solution (C_3H_8) at 77°K[8]

III. Reactions of "Energy-Rich" Molecules

A. Addition of $(NO^+)(NO_3^-)$ to isobutylene in alkane solutions at 77°K[9]

B. Chemical detonation of O_2-hydrocarbon solutions at 90°K[10]

---

can take place at these low temperatures and that from these examples evidence can be obtained of diffusion under cryogenic conditions.

The reactions outlined in Table 3 have been grouped to show three ways to supply energy other than by thermal activation. (The superscript numbers used in the table are literature references.) The use of radiation to initiate cryochemical reactions is a common and convenient method that can be used for a variety of reactions. Irradiation of solid CH_4 by γ rays leads to the formation of a polymer of composition $C_{20}H_{40}$, possibly by a carbonium-ion chain reaction initiated by a single γ ray. The photolysis of CH_2N_2 in N_2 or Ar matrices leads to the formation of ketene, cyclopropane, or allene, depending on the substrate. These studies show that at extremely low temperature an active species like carbene can diffuse some distance ($\sim$ 10 Å) in order to react. Whelan's review[6] describes the irradia-

tion of systems of more direct interest to cryobiology. In systems with hydrogen-bonding, such as neutral ice, both trapped electrons and OH have been observed. Organic glasses comprising molecules having low-lying orbitals have also been studied, including polycrystalline amino acids in which the species $R\dot{C}(O^-)OH$ is formed. Radical cations and anions can be formed from organic sulfur compounds. In the case of thioureas, these ions can be trapped at 4.2°K, but not at 77°K, indicating relatively rapid recombination rates at the higher temperature.

Reactive species can also be used to initiate cryochemical reactions. Examples of oxygen-atom reactions are given in Table 3 for both liquid and solid states. In the liquid case, O atoms from O_3 remove hydrogens to form water as well as unsaturated hydrocarbons; in the solid, epoxybutanes, butanone, and isobutyraldehyde are formed. Metastable species such as an ionic, reactive form of N_2O_4 can be generated *in situ* for addition to olefins, or such as O_3, which can trigger explosive oxidations. The examples shown in Table 3 show that, once initiated, chemical reactions can occur at very low temperatures, even in solids. Migration of chemical species can also take place. The facility with which radiation can initiate reactions indicates that biological samples should be protected from radiation during cryopreservation. Data presented by Prof. Foote at the conference provide experimental evidence for the degradative effects of visible radiation on bovine spermatozoa.

Physical Transformations

The events outlined in Table 4 have been cited to illustrate the physical transformations that can occur in condensed phases at cryogenic temperatures. Although it is useful as a rule of thumb to regard 123°K as "absolute zero" in biology, there are a variety of physical transformations that occur well below this temperature and that alter molecular environments significantly. The most obvious examples are the phase transitions of simple molecules such as O_2. The temperatures and heats of two transitions are given in the Table 4 that characterize intermolecular rearrangements in pure, condensed O_2. In solid solutions with Ar containing 1-20 percent O_2, athermal martensitic transformations from hexagonal to cubic close-packed phases have been observed at 80°-10°K, depending on the O_2 content. These remarkable transitions are more

TABLE 4 Physical Events at Cryogenic Temperatures

I. Phase Transformations of Simple Molecules (e.g., Oxygen)
   - A. Liquid-solid; first order, relatively rapid; 54.50°K, 106 cal/mol[11]
   - B. Solid-solid: first or higher order, fast or slow
     1. Thermal transformations: 43.77°K, 178 cal/mol[12]
     2. Athermal (martensite) transformations: Ar-O_2 solutions[13]

II. Transformations of Complex Molecules and Polymers
   - A. Glass transitions: higher order, gradual
     1. Ethanol: 90°-96°K[14]
     2. *trans*-2-hexene: 85°K[15]
     3. poly(dimethylsiloxane): 150°K[16]
   - B. Crystallization of glasses
   - C. Intramolecular rearrangements
     1. Higher order, small thermal effects
     2. Amorphous polystyrene[17]
        - a. Phenyl group motions: 38°-48°K
        - b. "Crankshaft" motion: 130°K

III. Molecular Reorientations and Diffusion
   - A. Site motions: rotation of H_2O trapped in Ar at 4.2°K[18]
   - B. Diffusion of energetic species
     1. Electrons and ions: irradiation of solids at 4.2°K[19]
     2. Radicals and atoms: reactions in solid matrices[6,20]
     3. Proton tunneling: proton transfer reactions at 150°K[21]

---

commonly observed in metals, are very rapid, and can be induced either thermally or mechanically.

Of more direct relevance to cryopreservation are the transformations characteristic of complex molecules and polymers at low temperatures. Many of these species

exhibit glass transitions, which are second order or higher thermodynamically. Three examples of unusually low glass-transition temperatures are shown in Table 4. Biological samples to be stored cryogenically typically contain three types of glass-forming substances; aqueous solutions of electrolytes, hydrogen-bonded molecules, and polymers. In the glass, certain molecular arrangements are "frozen in," the extent and kind depending on the cooling rate. The approach to equilibrium (crystallization) may continue very slowly. Experimental studies of devitrification at storage temperatures appear to be lacking, owing to relatively short storage times and the more immediate problem of avoiding crystallization on warming. For very long storage times (years), isothermal crystallization of glasses at cryostorage temperatures will become more important and merits experimental investigation.

Closely related to the glass transitions are the intramolecular rearrangements observed with many polymers below the glass-transition temperatures. These are thermodynamic transitions of second order or higher and are characterized by small (often negligible) thermal effects. The transitions are most easily detected by nuclear spin resonance or dynamic mechanical measurements, and they correspond to internal polymer chain motions rather than those of entire molecules. Amorphous polystyrene has been well studied. Below its glass-transition temperature (373°K), three types of chain motions have been observed: torsional phenyl group vibrations at 325°K and the two other motions cited in Table 4. Of special interest is the "crankshaft" motion attributed to rotations about single bonds in the grouping $(CH_2)_4$, observed at 150°K in polyethylene.[17]

Glass transitions and intramolecular rearrangements of polymers have special significance for cryopreservation. One must expect motions of biological polymer chains at storage temperatures, especially rotation and flexing of side chains. Such changes in configuration may be coupled or hindered and could alter local chemical environments. In this way, the local configuration and composition at specific sites on a membrane surface or within a cell might vary with time during storage.

In addition to transformations of phases, motion and transport of individual species have also been observed at cryogenic temperatures. A dramatic illustration of molecular reorientation is cited in Table 4. Rotational lines observed in the infrared were observed, indicating nearly free vib-rotors for H_2O, HDO, and D_2O trapped in

solid Ar at 4.2°K. As one would expect, diffusion at low temperatures is more difficult in solid than in liquid phases and is most often observed with energetic species. Kasai's review[19] describes the irradiation of Ar matrices containing small amounts of electron donors (such as Na atoms) and electron acceptors (such as HI or furan). Electrons generated by photoexcitation with visible or near ultraviolet light migrated distances of 7-8 Å before being captured and dissociating covalent bonds in the host species. In some cases, the products moved apart as evidenced by the character of the electron spin resonance signals used to detect them.

Other evidence for diffusion in condensed phases at cryostorage temperatures is derived from chemical reactivity. Whelan's review[6] cited earlier in this respect, also describes results from experiments in which neutral ices were irradiated with γ rays. H atoms could be trapped and detected at 20°K, but not at 77°K; it was concluded that H atoms were free to diffuse in the solid at 77°K. In a separate study,[20] the products observed when H and D atoms reacted with solid propylene at 77°K indicated that isopropyl radicals were rather free to diffuse in the solid. Finally, indirect evidence for quantum mechanical tunneling was observed in a study of proton transfer in the liquid phase.[21] In ethanol-ether solutions at -60° to -124°C, the kinetics of proton transfer from 4-nitrobenzyl cyanide to the ethoxide ion were interpreted in terms of quantum mechanical effects at the lower temperatures. Thus, although the evidence for transport is qualitative rather than quantitative, diffusion of small or energetic species can occur at low temperatures, even in solids. Electron transfer and quantum mechanical tunneling of protons have special significance for cryopreservation of biological substances, such as proteins, for example.

ADDITIONAL EVENTS IN EXTENDED CRYOPRESERVATION

The present understanding of the factors affecting cryopreservation is based upon a considerable body of previous research that has been the subject of periodic reviews.[22-25] Both chemical and physical effects have been examined, primarily from a biological point of view, including ice formation (both mechanical effects and concentration of solutes), osmotic effects, ionic strength and pH changes, and temperature changes (both extent and rate). It has also been recognized[25] that these factors are interrelated; not only

do they act in combination, but some can alter the susceptibility of biological systems to other, subsequent stresses. The purpose of the following discussion is to examine events, not discussed or emphasized in previous reviews, that occur on a molecular scale and may have biological consequences. For reference, these are outlined in Table 5.

Events Occurring During Cooling

Most earlier studies have focused on the cell wall as a protective enclosure or a barrier separating phases. Ho, Doebbler, and Rinfret[26] suggested that the action of cryoprotective agents involves the binding of these species to sensitive sites on the cell membrane. Greiff and Seifert[27] presented experimental evidence for this hypothesis in a study of mucopolysaccharide sites on red cell membranes protected by glycerol and dimethyl sulfoxide solutions. In addition to chemisorptive effects, physical adsorption on

TABLE 5 Additional Events in Extended Cryopreservation

I. Events Occurring During Cooling
   A. Adsorption of ions or cryoprotective agents on cell walls
   B. Separation of proteins and lipids in cell membrane
   C. Mesophase transitions of lipid regions
   D. Conformation changes: secondary, tertiary

II. Events Occurring During Storage
   A. Crystallization of glassy regions
   B. Conformation changes: tertiary
   C. Electron transfer and proton tunneling

III. Events Occurring During Warming
   A. Reversal of cooling events (listed above)
   B. Chemical reactions
      1. Rearranged molecular segments
      2. Increase in chemical rate constants faster than increase in physical rate constants

cell walls is highly probable; both the increasing solute concentrations and the decreasing temperature provide effective driving forces. Adsorbed layers may alter membrane properties.

A recent review of the physical state of cell membranes and its relation to function[28] described evidence for lateral separation of proteins and lipids within the cell membrane from electron microscopic examination of freeze-fractured membranes. Although these lateral phase transitions occur above 0°C, they bring about the association of globular proteins and ordering of lipid regions, resulting in changes in local environment composition and molecular arrangement. Concurrent with this separation are physical changes in the lipid regions. Changes between liquid and crystalline states have been observed using calorimetric, X-ray diffraction, and electron spin resonance (spin labeling) techniques.[28]

Individual lipids exhibit thermotropic mesomorphism, and mixtures of them with water and cholesterol form lyotropic mesophases near ambient temperature. The latter exist in biological systems, and their relationship to function, especially in the cell membrane, has recently become the subject of intense study.[28,29] Water induces the formation of lamellar and hexagonal mesophases, while cholesterol organizes lipids into single phases and prevents crystallization. The naturally occurring cholesterol is thought to control the fluidity of the hydrocarbon chains of the lipids and provide an ordered system that is stable over a fairly wide temperature range. Experimental evidence has been obtained using model systems and some microorganisms by means of thermal, spectroscopic, and structural measurements.[29] In addition to transitions between liquid and solid phases, mesophase transitions involving these "liquid crystals" have also been observed.

Lipid mesophases are found in many biological systems, often crucially associated with life processes, e.g., in myoglobin, cell membranes, mitochondria, and the Golgi apparatus.[30] The explicit role of mesophases, and the transitions they undergo, is still controversial, but changes in the physical states of these mesophases can cause the death of the organism. A relation has been established between mesophase state and β-glucoside transport in cell membranes.[28] Mesophase state may control the cell permeability to ions in the cell suspension during cooling. The other effects in sperm cryopreservation are likely to be indirect. Mesophase transitions can change the molecular arrangement in the lipid regions both in the membrane and

within the cell. Whether a given arrangement is quenched in or bypassed on cooling may determine the local chemical environment during storage or warming.

Events B, C, and D of Part I in Table 5 deal with physical effects that lead to changes in arrangement and order on an increasingly smaller scale: separation of regions, transitions within a region, and finally changes in molecular orientation. Calorimetric, spectroscopic, and X-ray diffraction studies of lipids in the solid, as well as the liquid, state[29] provide evidence for intramolecular rearrangements like those cited in Table 4 for polymers. These are associated with motions of the hydrocarbon chains of the fatty acid residues. Chain motions persist on cooling solid lipids; the more unsaturated the hydrocarbon chain, the lower the temperature at which the chains "freeze." Water and cholesterol tend to lower these temperatures to below ambient.

The possibility that conformation changes in enzymes on cooling could effect changes in specific catalytic activity was examined by Tappel.[31] The kinetics of the peroxidase-catalyzed oxidation of guaiacol in CH_3OH from +25° to -25°C were interpreted in terms of a distribution of conformational isomers of the enzyme that changed with temperature. The need to consider other factors, such as the association of enzymes (see I.B in Table 5), was also recognized. In frozen systems involving enzyme catalysis, the concentration of ionic solutes accompanying cooling can have the greatest effect. Salts that denature proteins also deactivate enzymes. Thus, the cooling step in cryopreservation can induce conformation changes in the larger molecules of biological systems, whether by temperature change or by solute concentration. Local environments in both lipid and protein regions may change as a result.

Events Occurring During Storage

Since cooling is crucial to achieving cryopreservation, experimental studies have been concentrated on this step, with somewhat less attention to the warming step. The anticipation of extended storage periods requires consideration for this step of physical and chemical processes analogous to those described under "Molecular Processes at Cryogenic Conditions" in this paper. Crystallization of glassy regions in biological systems is to be expected, but at very slow rates. Measurement of these rates would be difficult and are worth considering only because of the catastrophic effects of devitrification.

Conformation changes in the form of intramolecular rearrangements can be expected during storage, because the rapid cooling rates quench temporarily conformations characteristic of higher temperatures. Whether these driving forces can surmount the energy barriers described in Table 1 for appreciable rates has not been tested experimentally. As previously discussed, motions of polymer chains are possible, specifically of side groups or rotation about single bonds. The following studies based on experiments near ambient temperatures suggest that such rotation is likely at cryostorage temperatures.

Hindered rotation about single bonds is a basic mechanism by which proteins change conformation. In the calculation of conformational energies, Poland and Scheraga[32] have used experimental values of rotational barriers about single bonds in ethanelike molecules that have values from < 1 kcal/mol to 4.0 kcal/mol. These have been supplemented by other barriers arising from steric effects, as well as hydrogen and hydrophobic bonding. Such calculations have been used primarily to predict helix-coil transition energies for polyamino acids at ambient temperatures in water. Enthalpies for these transitions in the range -1.2 to +1.1 kcal/mol of residue have been reported.[33,34] The magnitudes of these values provide estimates of the minimum energy barriers expected at cryogenic temperatures.

Experiments with polymer solutions provide evidence that energy barriers determined for relatively small molecules may be extrapolated to those of biological interest. The purpose was to investigate the extent to which rotation about a single bond is hindered by being incorporated into the backbone of a polymer.[35] "Crankshaft" type motions were anticipated, which require correlation of several motions, and an increase in the energy barrier was anticipated but not observed. Rotations about the amide bond in relatively small amides were compared with those in polyamides. Neither the activation energies for rotation measured by nuclear spin resonance nor the cis-transisomerization rates differed significantly for the two types of molecules. Thus conformation changes at cryostorage temperatures seem possible--side-chain rotations in particular.

In Table 5, Event II.C, two simple "chemical" reactions are suggested as possible in unperturbed biological systems, most likely between nearby specific groups if they occur at all. As previously discussed, these reactions, as well as more complex ones, are readily initiated by radiation at very low temperatures. Exposure to ionizing

radiation must of course be avoided, but the evidence presented by Professor Foote at the conference indicates that visible light may also be effective.

Events Occurring During Warming

On warming, one expects the reversal of those physical processes that occurred during cooling, including those outlined in Table 5. The rate of warming for cryostored spermatozoa is susually fast so that ice crystallization can be avoided. It is unlikely that a sperm sample on warming follows the same thermodynamic path that it did on cooling. Not only will the temperature-time program and heat transfer be different, but, for example, the system may also have been altered during storage by conformation changes. During rapid warming, thermodynamic equilibrium will not be achieved. Thus, the reversal of a series of conformation changes can lag behind the temperature rise.

It is proposed that the physical and chemical changes that occur during cooling and storage can also alter the composition and arrangement of local environments in such a way that chemical reactions might take place on warming that were not possible or anticipated on cooling. For example, it is suggested that these reactions could involve specific groups brought into proximity by rearrangements of molecular segments on protein chains. In the following, the conditions favoring chemical reactions at low temperatures during warming will be discussed, and some evidence from the literature will be cited.

The relative kinetics of physical and chemical processes on the molecular scale can be viewed in terms of the activation energies, which are frequently higher for chemical reactions. As a result, the rate constants for chemical steps decrease more rapidly with temperature on cooling, so that reactions can often be quenched readily by fast cooling. However, during rapid warming, the rate constants for chemical steps will increase faster with temperature than those for physical processes. For this reason, two specific groups, each on a separate protein side chain, might interact chemically before a series of rotations could move them apart.

The factors favoring reactions on warming can be identified more specifically by examining the results of experimental investigations of chemical kinetics in partially frozen systems. Recently, Pincock[36] reviewed reactions in frozen systems and developed a phenomenological description

of fairly general validity. This formalism can account for kinetic effects observed when liquid phase reaction systems are cooled to the region of the phase diagram between the liquidus and solidus curves, where both solid solvent and concentrated solution phases are present. Examples of rate increases in frozen systems cited included the base-catalyzed hydrolysis of penicillin, the hydroxyammonolysis of some amino acid esters, Tappel's work,[31] and investigations by Pincock and his associates of nonaqueous systems.

The occurrence of maxima in rates on cooling, changes in kinetic order, and inhibition by inert solutes could be accounted for by a simple model. It assumes that reaction occurs only in the liquid phase and that the observed rate can be described by solution kinetics derived at ambient temperature, if the temperature dependence of the rate constant and the change in reaction volume are accounted for. Thus, the observed rate depends on reactant concentrations, which in frozen systems change both by reaction and by reduction of liquid phase volume. In dilute solutions, it is usually the pure solvent that solidified; in concentrated solutions, solid mixtures can be expected.

This model has been successfully tested with frozen systems and suggested for application to molten systems above ambient temperature. It is proposed here that it is also appropriate to describe conditions for reactions on warming cryostored biological samples. As melting occurs, the first-formed liquid is likely to have relatively high concentration of solutes, corresponding to some eutectic composition. In this case, both thermal and concentration driving forces act together, favoring reaction with species dissolved in or in contact with the liquid phase.

Some experiments reported in the literature might be explained in these terms. Levitt's review[37] of winter hardiness in plants outlines the sulfhydryl-disulfide theory of frost injury and resistance, in which injury is attributed to denaturation of proteins on warming. Denaturation is thought to occur by the unfolding of proteins due to strong disulfide bonds formed as a result of freezing to temperatures in the range 0° to -30°. Levitt proposed that disulfide bonds were formed during freezing by oxidation or exchange reactions between sulfhydryl groups brought together in the process of freezing. Progress toward quantitative confirmation of this mechanism might be made by applying Pincock's formalism, not only to the freezing, but also the thawing of plant proteins. The events outlined in Table 5 provide explicit molecular mechanisms for bringing

together sulfhydryl groups, and the concurrence of chemical driving forces during warming suggests that disulfide bond formation, as well as protein unfolding, could occur on thawing.

The study by Greiff and Kelley[38] on the effects of freezing on lactic dehydrogenase (LDH) solutions provides some additional, though less direct, evidence. LDH activity was lost by freezing, the extent increasing as the minimum temperature decreased from -20° to -192°C. Loss in activity was attributed to enzyme conformation change resulting from denaturation processes analogous to those occurring in thermal degradation at superambient temperatures. Retention of LDH activity was greater for slow freezing, high enzyme concentration, added protein present, and isothermal pauses during cooling, that is, those conditions leading to protection of the enzyme by the solvent or dissolved salts. It was possible to describe the losses in LDH activity observed after multiple freeze-thaw cycles in the same way as thermal denaturation. The results of this investigation are also consistent with the views presented here and summarized in Table 5. Thus, the molecular rearrangements occurring during cooling can be beneficial or deleterious, depending on the presence of a protective agent. In addition, the effects of minimum temperature and multiple freeze-thaw cycles may in fact be due to reaction on warming.

REFERENCES

1. Benson, S. W. 1960. *The Foundations of Chemical Kinetics*. New York: McGraw-Hill. p. 67.
2. Benson, S. W., op.cit., p. 148.
3. Dushman, S., and Lafferty, J. M., eds. 1962. *Scientific Foundations of Vacuum Technique,* 2d Ed. New York: John Wiley and Sons. pp. 10-11.
4. Davis, D. R., Libby, W. F., and Meinschein, W. G. 1966. J. Chem. Phys. *45*(12):4481.
5. Jacox, M. E., and Milligan, D. E. 1963. J. Am. Chem. Soc. *85*:278.
6. Whelan, D. J. 1969. Chem. Revs. *69*:179.
7. DeMore, W. B. 1969. J. Phys. Chem. *73*(2):391.
8. Scheer, M. D., and Klein, R. 1969. J. Phys. Chem. *73*(3):597.
9. Parts, L., and Miller, J. T. 1969. J. Phys. Chem. *73*(9):3088.
10. Mullhaupt, J. T., and Stern, S. A. 1965. Unpublished research, Union Carbide.

11. Rossini, F. D., Wagman, D. D., Evans, W. H., Levine, S., and Jaffe, I. 1952. *Selected Values of Chemical Thermodynamic Properties*. USNBS Circular No. 500. Washington, D. C.: U. S. Government Printing Office.
12. Orlova, M. P. 1966. Russ. J. Phys. Chem. *40*(12):1603.
13. Barrett, C. S., and Meyer, L. 1964. Proc. IX Int. Conf. Low-Temp. Phys., Columbus, Ohio. In: *Low Temperature Physics LT9 (Part B)*. Plenum Press. p. 1085.
14. Shen, M. C., and Eisenberg, A. 1967. Glass transitions in polymers. In: *Progress in Solid State Chemistry*, Vol. 3. Pergamon Press. p. 476.
15. Bondi, A., and Tobolsky, A. V. 1967. Phase transitions and vitrification. In: *Polymer Science and Materials*. Wiley-Interscience. p. 113.
16. Shen, M. C., and Eisenberg, A. 1967. Glass transitions in polymers. In: *Progress in Solid State Chemistry*, Vol. 3. Pergamon Press. p. 475.
17. Shen, M. C., and Eisenberg, A. 1967. Glass transitions in polymers. In: *Progress in Solid State Chemistry*, Vol. 3. Pergamon Press. pp. 470-472.
18. Redington, R. L., and Milligan, D. E. 1962; 1963. J. Chem. Phys. *37*(10):2162; *39*(5):1276.
19. Kasai, P. H. 1971. Acc. Chem. Res. *4*(10):329.
20. Scheer, M. D., and Klein, R. 1961. J. Phys. Chem. *65*:375.
21. Caldin, E. F., Kasparian, M., and Tomalin, G. 1968. Trans. Faraday Soc. *64*:2802.
22. Rinfret, A. P. 1963. Cryobiology. In: R. W. Vance, ed. *Cryogenic Technology*. New York: John Wiley & Sons. Chapter 16.
23. Mazur, P. 1966. Physical and chemical bases of injury in single-celled micro-organisms subjected to freezing and thawing. In: H. T. Meryman, ed. *Cryobiology*. New York: Academic Press.
24. Pegg, D. E. 1972. Cryobiology--A review. In: *Advances in Cryogenic Engineering*, Vol. 17. Plenum Press. p. 116.
25. Farrant, J., and McGann, L. E. 1975. Advances in cryobiology. In: *Advances in Cryogenic Engineering*, Vol. 20. Plenum Press. p. 417.
26. Ho, C., Doebbler, G. F., and Rinfret, A. P. 1962. Fed. Proc. *21*:77.
27. Greiff, D., and Seifert, P. 1968. Cryobiology *4*(6):295.
28. Linden, C. D., and Fox, C. F. 1975. Acc. Chem. Res. *8*(10):321.

29. Chapman, D. 1974. Liquid crystals and cell membranes. In: *Liquid Crystals and Plastic Crystals*, Vol. 1. Chapter 6, Significance of liquid crystals in biology. Halsted Press. pp. 288-307.
30. Stewart, G. T. 1974. The role of liquid crystals in life processes. In: *Liquid Crystals and Plastic Crystals*, Vol. 1. Chapter 6, Significance of liquid crystals in biology. Halsted Press. pp. 308-326.
31. Tappel, A. L. 1966. Effects of low temperatures and freezing on enzymes and enzyme systems. In: H. T. Meryman, ed. *Cryobiology*. New York: Academic Press. pp. 163-167.
32. Poland, D., and Scheraga, H. A. 1967. Theory of non-covalent structure in polyamino acids. In: *Poly-α-Amino Acids: Protein Models for Conformational Studies*. Marcel Dekker. pp. 393-405.
33. Scheraga, H. A. 1971. Chem. Rev. *71*(2):195.
34. Lotan, N., Berger, A., and Katchalski, E. 1972. Conformation and conformational transitions of poly-α-amino acids in solution. *Ann. Rev. Biochem. 41*;869. Annual Reviews, Inc.
35. Morawetz, H. 1970. Acc. Chem. Res. *3*:354.
36. Pincock, R. E. 1969. Acc. Chem. Res. *2*(4):97.
37. Levitt, J. 1966. Winter hardiness in plants. In: H. T. Meryman, ed. *Cryobiology*. New York: Academic Press. pp. 553-558.
38. Greiff, D., and Kelly, R. T. 1966. Cryobiology *2*(6):335.

MAINTENANCE OF FERTILITY OF SPERMATOZOA AT -196°C

R. H. Foote

INTRODUCTION

"Time marches on." When aging is defined in terms of time, then aging is a simple fact of life. What is important are the possible changes with time. Furthermore, there is no question but what changes may take place in cells, including spermatozoa, at low temperature. Low-temperature storage reduces measurable changes per unit of time, and biological consequences may not be manifested after low-temperature storage for a given period of time. For example, if a specific change is not critical to the spermatozoon, the change may not be detected by the particular test criterion employed. Secondly, if the change is minimal, it may be below the sensitivity of the assays used to assess change. Thirdly, if not all cells are affected equally and if there is a possibility for selection of certain types in the sample obtained, the result may not be representative of the original population. Such a selection may occur in the female reproductive tract. It is possible that sufficient selective filtration occurs to eliminate most defective sperm cells before they reach the egg, thereby mitigating, essentially, any aging damage that would contribute to embryo mortality.

Conditions of Storage

Obviously the conditions under which spermatozoa are stored will influence the outcome of storage.[1,2] These conditions include (a) temperature; (b) outside energy sources that may reach the cell, such as light; (c) storage media; and (d) the liquid, gaseous, and solid phases in the environment. Also, the storage conditions must be imposed rigidly

on a continuous basis because a fluctuating environment may be detrimental.[1,2]

Conditions under which spermatozoa have been stored have not always been well defined. The studies easiest to interpret, and perhaps the only valid ones, are those in which well-defined conditions have been imposed continuously throughout the storage period.

Criteria to Evaluate Aging

It is desirable that criteria selected to evaluate aging of spermatozoa have at least three characteristics in common as follows: (a) have high sensitivity, (b) be highly repeatable and little affected by possible changes in test conditions over extended periods of time, and (c) be of biological significance. No doubt the most import biological characteristic of a spermatozoon is its ability to fertilize an egg and initiate normal development. Unfortunately, it is virtually impossible to rigidly control conditions under which an egg is fertilized and develops in species where most information is available on stored semen--namely cattle. The conditions *in utero* of individual females certainly vary. However, if conditions of storage *in vitro* can be rigidly controlled, and they can be, then various morphological and biochemical tests on spermatozoa during prolonged storage can be compared with their fertility.

REVIEW OF LITERATURE

This report is based primarily on cattle. Popular reports and unsubstantiated observations are not included in this review. However, not all studies cited had the best possible controls.

Storage at -79°C

Stewart[3] diluted bull semen 1:10 with an egg yolk-citrate-glycerol medium containing antibiotics and stored the spermatozoa in glass ampules in a chamber of solid carbon dioxide at approximately -79°C. Results over a 9-yr storage period are summarized in Table 1. Fertility was measured by a 112-day nonreturn rate. The nonreturn rate (NR) simply is the proportion of cows that are not reinseminated by a

TABLE 1 Fertility of Bull Spermatozoa Stored at -79°C in Glass Ampules[a]

Storage Time	No. of 1st Services	112-Day, % NR
1-4 wk	209	66.5
1 yr	272	61.8
2 yr	298	64.4
4 yr	276	62.0
9 yr	255	58.0

[a]Mixner.[4]

certain time and are assumed to be pregnant. The nonreturn rates are higher than the true pregnancy rates. The fertility of spermatozoa stored at -79°C in this study by Stewart decreased 8.5 percentage units over a 9-yr period.

Mixner[4] stored bull semen frozen in an egg yolk-citrate-glycerol medium at -79°C for 8 yr and in liquid nitrogen 4 more yr. The fertility of the semen used initially after freezing was 65.6 percent (on a 60- to 90-day nonreturn basis), and after 0.5, 1, 2, 4, and 8 yr it was 69.8, 66.2, 56.1, and 59.2 percent. The proportion of motile spermatozoa declined from 63 to 9 percent during the 12 yr. Thus, solid CO_2 does not provide suitable conditions for prolonged storage of bull semen.

Storage at -196°C

Many researchers have reported -196°C storage to be more satisfactory than -79°C[1] on both practical and theoretical bases. The remaining results are based upon storage of bull spermatozoa in liquid nitrogen at -196°C.

Clegg and Pickett[5] analyzed 21,488 first inseminations resulting from semen stored in *glass ampules* for 1 to 2 yr. The 60- to 90-day nonreturns were 69.4 percent for semen

stored less than 3 mo versus 71.9 percent for semen stored 1 to 2 yr. Strom[6] analyzed 55,378 inseminations with semen stored in *pellet* form and found no decline in 56-day nonreturn rates over a period of 1 to 2 yr.

Cassou[7] analyzed 104,368 first inseminations in France with semen stored in *straws* and found no change in the 60- to 90-day nonreturn rate for spermatozoa stored up to 66 mo (69.8 percent at the beginning versus 69.1 percent at the end of the study).

These three studies in three countries with three types of packaging all yielded results supporting the concept that semen can be stored at -196°C for considerable time without loss of fertility. These results seemingly are somewhat in conflict with the extensive studies of Salisbury and colleagues,[8,9] who found a decline after an initial rise in fertility (nonreturn rate) of bull semen stored for various periods.

Part of the difference may be accounted for by the interval at which the fertility was computed,[10] particularly if the age at which an embryo dies is affected by storage of the sperm cell. However, most of the studies referred to so far are based primarily on routine use of bull semen by artificial insemination organizations. Most of the inseminations are with semen stored for a short period of time. The oldest semen tends to be from those bulls and ejaculates that, in our experience, are in least demand. Therefore, a number of potential biases arise, some of which may not be recognized or, if recognized, do not permit statistical corrections to be made by new or "age-old" techniques.

In fact, the perfect experiment, testing aging effects on spermatozoa, while holding everything else still, of course is impossible. Instead, we have the practical dilemma of trying to hold the spermatozoa constant and measuring any inconsistencies in these cells, while everything else may be changing.

EXPERIMENTAL STORAGE OF BULL SPERMATOZOA

At -196°C for up to 2 Years

Because of the many problems previously encountered, a trial was undertaken in our laboratory[11] in 1968 to compare semen frozen in both summer and winter and used simultaneously over a period of 2 yr. The summer and winter components were included because of previous re-

ports[8,12] that seasonal fertility of cows and bulls differed. Semen was extended with egg yolk-citrate medium, plus glycerol and antibiotics, and frozen in glass ampules containing 12 million motile spermatozoa before freezing. Every ejaculate received a distinctive code, and each was used for insemination after continuous storage under liquid nitrogen for various intervals, regardless of the number of surviving spermatozoa. The bulls aged during the trial, and the percentage of motile spermatozoa collected and frozen at the end of the trial averaged 26 percent, whereas the spermatozoa collected at the start and stored for 2 yr averaged 36 percent progressively motile.

The fertility results are summarized in Tables 2 and 3. Fertility of the semen frozen in the winter tended to be higher after 6 mo of storage, as reported by others.[8,13] However, this was not statistically significant and the trend was not repeated in the semen collected and frozen in the summer. Fertility of semen obtained in summer, based upon 150- to 180-day nonreturns, was 2.9 percentage units lower than semen frozen in the winter. Throughout this time period 12,779 inseminations with freshly frozen semen averaged 66 percent on a 150- to 180-day basis.[11]

The major point is that none of the storage intervals were significantly different. Detailed regression analyses revealed that storage time contributed nothing to the level of fertility. The conclusion[11] was that with continuous storage at -196°C in the dark "there was no demonstrable impairment of spermatozoan fertilizing capacity or ability to sustain pregnancy."

The possible increase in fertility during the first 6 mo of storage reported by others[8,13] recently was investigated in our laboratory. A carefully controlled field trial was conducted in which semen was frozen, held centrally at -196°C in 6 aliquots, and released into the field each month for 6 mo. The fertility results are given in Table 4. The statistical analysis revealed that there was no effect of storage. Part of the monthly fluctuations reflected fluctuations in fertility in the field, which affected ejaculates of semen collected and used with minimal storage. Therefore, the deviation from breed average also is given in Table 4. From these data it is concluded that no pronounced changes in fertility, such as those reported previously,[13] occur during the first 6 mo when semen is stored under carefully controlled conditions.

TABLE 2 Fertility of Bull Semen Frozen in the Winter and Stored in Glass Ampules at -196°C[a]

Storage Time (mo)	No. of 1st Services	% Nonreturns			Decrease: 30-60 to 150-180 Days
		30-60 Days	60-90 Days	150-180 Days	
1	4,130	79.5	71.5	67.3	11.2
6	1,201	82.5	76.9	73.8	8.7
12	1,127	77.5	70.6	67.4	10.1
18	941	80.8	74.0	71.3	9.5
TOTAL OR MEAN	7,399	79.9	73.3	70.0	9.9

[a]Foote.[11]

TABLE 3 Fertility of Bull Semen Frozen in the Summer and Stored in Glass Ampules at -196°C[a]

Storage Time (mo)	No. of 1st Services	% Nonreturns 30-60 Days	60-90 Days	150-180 Days	Decrease: 30-60 to 150-180 Days
1	2,840	77.8	72.1	65.2	12.6
6	1,460	79.7	71.0	66.3	13.4
12	1,253	79.2	72.6	68.1	11.1
18	1,197	75.6	69.6	65.8	9.8
24	1,084	80.2	73.4	70.0	10.2
TOTAL OR MEAN	7,834	78.5	71.7	67.1	11.4

[a]Foote.[11]

TABLE 4 Fertility of Bull Semen Stored Centrally in Straws at -196°C and Used Each Month[a]

Storage Time (mo)	No. of 1st Services	60- to 90-Day NR %	60- to 90-Day NR Deviation from Breed Average
1	1,515	73.3	4.4
2	2,600	75.0	3.9
3	2,660	73.4	2.2
4	3,348	76.7	4.7
5	3,463	73.8	1.2
6	3,220	75.6	2.4

[a]Foote, unpublished data, 1975.

At -196°C for up to 5 Years

Pellets. In Scandinavia and several other countries semen from young bulls is frozen as pellets. The bulls are slaughtered while still young, and pellets of semen are stored for use after these bulls have become progeny tested. Coulter and Foote[14] have reported that this actually may improve fertility because of a greater aging effect in bulls than in frozen spermatozoa.

Lindstrom[15] reported results of two trials in Finland with pelleted semen frozen in an egg yolk-lactose medium. In Study 1 (Table 5) young and old stored semens (collected at different times) from the same bulls, were used simultaneously. There was a significant difference in favor of the semen used within 10 mo after collection. The author believes the difference may be due to better methods of pellet processing of the semen stored for a shorter period of time. In Study 2 the same source of semen was used in 1968 and 1971. The significantly higher fertility with semen stored more than 3 yr (1971) may reflect, in part,

TABLE 5 Fertility of Spermatozoa After Storage at -196°C in Pellets[a]

Study No.	Storage (mo)	No. of 1st Services	Nonreturns, %		
			60-Day	90-Day	120-Day
1	≤ 10	2,784	73.2	69.1	67.5
	≥ 36	4,603	69.9	66.3	65.1
TOTAL OR DIFF.		7,387	+3.3[b]	+2.8[c]	+2.4[c]
2	<12	4,477	69.9	66.6	65.4
	>36	12,363	72.9	69.8	68.8
TOTAL OR DIFF.		16,840	-3.0[b]	-3.2[b]	-3.4[b]

[a] Lindström.[15]

[b] $P < 0.01$.

[c] $P < 0.05$.

better reproductive rates among the cows in 1971. In both tests the tendency was for old semen to decline less in nonreturn rate between 60 and 120 days than was true of semen used within 1 yr of collection. This finding is opposite of the results obtained by Salisbury.[8] Lindström concluded that "pellets maintain their fertilizing ability to a high degree after three years of storage."

Andersen and Pedersen[16] processed bull semen with egg yolk-lactose by the pellet method in 1969. The semen was stored immersed in liquid nitrogen for up to 5 yr (Table 6). The 60- to 90-day nonreturns were 0.8 percentage units lower after 5 yr of storage, but control pelleted frozen semen used within a few months of collection was 3.4 percentage units lower the fifth year. Thus, it would appear that the test conditions had declined slightly in 5 yr and that semen stored for 5 yr was at least equal in fertility to semen used with minimal storage.

Ampules

The Milk Marketing Board in England has reported on two trials with semen stored in glass ampules.[17,18] In the first trial semen was distributed to 24 inseminators after immersion in liquid nitrogen for 1, 6, 12, and 24 mo. The proportion of cows requiring reinsemination after various intervals up to 84 days was not affected by storage time of the spermatozoa regardless of whether 20×16^6 or 40×10^6 spermatozoa were packaged per insemination dose of 1 ml.

In a second trial bull semen was again frozen in glass ampules and distributed to six technicians 2, 3, and 5 yr later (Table 7). Two technicians had to be replaced at 5 yr by two with equivalent records. There was no detectable effect of storage on fertility during the 5 yr.

Straws

Andersen and Pedersen[16] also studied 0.5 ml-capacity straws as a packaging system for banking bull semen in Denmark (Table 8). Remarkably good fertility (60- to 90-day nonreturn rates) was obtained over a period of 5 yr. Whereas the general level of fertility in this artificial breeding unit appeared to be about 3 percentage units lower in 1974 than in 1969, the semen stored for 5 yr partially reversed this trend, being 2.9 percentage units higher than the recently frozen control in 1974.

TABLE 6 Fertility of Bull Spermatozoa Stored as Pellets at -196°C for 5 Yr[a]

	Stored Semen		Frozen Control		
Years Stored	No. of 1st Services	60- to 90-Day % NR	No. of 1st Services	60- to 90-Day % NR	Difference
0-1	2,582	66.3	8,801	66.1	0.2
1-2	2,398	67.3	8,861	66.0	1.3
5	2,034	65.5	8,123	62.7	2.8
Difference	--	0.8	--	3.4[b]	

[a] Anderson and Pedersen.[16]

[b] $P < 0.01$.

TABLE 7 Fertility of Bull Spermatozoa Stored in Glass Ampules at -196°C for 5 Yr[a]

Years Stored	No. of 1st Services	84-Day % NR
2	692	69.9
3	729	68.5
5	702	71.0

[a]Milk Marketing Board Report.[18]

RESULTS OF STUDIES

The results of using bull spermatozoa packaged in three systems and stored experimentally, immersed in liquid nitrogen, for 5 yr provide compelling evidence that the fertility of these stored cells is not reduced during this period of "aging." A limited number of inseminations with ram spermatozoa frozen in pelleted form and stored for up to 5 yr also has given as good lambing rates[19,20] as freshly frozen ram semen (2 wk = 54 percent and after 5 yr = 53 percent lambing).

At the same time Salisbury and colleagues[8,9] have reported that several biochemical changes occur in bull spermatozoa at -196°C. Our laboratory (Coulter and Foote, unpublished, 1973) found oxygen consumption of spermatozoa to be higher after 6 mo of storage ($P < 0.01$) than after 0 and 18 mo of storage (Table 9). Exposure to light (4,800-11,800 lux) for 60 min at 5°C prior to freezing had a detrimental effect ($P < 0.01$). The proportion of motile spermatozoa also declined during storage in the egg yolk-citrate medium ($P < 0.01$).

These studies were carried out simultaneously in glass ampules as well as straws. There were several significant interactions ($P < 0.01$) among storage time, extenders, and packaging for the various criteria used to assess storage effects (motility, acrosomal normality, and actinomycin-D binding). These results provide evidence that varying storage conditions within the same temperature

TABLE 8 Fertility of Bull Spermatozoa Stored as Straws at -196°C for 5 Yr[a]

Years Stored	Stored Semen: No. of 1st Services	Stored Semen: 60- to 90-Day % NR	Frozen Control: No. of 1st Services	Frozen Control: 60- to 90-Day % NR	Difference
0-1	2,035	68.9	5,884	67.6	1.3
1-2	2,016	67.2	5,717	65.5	1.7
2-3	1,917	66.7	6,303	64.7	2.0
5	2,035	67.5	8,708	64.6	2.9
Difference	--	1.4	--	3.0[b]	

[a]Andersen and Pedersen.[16]

[b]$P < 0.01$.

TABLE 9 The Effect of Light and Storage on O_2 Consumption of Bull Spermatozoa Stored at -196°C in Different Extending Media (expressed as ml of $O_2/h/10^8$ cells)[a]

Months Stored	Yolk-Citrate		Promine-D[b]	
	Control	Light	Control	Light
0 Fresh	19.2 ± 1.2[c]	14.2 ± 2.1	16.1 ± 1.2	14.1 ± 0.7
0 Frozen	9.6 ± 1.3	7.8 ± 1.2	6.5 ± 1.0	5.6 ± 0.8
6 Frozen	14.8 ± 1.5	14.6 ± 1.6	10.9 ± 1.1	10.6 ± 0.8
18 Frozen	9.7 ± 0.8	9.2 ± 0.6	8.4 ± 1.0	7.7 ± 0.6

[a]Coulter and Foote, unpublished data, 1973.

[b]Promine-D is an extender containing soybean protein.

[c]Means ± SEM.

range can affect the biochemical and morphological characteristics of spermatozoa.

Another study was done on acrosomal proteinases in cooperation with Duane Garner, then at the University of Illinois (Garner and Foote, unpublished, 1972). Bull semen stored in standard egg yolk-citrate at -196°C for 6 yr was compared with semen collected from the same bulls and processed in the same way, but examined within a year of freezing. Careful isolation of the acrosomal pellet by repeated density gradient centrifugation was followed by disc gel electrophoresis. The pattern of bands on 6-yr-old semen was slightly different from semen held less than 1 yr. Whether or not this has any biological significance remains to be determined.

Salisbury[8] reported that the nonreturn rate tended to remain high or even rise until spermatozoa has been stored at -196°C for 11 mo. Fertility declined thereafter. Sullivan[21] compared the fertility of bull spermatozoa processed to provide 5, 10, or 15 million motile cells per insemination. Fertility during the 1-yr trial only declined with the low sperm numbers ($P < 0.01$), and Sullivan attributed this to the loss of motile cells below the critical number required for optimum fertility. This seems reasonable, but it is surprising that the fertility (60- to 90-day nonreturn rate) was declining within 6 mo of freezing. In the trial by Foote,[11] no decline was found during 2 yr when the glass ampules contained an average of about 7×10^6 motile sperm per insemination dose.

We also examined fertility of bull spermatozoa stored in inseminators' field units (Oltenacu and Foote, unpublished, 1976) for periods ranging from less than 2 mo to more than 14 mo (Table 10). All semen samples in the cooperating AI organization (Eastern A. I. Coop., Inc.) have the exact collection date and bull identified on each breeding unit. The semen was processed in a manner similar to the experimental field trial,[11] but the frozen semen for the present study was stored in the field. The uncorrected nonreturn rates suggest that a marked decline has occurred in the field. However, when the data are corrected for certain changes in procedure, and particularly the fact that the bulls with lower fertility tend to be in less demand and remain in the field units longer, the storage effect becomes very small (Table 10). Under field handling conditions of possible brief multiple exposures to ambient temperatures and light, a real handling effect rather than a true unbiased age effect at low temperature may be produced.[1,2] In this field data there is a small residual

TABLE 10 Fertility of Semen Stored in Field Units with Potential Exposure to a Variety of Handling Problems[a]

Months Stored	No. of 1st Services	60- to 90-Day % NR	Extraneous Influences
≤2	13,769	75	1. Unequal use of bulls
3-4	28,713	75	
5-6	26,357	74	
7-8	19,345	72	2. Extender change
9-10	19,225	71	
11-12	13,518	70	3. General rise in NR rate
13-14	10,516	72	
>14	8,844	69	

[a]When appropriate corrections were made for known sources of variation most (but not all) of the change in nonreturn rate was eliminated.

effect that may be a real effect of aging under field-handling stresses. These results emphasize the hazards of using field data obtained outside of rigorously conducted experiments with suitable controls.

Although data are scarce on this topic, no increase in frequency of abnormal calves have been observed by organizations breeding cattle with frozen semen.

CONCLUSIONS

1. Fertility of bull semen stored in liquid nitrogen under routine use in the field is influenced potentially and practically by intermittent exposure to undesirable storage conditions. Thus, such data are unsuitable for estimating true age effects on fertility of spermatozoa attributable to storage per se.

2. Experiments designed to keep bull semen *continuously* protected at -196°C for up to 5 yr consistently and conclusively reveal no decline in fertility, as measured by nonreturn rate.

3. The biochemical changes reported during storage, and apparent slight loss of motile spermatozoa during storage without a concomitant reduction in fertility, is a scientific puzzle. These results have led the author to suggest that some of the changes monitored may have little significance for fertilization or the developing embryo. Secondly, and perhaps more importantly, it is postulated that any cells damaged by the storage process may not participate in fertilization. It is conceivable that the female tract acts as a selective filter, and such spermatozoa may not be transported to the vicinity of the egg. Any cells that are transported may be at a competitive disadvantage in penetrating the investments of the egg, at least in cattle. Thereby, fertility of stored bull spermatozoa would be unimpaired, given a sufficient number of spermatozoa initially.

These conclusions are supported by controlled field trials with bull spermatozoa stored for 5 yr. Extrapolation always is dangerous, but these data give no hint of any precipitous decline for extended periods of storage. Also, sporadic surveys and monitoring of births by cattle inseminating organizations have not uncovered any increase in "birth defects" resulting from the use of frozen semen.

ACKNOWLEDGMENTS

The author thanks the investigators who provided data, and particularly Eastern A. I. Cooperative, Inc., for supporting studies conducted by the author.

DISCUSSION

E. S. E. HAFEZ: We must ask ourselves what is the biological definition of "aging of spermatozoa"? Various authors use the term differently. In my opinion aging of spermatozoa is biological injury without killing the sperm, so the sperm can participate in fertilization with subsequent embryonic mortality or birth defect. Spermatozoa that die during the freezing are not biologically significant, because they do not participate in fertilization.

REFERENCES

1. Pickett, B. W. 1971. Factors affecting the utilization of frozen bovine semen for maximum reproductive efficiency. Artif. Insemination Dig. *19*(2):8-23.
2. Pickett, B. W., and Berndtson, W. E. 1974. Preservation of bovine spermatozoa by freezing in straws: A review. J. Dairy Sci. *57:*1287-1301.
3. Stewart, D. L. 1964. Observations on the fertility of frozen semen stored at -79°C and -196°C. V Int. Congr. Anim. Reprod. Aatif. Insemination *4:*617-623.
4. Mixner, J. P. 1968. Fertility of bull semen frozen for 12 years. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1095-1098.
5. Clegg, E. D., and Pickett, B. W. 1966. Effect of storage at -196°C on fertility. Artif. Insemination Dig. *14:*12-13.
6. Strom, B. 1968. Influence upon fertility of bull semen of storage time in liquid nitrogen. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1171-1174.
7. Cassou, R. 1972. Development of fertility rates in terms of age of semen. VII Int. Congr. Anim. Reprod. Artif. Insemination *2:*1421-1425.
8. Salisbury, G. W. 1968. Fertilizing ability and biological aspects of sperm storage in vitro. VI Int. Congr. Anim. Reprod. Artif. Insemination *2:*1189-1204.
9. Salisbury, G. W., and Hart, R. G. 1970. Gamete aging and its consequences. Biol. Reprod. Suppl. *2:*1-13.
10. Salisbury, G. W., Bratton, R. W., and Foote, R. H. 1952. The effect of time and other factors on the non-return to service estimate of fertility level in A. I. of cattle. J. Dairy Sci. *35:*256-260.
11. Foote, R. H. 1972. Aging of spermatozoa during storage in liquid nitrogen. Proc. IV Tech. Conf. Artif. Insemination Reprod. pp. 28-30.
12. Sullivan, J. J., and Elliott, F. I. 1968. Season and fertility in artificial insemination. VI Int. Congr. Anim. Reprod. Artif. Insemination *1:*329-331.
13. Hafs, H. D., Boyd, L. J., Cameron, S., Johnson, W. L., and Hunter, A. G. 1971. Fertility of bull semen with added Beta-glucoronidase. J. Dairy Sci. *54:*420-422.
14. Coulter, G. H., and Foote, R. H. 1974. The economics of selected systems of banking semen vs. maintaining bulls. Proc. V Tech. Conf. Artif. Insemination Reprod. NAAB. pp. 67-72.
15. Lindström, U. 1972. Pellets: Experiences and results

of storage over 3 years. VII Int. Congr. Anim. Reprod. Artif. Insemination *2:*1427-1435.

16. Andersen, J. B., and Pedersen, H. 1976. Breeding efficiency of frozen bull semen after 5 years storage at -196°C in liquid nitrogen. VIII Int. Congr. Anim. Reprod. Artif. Insemination, Cracow. In press.
17. Milk Marketing Board Report. 1967-68. The effect of long-term storage of semen in liquid nitrogen on the non-return percentage. *18:*117-119.
18. Milk Marketing Board Report. 1973-74. Long term storage of semen. *24:*68-69.
19. Salamon, S. 1972. Fertility of ram spermatozoa frozen-stored for 3 years. VII Int. Congr. Anim. Reprod. Artif. Insemination *2:*1493-1495.
20. Salamon, S., and Visser, D. 1974. Fertility of ram spermatozoa frozen-stored for 5 years. J. Reprod. Fertil. *37:*433-435.
21. Sullivan, John J. 1970. Sperm numbers required for optimum breeding efficiency in cattle. Proc. III Tech. Conf. Artif. Insemination Reprod. pp. 36-43.

THEORETICAL AND PRACTICAL SIGNIFICANCE OF FROZEN SEMEN BANKING IN THE ANIMAL BREEDING INDUSTRY

F. I. Elliott

At the outset, I should say that this discussion will be limited largely to consideration of the bovine spermatozoan. We have had a significant amount of experience with equine sperm cells and a limited amount with those of some other mammalian species. In our estimation, extending media and procedures have not yet been fully elaborated with those species, nor are we aware of storage data of significance with them.

Our first significant attempt to study the effect of storage on viability of bovine sperm cells was conducted in 1955 by Dr. Jerry Sherman, who was with us at that time in Madison. Semen from 20 bulls was included in the trial, and the frozen ampules were stored in one of the original Linde containers with a 1½-in neck, with the nitrogen being replenished weekly. This means that most of the ampules were at liquid nitrogen temperature most of the time, but not necessarily all of the time. This treatment was compared with semen stored in dry ice-alcohol at approximately 78°C, and a third treatment involved storage at approximately -103°C, in a mechanical refrigeration box (Figure 1).

All post-thaw evaluations were by means of dark-field time-exposure photography as reported by us at Milan in 1973.[2] We believe the photographic method to be the most practical method of obtaining reasonably objective motility information.

Worthy of note is the fact that the ampules were attached to fiberglass canes. This means that probably, as nitrogen level dropped in the liquid nitrogen container, the top ampules on the canes tended to warm more than would have been the case had they been stored on metal canes. There is practically no warm-up of top ampules on metal canes in a well-insulated nitrogen container if those canes are standing in the liquid itself, even if the tops of the

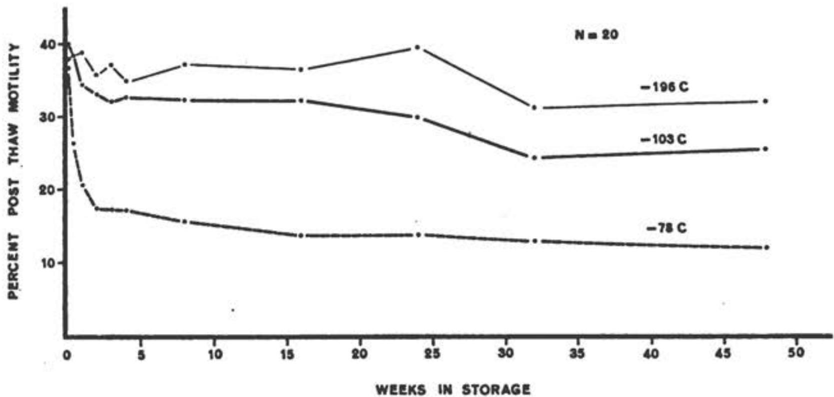


FIGURE 1 Effect of storage temperature on post-thaw motility. J. K. Sherman and F. I. Elliott, 1955, unpublished data.

canes are in the vapor. The same is not necessarily true with respect to fiberglass canes.

Note in Figure 1 the loss of motility in the semen stored at dry-ice temperature is more rapid than has generally been reported to be the case. We believe this is a reflection of the greater accuracy of our photographic technique of measuring motility. The intermediate temperature gave intermediate results.

In liquid nitrogen there was no loss until after 6 mo. The first significant drop in motility appeared at the eighth month observation, and Dr. Jim Torrie, of the University of Wisconsin, our statistical consultant, told me that we must assume that something happened after 6 mo resulting in the decrease in motility of the nitrogen-stored semen.

We have recently completed a much larger trial, in connection with other studies, involving 444 samples (Figure 2). In this case, semen was stored in an MVE A3500 container, at least the bottom of each cane was always bathed in liquid nitrogen, and this time the canes were made of tinplate. Note that we detected no change in percent progressively motile cells over a period of 1 yr.

Our current computer program does not make it simple to obtain fertility data as related to the age of the semen. As a matter of fact, it makes it practically impossible to do so! Early experience made us confident that

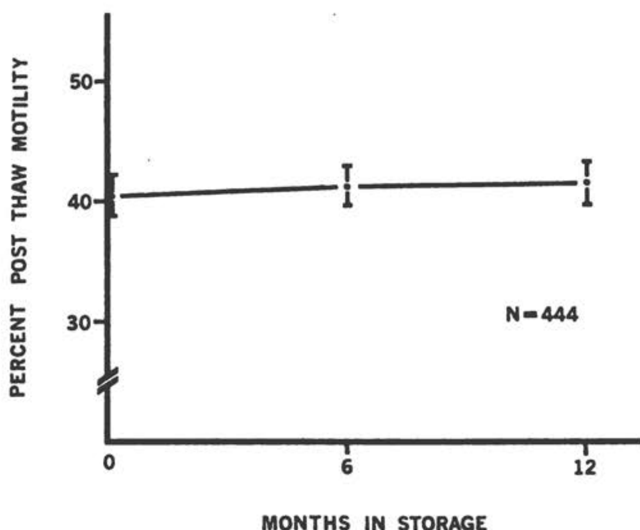


FIGURE 2 A further study of storage time and motility. Storage at liquid nitrogen temperature.

carefully stored semen could be used with impunity, and we process 10,000 to 15,000 separate batches of semen per year. Such volume taxes our data storage, especially on the IBM 1400 we used for several years, and we elected to drop each collection at a certain point. Data after that point were so few as to be unimportant anyway.

I was able to go back to the years of 1957 and 1958, when this type of information was recorded by hand from monthly IBM reports. Figure 3 shows the nonreturns obtained with semen used during the first 6 mo after collection as compared to the nonreturns where semen from the same bulls was used from 7 to 12 mo after collection. There was no detectable change in the nonreturn level.

Seventeen of the 29 bulls that were in this first group also had a significant number of services where the semen was from 13 to 18 mo of age. This involves a total of approximately 200,000 services, and still there is no detectable change in nonreturn rate.

Four of the bulls involving 55,000 services also had semen used in all three of the aforementioned periods, plus the period of 19 to 24 mo. A total of about 900 services were involved in the 19 to 24 age block. I would attach

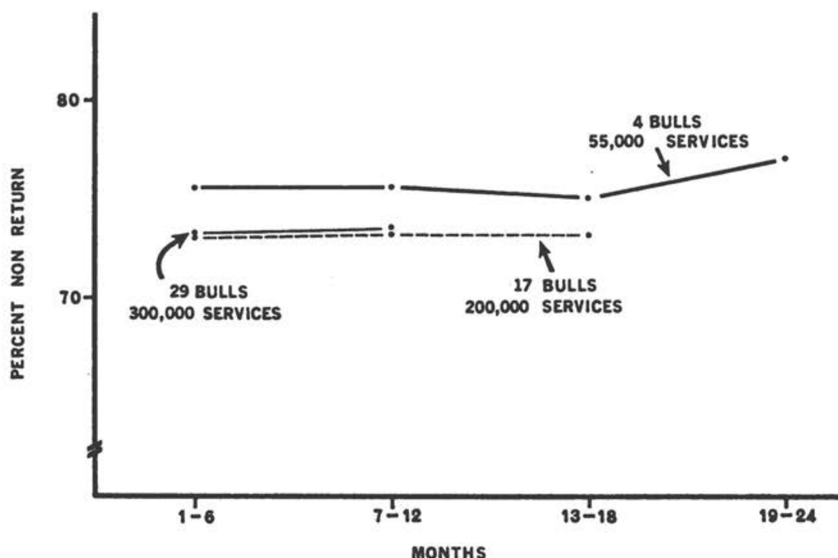


FIGURE 3 Time in storage and 60- to 90-day nonreturns. Routinely-used semen, 1957-1958.

no significance to the fact that the curve seems to go up at this point.

We think this lends further support to the belief that there is no loss in fertility with time when the semen has been carefully stored at liquid nitrogen temperature and where the live or motile cell count is above minimums. In this case, the average motile cell count was in the range of 12×10^6 to 14×10^6 per inseminating unit. The total cell count per unit was 35×10^6 to 40×10^6.

It is our belief that if semen is to be stored for a long time, especially under conditions where it may be beaten up a bit, it is important to have a generous supply of motile cells[3] (Figure 4).

Note that the semen which was packaged at an average of 10×10^6 or 15×10^6 progressively motile cells per unit shows no loss in nonreturn rate through a period of 12 mo. On the other hand, semen packaged at 5×10^6 progressively motile cells shows a somewhat gradual loss in nonreturn rate through the period of the year, although that loss would not appear to be a tremendously large one. These data are taken from a field trial involving some 57,000 first services, all in the Holstein breed.

Our reporting system does not readily give us information on late nonreturns or embryonic losses. Again, our early experience led us to the conclusion that there was no serious problem in this area. Our Veterinary Department did establish a reporting system for abnormal calves, and our representatives and customers have been encouraged to report all instances of this kind.

When such a report comes in, and it has been established beyond reasonable doubt that the calf actually was sired by the bull in question, a black mark goes up against that bull's name. If the abnormality is a generally recognized, simply inherited recessive lethal or its equivalent, and one or more additional reports are received, the bull goes to slaughter. Examples of these abnormalities are porphyria, syndactylism, dwarfism, etc. These cases of abnormalities have been removed from the report I am going to discuss.

Figure 5 shows number of first services reported by age of semen in months after the semen was collected and frozen. Month 1 is month of collection. The data were taken from the years 1957 and 1958, the same years used earlier for the nonreturn information. A total of approximately 500,000 first services were involved.

Note that approximately 96 percent of the services were made within the first 12 mo. Note also that of the

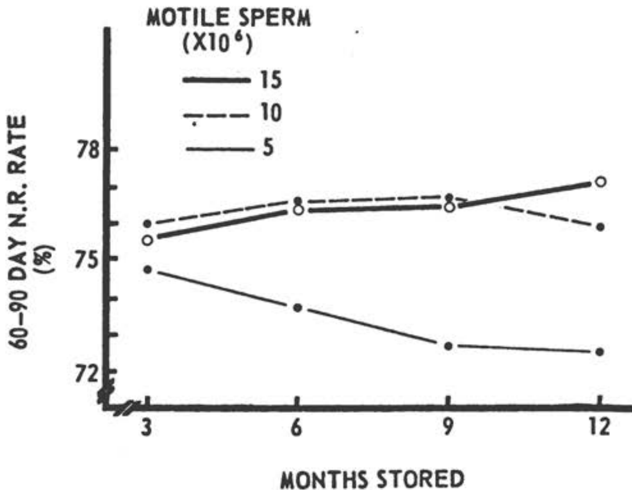


FIGURE 4 Effect of motile sperm concentration of stored semen on nonreturn rates.

abnormal calves reported as having come from semen produced during these 2 yr, which abnormalities were not classified as hereditary in the conventional sense, five occurred as a result of insemination with semen that was less than 12 mo of age and one occurred with semen that was 14 mo of age when used.

Of further interest is the fact that among the total of 54 such reports of abnormal calves produced with semen frozen during the years 1957 through 1972, inclusively, 46 or 85 percent resulted from semen that was less than 12 mo

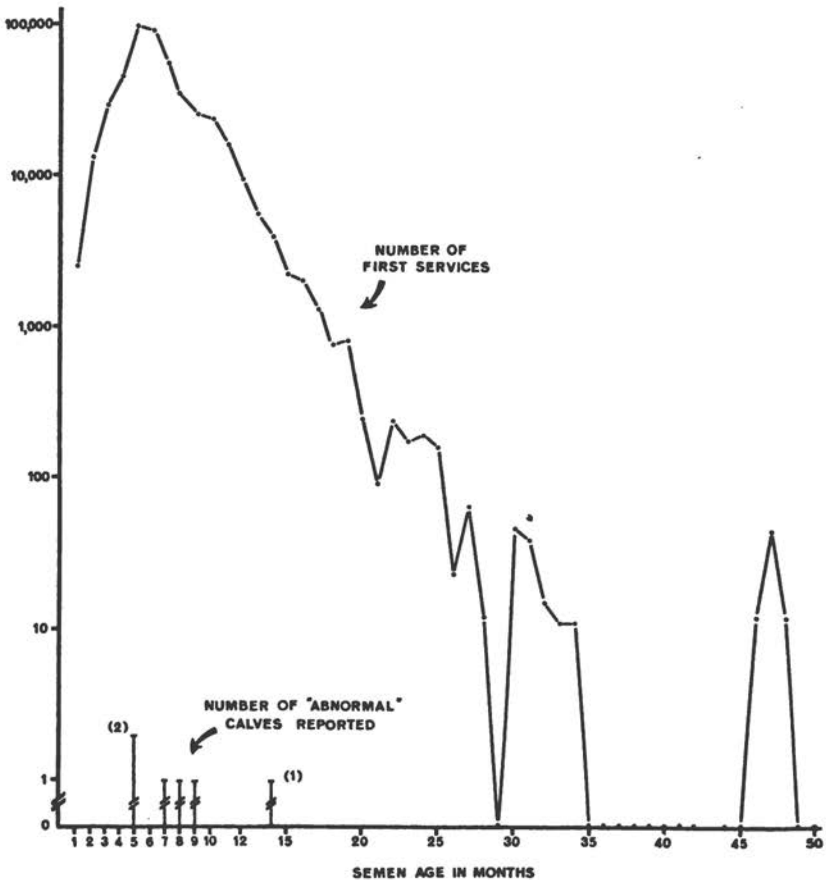


FIGURE 5 Number of birth defects as related to age of semen.

of age and 8 or 15 percent resulted from semen that was more than 12 mo of age.

I should warn that these are based on a reporting system that we recognize to be substantially less than 100 percent efficient. We have plans for improving the reporting, but these are the data we have at the present time. In our opinion, there is no support here for substantial concern for an increase in the percentage of abnormal calves born at term as the result of the use of semen that has been stored for a considerable period of time.

What I have said to this point refers to bovine semen only. It has occurred to me that we should have some equine semen in storage that has been there for a substantial period of time. Figure 6 compares the percent progressively motile cells in stallion semen immediately after freezing and after storage periods of 2 and 3 yr. You will note that semen from four stallions is involved. Each point on the chart represents the mean of three or four separate collections of semen, the same collections being examined at both intervals.

There would seem to be little doubt that there is loss of motile cells in equine semen that has been stored in liquid nitrogen for periods of up to 2 or 3 yr in *tris-*

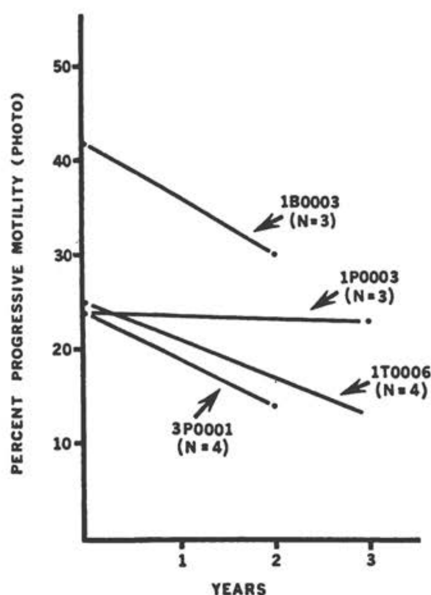


FIGURE 6 Effect of storage on motility--stallion semen.

buffered extender. This points up a problem to which we readily admit: We do not yet have a completely satisfactory extender nor optimum procedures for equine semen. We know fertility to be suboptimal even where the semen was used reasonably soon after it was frozen. Several hundred foals resulting from the use of ABS frozen equine semen have been reported, however, and we have had no reports of abnormal foals.

Now to turn briefly and more specifically to the theoretical and practical significance of frozen semen banking. As I have pointed out, most of the semen is used, in our case, within the first year. While this statement is based on experience dating back a good many years, I do not believe more recent data or data from other North American AI organizations would be grossly different. On the other hand, a minimum storage time of some weeks or months provides real advantages to a frozen semen operation: It provides the necessary time for biological, microbiological, and clinical testing. It permits delivery of the viable product to practically any point in the world, and it permits the development of necessary working inventories. In the case of our company, a Holstein bull has to have produced in the range of 5,000 ampules before we have enough semen to fill the distribution pipelines and deliver semen to the ultimate consumer. A smaller artificial insemination organization might require a smaller inventory in hand before distribution can begin, hence their maximum usage might be at 2 to 4 mo instead of 5 to 6 mo.

On the other hand, long-time storage of bovine semen plays a very minor part in the industry in North America. Because of the upward genetic trend in the cattle industry, a bull that was an outstanding herd improver 10 yr ago tends to be a very average bull today. An example is our bull Cottonade Emmet, whose last collection was frozen on November 19, 1953. His predicted difference was about +500 pounds of milk in 1953. The times have passed him by, although we still have a few ampules in storage 22+ yr later.

There is thus no need for long-time storage of the semen of a very large majority of the bulls in use today. There are, of course, exceptions, probably of the order of 1 bull in 100 or maybe even 2 or 3 in 1,000. For the most part, however, those bulls cannot be identified as the semen is being frozen and stored.

Most of the progressive AI units are continually making the planned matings for the next generation of bulls. In these planned matings they are primarily using semen from the outstanding bulls of the day, but in some cases bulls

that are already dead. Only in rare cases is the semen of a bull long dead used for this purpose. These services make up a very small percentage of semen requirements, and the geneticists who do the planning report no obvious problem with fertility, late returns, or abnormal embryos.

Substantial selection pressure is applied as the offspring of the bulls complete their first lactation records. Normally, one in five or six of the young bulls started in the testing program graduates to take part in the routine operations in the AI unit. A minimum interval of 4 to 5 yr transpires between the time the young bull's semen is first used in the field and production information on his daughters is available.

Several European countries are using a system whereby semen is collected and stored from young bulls during this interval. Once enough semen has been produced, the bull is sacrificed. When production information from the daughters is available, the semen from those bulls selected is put into use, and the semen from the culled bulls is discarded.

Coulter and Foote[1] have made an economic study of this system for use in North America. They have shown that theoretically there should be some savings and further that one would be taking advantage of the bull's peak reproductive years to produce his quota of semen.

This system works satisfactorily, apparently, in some European countries where often by law a certain maximum number of cows may be bred to any one particular bull. Our North American geneticists have not registered concern about too extensive usage of individual sires in the large North American cattle population.

In our hands, the average Holstein bull remains in service something like 3½ yr after he "graduates," and during that time, on the average, he will produce about 180,000 ampules. The maximums we have thus far obtained are in the range of 460,000 to 490,000 ampules. In those cases the bulls continued in service for substantially longer than 3½ yr.

Coulter and Foote have calculated that a bull might be expected to produce 200,000 to 300,000 ampules during the interval between completion of production of semen for the progeny test and the time production information is available on the bull's daughters. Since ABS tests in the range of 125 young Holstein bulls each year, plus substantial numbers of other breeds, we are talking about adding in the range of 600 to 800 bulls to the roster to be collected from each week. At the present time, we collect from about 150 bulls per week. The housing and labor requirements for

such a program boggle the imagination. While our young bulls are in waiting, they are group housed. If we were to collect from them regularly they would have to be individually housed and we would have a tremendous increase in labor requirements to collect, process, freeze, and store the semen.

The situation could be different with respect to minor breeds or possibly to smaller AI units. Where the lifetime requirements of semen from a bull are lower, it could make sense to freeze and store that number of ampules, acknowledging that a majority of the semen will have to be discarded when tests data are available.

In addition, geneticists have improved substantially their ability to predict from pedigree information which bulls should be outstanding when progeny data become available. For this reason, in addition to the bulls of the minor breeds (which really in effect means all breeds except Holsteins), it might be feasible to freeze and store semen from a select number of young Holstein bulls who offer particularly outstanding promise based upon the pedigree index.

In any case, I think you would get pretty general agreement that were this to come about we do not have a serious concern about storing the semen for those 5 yr or more.

REFERENCES

1. Coulter, G. H., and Foote, R. H. 1974. The economics of selected systems of banking semen vs. maintaining bulls. Proc. V Techn. Conf. Artif. Insemination Reprod. National Association of Animal Breeders, Columbia, Mo. p. 67.
2. Elliott, F. I., Sherman, J. K., Elliott, E. J., and Sullivan, J. J. 1973. A photographic method of measuring percentage of progressively motile sperm cells using dark-field microscopy. Eighth *Simposio Internazionale di Zootechnia,* Societa Italiana per il Drogresso della Zootecnica, Milan. pp. 160-166.
3. Sullivan, J. J., and Elliott, F. I. 1968. Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination. Proc. VI Int. Congr. Anim. Reprod. Artif. Insemination, Paris *2:*1307-1312.

MICROBIAL CONTAMINANTS IN FROZEN SEMEN

David E. Bartlett

My associate, Dr. F. I. Elliott, whose paper dealt with the subject of frozen semen banking in animal breeding, and I accept the label of pragmatists. However, it is reasonable that this label be as pragmatists with experience since our organization has processed between 40×10^6 and 50×10^6 of the frozen bovine semen ampules of the 200×10^6 produced and used in the United States during the past 20+ yr. Additionally, it was in our organization that the use of liquid nitrogen for the preservation of frozen bovine semen was first conceived, and it was our organization that paid the Linde Company to develop the prototype of all modern liquid nitrogen refrigerators.

From the purely technical standpoint, the problem of microbial hitchhikers in frozen human semen seems relatively simple as compared with the technological problems that have confronted us with pathogens in frozen bovine semen.

A review of the subject of pathogens and potential pathogens in frozen bovine semen was recently presented by my colleagues and me and will appear in the forthcoming Proceedings of the VI Technical Conference on Reproduction and Artificial Insemination of the National Association of Animal Breeders.

I will begin with a basic statement that I believe, the principal for which was established by MacPherson and Fish in 1954: As a working concept for the purpose of this discussion, it seems reasonable to accept that microorganisms contaminating semen will survive processing, freezing, and storage at least as well as sperm cells.

Differing from frozen bovine semen, use of human semen implies a relatively small infection potential. The human semen donor-to-recipient infection potential ratio is very

close, i.e., one to one, or perhaps, occasionally, one to a very few. Also, it is probable that human semen will be used at only one location.

For bovines, a single frozen ejaculate may be used for insemination of hundreds or, sometimes, more than a thousand females--in 50 states, on several continents, in many countries, extending over several years.

Ejaculates collected from one bull during one month may be used in several thousands of females. Ejaculates collected during 1 yr from one bull will be used in tens of thousands of females and sometimes in more than a hundred thousand females (Figure 1).

In men the typical venereal diseases are usually associated with obvious symptoms or lesions. The clinical history to be elicited from the semen donor is valuable. With one possible exception, the venereal diseases of humans are easy to diagnose and treat.

In bulls the typical venereal diseases are asymptomatic upon clinical examination. The history to be elicited from the owner or manager is too often worthless. The bovine venereal diseases are all difficult to diagnose and all

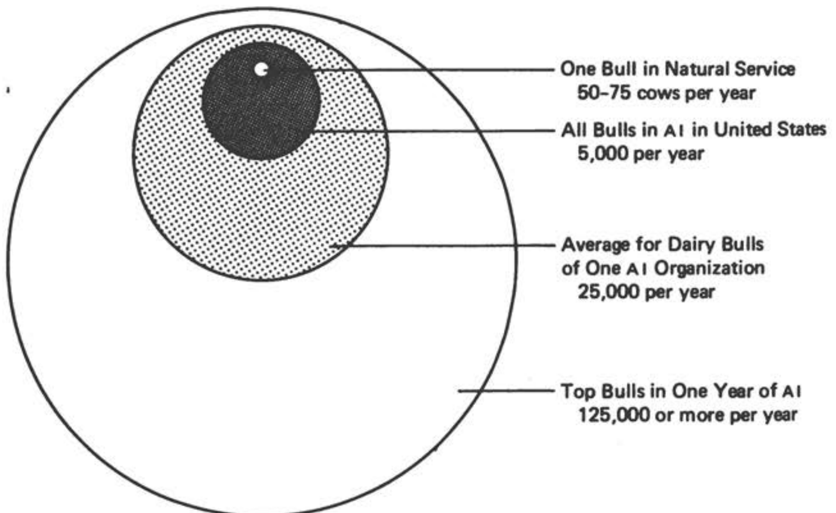


FIGURE 1 Animal contacts--natural service and artificial insemination.

difficult to treat. Several of the diseases aberrantly transmissible venereally are not treatable.

The placement of semen at AI of the human female is at the external cervical os. In the bovine female, placement of semen at AI is through the internal cervical os at the body of the uterus. This location affords lessened opportunity for the natural forces of resistance to act.

Human semen donors present themselves as individuals and on few occasions. Typically, it is necessary to deal with bulls as members of populations of considerable size--from, sometimes, approximately 50 to several hundred. Semen may be collected from bulls repeatedly, often twice-a-week, over a period of several years.

The number of human diseases venereally or aberrantly venereally transmissible is relatively few (Table 1) as compared with the number of such diseases in cattle.

For the purposes of orderly consideration, the bovine AI/frozen-semen-borne diseases may be placed in four categories in recognition of the procedures and/or techniques with which they must be dealt.

Relative to Table 2, List III, it should be noted that, in contrast to a bull, a human male is not only psychologically but anatomically better adapted to the simple and effective procedures of thorough washing of the external genitalia. The glans penis of the bovine is maintained in

TABLE 1 Human Pathogens, Potentially AI/Frozen-Semen-Borne

	Pathogen	Disease
1.	*Treponema pallidum*	Syphilis
2.	*Neisseria gonorrhea*	Gonorrhea
3.	Herpes virus hominis I and II	Genital herpes
4.	*Hemophilus ducreyi*	Chancroid
5.	*Chlamydia (Bedsonia)*	Lymphogranuloma venereum
6.	*Donovania granulomatis*	Granuloma inguinale
7.	*Mycoplasma hominis*	Mycoplasmosis
8.	"Virus?"	Genital warts
9.	Diseases aberrantly venereally transmissible.	
10.	Vagrant, nonspecific, opportunistic, ubiquitous microorganisms, i.e., staphylococcus, streptococcus, *E. coli*, yeasts/molds/fungi.	

a cavernous prepuce ideal for the harboring of a swarming, microbial menagerie.

Relative to the use of antibiotic additives, Table 2, List IV, it should be specifically pointed out that bovine artificial insemination is not antibiotic dependent for effective disease control. Antibiotics have been tested

TABLE 2 Bovine Pathogens, Potentially AI/Frozen-Semen-Borne

I. Specific Pathogen-Free (SPF) List
   - A. SPF by Territorial Status (United States only)
     1. Virus of foot and mouth
     2. Virus of rinderpest
     3. *Mycoplasma mycoides* (contagious pleuropneumonia)
   - B. SPF by Programmed Test and Retest to Reprove SPF Herd Status
     1. *Mycobacterium bovis*
     2. *Mycobacterium paratuberculosis*
     3. *Brucella abortus*
     4. *Trichomonas foetus*
     5. *Vibrio fetus* var. *venerealis*
     6. *Leptospira* spp. *pomona, hardjo, canicola, icterohemorrhagiae, grippotyphosa*

II. Control by Surveillance List
   1. Virus of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis/infectious pustular balanoposthitis-IBR/IPV/IBP
   2. Virus of bovine virus diarrhea
   3. Virus of parainfluenza$_3$
   4. Virus of fibropapillomatosis
   5. Virus of blue tongue
   6. Virus of bovine leukemia (probably not AI transmissible)
   7. Chlamydia/Bedsonia/Miyagawanella
      Agent of psittacosis-lymphogranuloma-venereum group
      Agent of psittacosis-lymphogranuloma-trachoma group
      (epizootic/enzootic/sporadic bovine abortion)
      (genital bedsoniasis)
   8. *Coxiella burnetii* (Q fever)

TABLE 2 (Continued)

III. Control by Sanitation/Hygiene List
1. *Pseudomonas aerugiosa*
2. *Corynebacterium pyogenes*
3. *Staphylococci* spp.
4. *Streptococci* spp.
5. *Escherichia coli*
6. Molds/yeasts/fungi

IV. Control by Antibiotic Additives List
1. *Vibrio fetus* var. *venerealis* (SPF procedures should supersede control by antibiotics)
2. *Leptospira* spp. (SPF procedures should supersede control by antibiotics)
3. *Mycoplasma bovogenitalium* and *agalactiae* (Experimental: bacteriostatic *not* bacteriocidal)
4. Miscellaneous/unknown antibiotic sensitive organisms

---

and found effective in control of only two organisms, *Vibrio fetus* var. *venerealis* and *Leptospira pomona*. However, there is no longer excuse for either of these organisms to be present in bull populations used for AI, since technology has so advanced as to make readily possible coping with these two infections on an SPF basis. Antibiotic additives have no value against protozoa or viruses. The antibiotics that should be employed in bovine semen processing are penicillin, streptomycin, polymyxin-B sulfate, lincomycin, and spectinomycin. The latter two have been included experimentally for their activity against mycoplasma. However, it has recently been determined that lincomycin and spectinomycin are mycoplasmastatic and not mycoplasmacidal.

To us it seems that the solution of the problem of microorganisms in semen, human or bovine, involves the establishment of a rigid program of surveillance. This must involve the development of a system combining clinical monitoring and laboratory testing. There must be a firm commitment to the system and rigid adherence to the established principles. All scientific and technical facts available must be used diligently. New facts must be con-

tinually sought in order to improve upon the available technology.

In operating a bank of frozen semen, it is imperative to keep in mind that it has been repeatedly demonstrated that most microorganisms contaminating liquid nitrogen readily survive, even though unprotected in this medium. More attention needs to be paid to this phenomenon than has been provided in the past.

In closing, I must state that I am more than a little confused by a paradoxical attitude evident within government. The expressed concern by the U.S. Department of Health, Education, and Welfare's Food and Drug Administration regulators at this meeting in respect to human semen is in marked contrast to the attitude of the U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection regulators toward bovine semen.

In the animal field, we have not been successful in developing a discriminating public attitude, differentiating well-ordered semen banks from poorly run semen banks. Five states--Washington, Virginia, Montana, Wisconsin, and Mississippi--have established specific health regulations in respect to bovine semen. There are no federal or interstate regulations governing the movement of bovine semen within the United States.

The Animal and Plant Health Inspection Service of the USDA has elected for at least 15 yr to ignore AI and its potential for disease transmission. Their attitude has been consistently indifferent, and actions taken have been ineffective. It should be pointed out that the import regulations into the United States for frozen bovine semen from western Europe concentrate unilaterally on foot and mouth virus and ignore other pathogens. In fact, semen legally entering the United States under the USDA regulation may not be qualified for use in the regulating states. To the USDA regulators, this subject of frozen bovine semen seems to lack political sex appeal.

K. W. Sell, in his discussion of blood and other tissue banks, indicated his philosophy that industry guidelines should precede governmental regulations and that the concepts should be developed by peer groups. With this we agree completely, and such steps are consistent with historical fact. Recognizing that there was need for simple, minimal, practical standards, beginning in the early 1950's a code of standards for bovine AI was established within the American Veterinary Medical Association. This code was adopted by the National Association of Animal Breeders. A model regulation for states was developed in 1962 by a

special committee within the Infectious Diseases of Cattle Committee of the U.S. Animal Health Association. Beginning in 1968, a subcommittee of the Infectious Diseases of Cattle Committee, combining scientific and technical participants within industry and USDA, has endeavored to develop a suitable regulation for the industry. This regulation, in principal, has been repeatedly endorsed by the Infectious Diseases of Cattle Committee and the entire body of the U.S. Animal Health Association, with its recommendation submitted to the Secretary of Agriculture for action. The proposed regulation has been endorsed by the Sire Health Committee of the National Association of Animal Breeders, by the Board of Directors of the National Association of Animals Breeders, and by the entire body of the National Association of Animal Breeders, with communication of its resolution of support to the Secretary of Agriculture. The proposed regulation has received the endorsement of the American Association of Bovine Practitioners. These steps seem to qualify as peer involvement.

DISCUSSION

R. H. FOOTE: Would you comment on the incidence of shedding IBR virus in bull semen and the relationship of virus shedding in semen to stress and experimental treatment of bulls with ACTH or corticoids.

DAVID E. BARTLETT: This is a fascinating phenomenon. Understanding seems to have followed recognition that in humans the herpes simplex virus resides permanently, quietly, in the trigeminal ganglion. Under stress, there is migration down the nerve fibers to the lips where vesiculation or "cold sores" develop. Experimentally, IBR carrier bulls have been found to shed virus in many secretions/excretions under the simulated stress induced by ACTH treatment. As with herpes simplex in humans, it appears similar with IBR/IPV/IBP in bovines that those infected remain permanently so. It is of interest to note, however, that the two laboratories (N.Y. State Veterinary Diagnostic Laboratory at Cornell University and Central Animal Health Laboratory, Madison, Wis.) with most experience in monitoring individual semen collections for virus presence have yet to recover IBR virus from the semen of serologically positive bulls in regular semen production upon routine tissue culture of their semen.

CURRENT CLINICAL APPLICATIONS OF SPERM BANKING

Joseph Feldschuh

Sperm banking and artificial insemination in the field of animal husbandry is a major method of reproduction. At the present time, sperm banks play only a small role in human reproduction. Approximately 5 yr ago, it was thought that sperm banks would have widespread growth and also have significant commercial impact. In point of fact, sperm banking is not a commercial success, and the few commercial sperm banks in existence are primarily supported by other activities of their parent companies. Noncommercial sperm banks are usually also sustained by university and research funding. For practical purposes, therefore, at the present time there is little to distinguish so-called commercial sperm banks from the nonprofit variety.

The following presentation is intended to provide some information about the current clinical status and utilization of sperm banks. I draw upon my experience as Medical Director of IDANT, which is believed to be the world's largest human sperm bank.

It was originally thought that sperm banks would primarily provide services for men undergoing vasectomy. At the present time, there are over 1 million males who have undergone voluntary sterilization in the United States. The storage of frozen semen, it was felt, would provide these men with a form of "fertility insurance" for what is considered to be essentially an irreversible operation. Because of the large number of males undergoing vasectomy, it was thought that there would be a widespread utilization of sperm banks. This, in fact, was the primary motivation behind the commercial sperm banks that were founded. This widespread utilization never developed, and probably less than 2 percent of all males undergoing sterilization actually store sperm, despite the low annual storage fee of $22.00. This is unfortunate in view of the many attempts currently under way to perfect surgical techniques to re-

verse vasectomy. At the present time there is only very limited success in reversing vasectomies.

The main reason for the very low rate of utilization of sperm banks in vasectomy can primarily be attributed to the belief by physicians and clinics performing vasectomies that these men have definitely decided not to have any additional children. At the present time, the withdrawal rate at IDANT would indicate that about 5 percent of those men storing semen will attempt to repeat paternity. This would appear to be corroborated by the experiences of physicians who are prominent in the field of surgical reversal of vasectomies. There has thus been a serious failure of communication between that part of the medical profession that performs sterilization and the patient--in terms of clearly stating to the patient the options available for fertility insurance.

Another group of patients who are utilizing the sperm bank in increased numbers are men who are undergoing partial or total sterilization as a result of their therapy for malignant disease. These patients are almost invariably under 35 yr of age and usually in their twenties. They encompass patients with both curable and incurable malignancies. There has been a growing awareness by oncology specialists, especially those at Sloan-Kettering Memorial Hospital in New York and New York Hospital, that it is possible to preserve fertility for some of these men where it would otherwise be irrevocably lost. This, however, is another situation where communication at a professional level is still inadequate. A significant number of patients are unfortunately referred after therapy has been initiated, when the sperm count may be too low for effective fertilization.

Where oligospermia is a factor in the infertility problem of a couple, a sperm bank may be of use. It is possible to freeze and store several ejaculates of the husband and then combine and concentrate them for use at a later date. The wife may be inseminated during her most fertile period. There has been only very limited success with this procedure, and fertilization depends on the presence of normal sperm cells.

A sperm bank may also be of use in other cases where artificial insemination with the husband's semen (AIH) is performed. Namely, if the husband travels or is not available during the wife's fertile periods, he may bank his semen and the gynecologist may inseminate at the appropriate time. Where anatomical anomolies preclude normal sexual relations, sperm banking may be used.

The other major function of the sperm bank is to provide semen for anonymous artificial donor insemination (AID). This has been a continuously growing area of utilization. The change in abortion laws in the United States has caused a marked decrease in adoptable babies. This has resulted in heightened interest in treating male infertility. An estimated 10 percent of males are either sterile or minimally fertile. The use of anonymous donor semen has been one of the methods used to overcome male sterility as the cause for infertility. At the present time, IDANT has had over 400 known births since 1971 from semen supplied from its bank. The actual number of pregnancies is higher, because not all physicians utilizing these services have responded to the questionnaires submitted. There have been varying estimates ranging as high as 50,000 babies born in the United States from the use of anonymous donor semen. This would indicate that the use of fresh semen is probably the major source at the present time when donor semen is used with effectiveness.

The use and effectiveness of frozen semen has been amply demonstrated in animal breeding. There have been questions raised about the effectiveness of frozen semen as opposed to fresh semen in human infertility. One of the problems in this area is that there are multiple techniques for freezing and storing semen. Comparison of the results of frozen versus fresh sperm depends heavily on the effectiveness of the freezing technique. The method in use at IDANT was developed by Dr. Jerome Silbert and is a modification of basic techniques developed by Dr. Jerome R. Sherman. In our hands, these methods, which do not involve the use of egg yolk diluent, have resulted in highly satisfactory living sperm recovery in the order of 50 percent to 75 percent per normal specimen. The factors responsible for variable results from different investigations relates to the multiplicity of freezing techniques in use. An additional variable often not mentioned is the adequacy of the frozen storage systems. Even limited thawing and refreezing will change the specimen. At IDANT a 24-h alarm system will be activated to the Holmes central alarm system in New York City in the event of a drop in the liquid nitrogen system and will result in an immediate response by on-duty personnel. This precaution assures that specimens frozen for years will not be exposed to even small temperature fluctuations prior to use.

The paper presented by G. W. Salisbury of the University of Illinois shows definite evidence for an "aging" effect on sperm populations as evidenced by a decreased

ability to produce conception in animals over a period of 12 to 18 mo. R. H. Foote of Cornell University has also presented evidence that semen stored in liquid nitrogen for periods of up to 5 yr showed no detectable loss of potency. These two papers might appear contradictory and demonstrate a very important point. Dr. Salisbury's data derives from semen data from technicians inseminating in the field and storing their specimens in small portable nitrogen containers. Dr. Foote's data derives from work with specimens stored at a central facility under carefully controlled conditions and not removed until ready for use. I believe that this difference in results provides dramatic proof of the necessity that long-term storage be maintained only under the type of strict careful standards that have been described. Even without thawing, considerable cumulative damage can be done to cell populations that are subjected to moderate temperature fluctuations occurring over long periods of time.

There are several important aspects to the question of frozen versus fresh semen. There is no doubt that for a given specimen to be frozen and thawed results in a reduction of living sperm. The question may be considered from another point of view. If one has the time to prepare a group of highly fertile sperm specimens, freeze and store them, and then match these specimens against a group of so-called average, normal, fresh specimens, what will be the result? To the best of my knowledge, this type of comparison has not been done, but I believe that under these circumstances the frozen specimens would be at least as effective, if not more so, than the fresh semen. This question assumes great importance when it is realized that the use of a sperm bank such as IDANT enables a physician to obtain a specimen from over 500 donor profile types. It thus becomes possible for the first time to match effectively a donor with the sterile father's physical characteristics. This is obviously not the case where fresh semen is used and a gynecologist's "pool" of donors may consist of two or three males who can be available at the convenient time. An additional advantage of the use of frozen semen is the physician's ability to inseminate his patient on three successive days with a highly fertile specimen each day, thus increasing the probability of conception. In cases where a woman's ovulation is irregular, frozen semen can be maintained in the doctor's office and used only if, after examination, the physician feels that this is an optimal time for fertilization.

The question of physician responsibility and the law

in regard to artificial insemination is important. A growing number of states have enacted laws regarding the legality of children conceived by artificial insemination. In New York State, where IDANT is the only licensed sperm bank, laws have been enacted regarding the testing and selection of donors. One of these laws pertains to the culture of semen donor specimens at least 1 wk prior to use. In the case of frozen semen, it is possible to culture the actual specimen to be used for insemination. In the case of fresh donors, because a separate specimen would be required for culture, the procedure is usually not performed--although legally it should be. It is well-known that venereal disease is not limited in any group or subgroup, such as medical students, and the legal requirement for culturing semen donors is a correct one. I am personally aware of two cases of gonorrhea that have resulted from the use of fresh semen. We have, among our own donor population, occasionally discovered gonorrhea in asymptomatic donors who had previously been negative. This illustrates the necessity for continuous testing of all specimens and the inadequacies of occasional testing of donors.

Most of the donors at IDANT are obtained from the New York City university community, which provides an unusually diverse group of ethnic backgrounds. Potential donors have medical and physical examinations and are given blood-screening tests. At the present time, the combination of standards in use results in only 15 percent of all candidates who apply being accepted. All donors are paid $20 per acceptable specimen. Physicians utilizing the semen are charged $35 per specimen, plus shipping costs. All specimens are subjected to a logging system with six cross-check labeling points to insure proper identity. All donor names are kept separate and known only to the Medical director. Donors are known to the staff by a code number. Recipient physicians who wish to use a donor for repeat insemination do so by the code number. This insures confidentiality. This is most important, as this arrangement assures that the recipient is unknown to the sperm bank and the donor is unknown to the administering physician and his staff. In some instances, where fresh semen is used, the administering physician and some of his staff know the identity of the donor, which may lead to a break in confidentiality.

It is our feeling that in due course, the obvious advantages of appropriate semen selection will become more

widely accepted, and this may become the prime method of artificial insemination.

The laws regarding the operation of sperm banks have not been clarified in most states. The lack of commercial success of sperm banks has resulted in a lack of interest in further commercial development. It is important that the public be protected. IDANT uses FDA-approved containers and inseminators. IDANT operates under the laboratory laws of New York City and New York State--possibly the strictest in the United States. There is a Medical Director and a Medical Board consisting of physicians certified in the specialties of gynecology, urology, pathology, and oncology. This Board, with the addition of two lay members, comprises an ethics committee, which deals with policy decisions on matters not clarified by existing standards.

The IDANT experience in sperm banking will illustrate the problems faced by commercial pioneers in this field. At one time, IDANT had six branches in different cities. Between 1971 and 1974 it lost approximately $1 million of its original capital. It was saved by the efforts of several physicians and scientists who provided services without monetary remuneration. The sperm bank was restructured: Its branches were reduced to two outside New York City, and additional laboratory services were provided at a central facility in New York.

One of these branches is in Buffalo, N.Y., which serves the southern Canadian region and upper New York State area. This branch was established in conjunction with the Erie Medical Center, which specializes in gynecology. This branch, which provides an insemination service at a low cost, has recently completed its first year of service and has provided us with an effective model of how services might be expanded to other areas of the country. The Center has a Medical Director who supervises, a registered nurse who performs the inseminations, and a social worker with an M.S.W. degree who provides counseling assistance to the couples. The Center accepts referrals from physicians within its region for insemination services.

The fee for a series of three inseminations per month is $135, which includes the cost of the semen. In addition, processing services and facilities are maintained. Processed frozen semen is shipped for permanent storage to New York City. The processing service is particularly important because of its physical proximity to the Rosewell Park Memorial Hospital, which specializes in oncology. Because of its link to the central bank in New York City, the local

branch is able to offer the full donor profile of the central bank. This arrangement has also afforded us with an opportunity to control other variables, such as techniques of insemination. (We plan to publish our findings at a future date.) At the present time, I would merely wish to comment that we have discovered significant variability in insemination technique from physician to physician. This is an important variable in analyzing success rates from different sources.

The Erie Medical Center and Sperm Bank combination has also allowed us a closer follow-up of patients. Although the number of cases seen are small, results are very promising. In the year that sperm banking has been instituted (February, 1975-March, 1976), 21 patients have been inseminated. Eight became pregnant; three have already delivered, (two boys and one girl); one miscarried; four are awaiting delivery. Six women are still in treatment, five of which have only been inseminated for one cycle and one for two cycles. Four women have been referred for further testing to determine a possible female component to the infertility problem. Two women were found to have definite fertility problems. One discontinued due to personal problems, and one woman was lost to follow-up. Of the eight conceptions, four took place in the first cycle, two in the second cycle, and one each in the fourth and fifth cycle. These results were achieved at a low cost to the public by the skillful use of physician-directed paramedical personnel. We feel that this clinic has provided us with a possible model to provide similar services in other areas of the country.

In summary, while sperm banking has not been a commercial success at this preliminary phase, it has been a scientific success. The major changes in the abortion laws of the past few years have focused attention on the necessity for advancement in the therapy of infertility. Sperm banking would appear to be a most hopeful area for treatment of otherwise insoluble infertility problems.

ACKNOWLEDGMENTS

I wish to acknowledge the assistance of Constance English, R.N., Marcia Buhl, M.S.W., and Marilyn Buckham, Administrator, for their help in obtaining the data from the Erie Medical Center.

DISCUSSION

R. H. FOOTE: Testicular tumors may cause a reduction of semen quality through local and/or systemic effects. In cooperation with Dr. McEntee we have observed unilateral tumors that have reduced sperm numbers and increased the proportion of abnormal spermatozoa on the affected side. The contralateral side can be normal. Perhaps spermatozoa could be ejaculated from the normal side in cases of unilateral tumors by compression of the ductus deferns on the affected side.

WILLIAM N. FISHBEIN: It appears that, despite the clear efficacy of frozen-thawed sperm in producing fertilization, 20 to 50 percent sperm motility is lost after a single freezing cycle. To me this indicates that motility is a much more sensitive indicator of damage than is fertility (as presently assessed) and that there is much more room for improvement in procedures for the freeze preservation of spermatozoa.

A COMMUNITY'S HUMAN TISSUE RESOURCES AND THE ROLE OF THE COMMUNITY BLOOD BANK--CUSTODIAL OR DEVELOPMENTAL?

Victor H. Muller

It has now been just over half a century since the transfusion of blood has become an acceptable and useful procedure. Less than 40 yr have elapsed since the first blood bank was established and only about 30 yr since the first community blood bank was formed. In that time span the use of whole blood and/or its component red cells has grown to a national figure of between 8 and 9 million half-liter units annually. Data from the National Heart and Lung Institute Blood Resource Study indicates that of the 8,799,700 units of blood for transfusions drawn in 1971, 6,038,700, or 69 percent, were recruited, drawn, stored, processed, and subsequently shipped to transfusion service units by regional or community blood centers. Three points well established then are: (1) current medical practice requires extensive use of homologous tissue, (2) the requirement for homologous tissue is growing in amount and in kind, and (3) community blood banks are already playing a major or even a dominant role in the solution of the logistical problems encountered in the procurement, storage, and distribution of the tissue and cells used at present.

What factors operate in community blood banks as they exist or as they soon might be expected to exist in response to the Department of Health, Education, and Welfare proposal for a National Blood Policy? Factors that might lead one to believe that a community blood bank is uniquely suited to a broadened role in the area of cell, tissue, and organ banking. First of all, it is the organization of the community blood bank itself. This is generally a not-for-profit but self-supporting corporation. It stands free of local differences in those areas in which competing health care facilities develop strong but divisive loyalties. It is not just *a* community organization, but it is *the* community organization in relation to its own specific activity.

Secondly, the community blood banks are already related to each other so as to form a basis for regionalization. Even though regionalization is not yet universal in a geographical sense, it is better developed than many realize in that national organizations of community blood centers already exist and that through these organizations universal policies, standards of operation, and use of technical methods are already in effect. Voluntary acceptance of certain inspection and accreditation programs is widespread, and, at the risk of being presumptuous, I say that most blood bank personnel are comfortable with licensing requirements and procedures. Communication in the field is rapid and effective through existing journals and newletters. There is a definite, although not unlimited, tendency toward a cooperative sharing of resources.

Governance of the progressive community blood bank is broadly based and representative of both professional and lay view points. It provides an open view of operation to the community it serves and thereby enlists community support.

At the functional level the typical community blood bank brings administrative, medical, technical, and volunteer help to bear upon the work to be done. Through these various disciplines the necessary activities related to public (donor) education and motivation can be effected. The problems of informed consent and donor recruitment are well known. The community blood bank employee who interfaces with the donor is trained and suited to the development of the special bond that forms between them. At the administrative level, common language allows data sharing and comparison studies.

Additionally, the community blood bank is already staffed by the professionals with the knowledge (or with a background that allows for its development) of cell and tissue collection and storage. The entire story of donor recruiting, record keeping, identification, confidentiality, uninterrupted service, quality control, precision processing, controlled and monitored storage, and shipping is repeated daily. Equipment for refrigerated and frozen storage is standard, and because of the bank's organizational free-standing structure the community is able to draw upon the entire range of talent available in the community.

In short, all of the mechanisms that need to exist to bring about the willing donation of an individual's own tissue and the proper acceptance of that donation are already present, although perhaps not fully developed,in the community blood bank.

The institution now generally recognized as a community blood bank or regional blood center represents a logical repository for a community's cell and tissue donations. It does so because of its free-standing, community-owned nature and governance and because of the ease with which regionalization could be affected. It does so because its development has been oriented to the purpose of tissue collection and storage. It does so because it has a staff attuned to the need for public information and education in the area of volunteer donation, and it does so because of its existing professional and technical staff.

The role of the community blood bank should be both custodial and developmental.

SEMEN BANKING TODAY: A REVIEW OF CURRENT TECHNOLOGY IN EQUIPMENT FOR THE STORAGE OF SEMEN AT LIQUID NITROGEN TEMPERATURE

R. R. Conte and K. A. Rensberger

CRYOGENIC REFRIGERATOR CHARACTERISTICS

A cryogenic refrigerator is designed to store products in sealed packages (Figure 1) such as glass ampules, plastic straws, or specimen cans (blood, tissue, culture, etc.). The storage capacity of a container for any particular package obviously depends upon the package shape and the method of racking individual samples for inventory purposes. A portable cryogenic refrigerator generally utilizes cylindrical storage canisters, which are lowered through a small neck opening (less than 5 in.) into the liquid nitrogen reservoir. The storage capacity of a portable container thus is a function of the neck tube opening, for any given package and rack combination (Figure 2).

All cryogenic refrigerators use a liquified gas (usually nitrogen) as a refrigerant. They also consume the cryogen at a relatively constant rate, which maintains the cold-storage temperatures. The magnitude of the consumption rate (usually expressed in liters per day) is a result of the thermodynamic heat leak reaching the fluid by a combination of conduction, convection, and radiation transfer. The most effective method of insulating cryogenic reservoirs is by superinsulation, because this method minimizes all three modes of heat transfer. Superinsulation consists of alternating layers of paper and foil in an evacuated annular space. Solid conduction is reduced by the paper until almost all heat transferred by conduction travels down the neck tube. The evacuation eliminates convection, with only a small amount of free molecular conduction remaining. Highly reflective foil is chosen, and these layers act as barriers to radiative energy. The most critical aspect of superinsulation is the maintenance of the vacuum; this is accomplished by

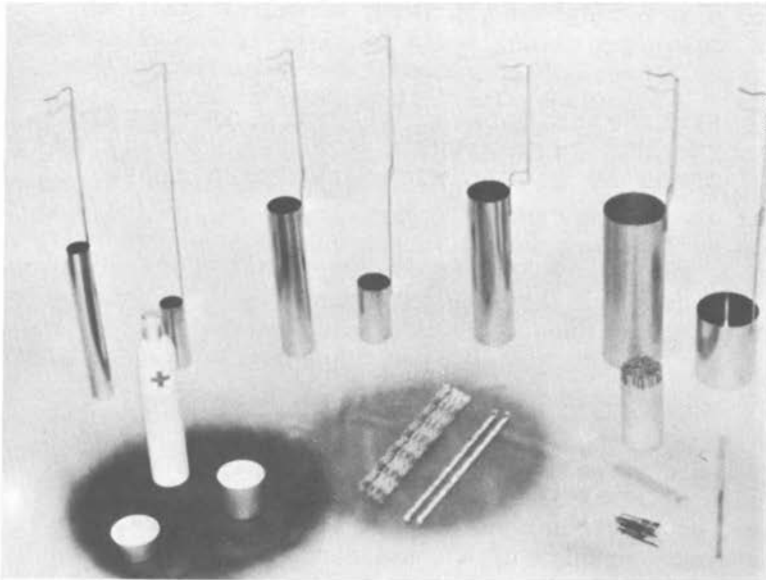


FIGURE 1 Cryobiological packages.

depositing quantities of molecular absorbents and getters in the annulus, which trap or react with outgassing molecules for many years.

Storage temperatures inside a cryogenic refrigerator are not constant. The reservoir always has a level of cryogenic fluid under a column of vapor, and the fluid maintains a constant temperature by continually boiling away more vapor. The vapor has a temperature gradient due to loss of sensible heat as it travels upward and out of the refrigerator.

There are three points of constant temperature in cryogenic refrigerators: the top neck, the bottom neck, and the liquid surface. Between these relatively constant points, the vapor temperatures vary, as shown in Figures 3 and 4.

SEMEN BANKING AT LIQUID NITROGEN TEMPERATURE

There are four fundamental components necessary to equip a semen bank (Figure 5) for safe, long-term storage at liquid nitrogen temperatures: a bulk liquid nitrogen

supply, cryogenic transfer lines, the bulk capacity cryogenic refrigerator, and liquid nitrogen level control and alarm equipment.

The bulk liquid nitrogen container is usually a low-pressure liquid withdrawal vessel with a capacity large enough to maintain refrigerator liquid levels for practical lengths of time. Optimum storage efficiency can be obtained if consideration is given to the balance between liquid nitrogen delivery costs and losses during the storage and transfer of liquid nitrogen.

Bulk liquid nitrogen containers most often are located outside of buildings to facilitate delivery of liquid nitrogen; this location requires some form of insulated transfer system for carrying the liquid to the

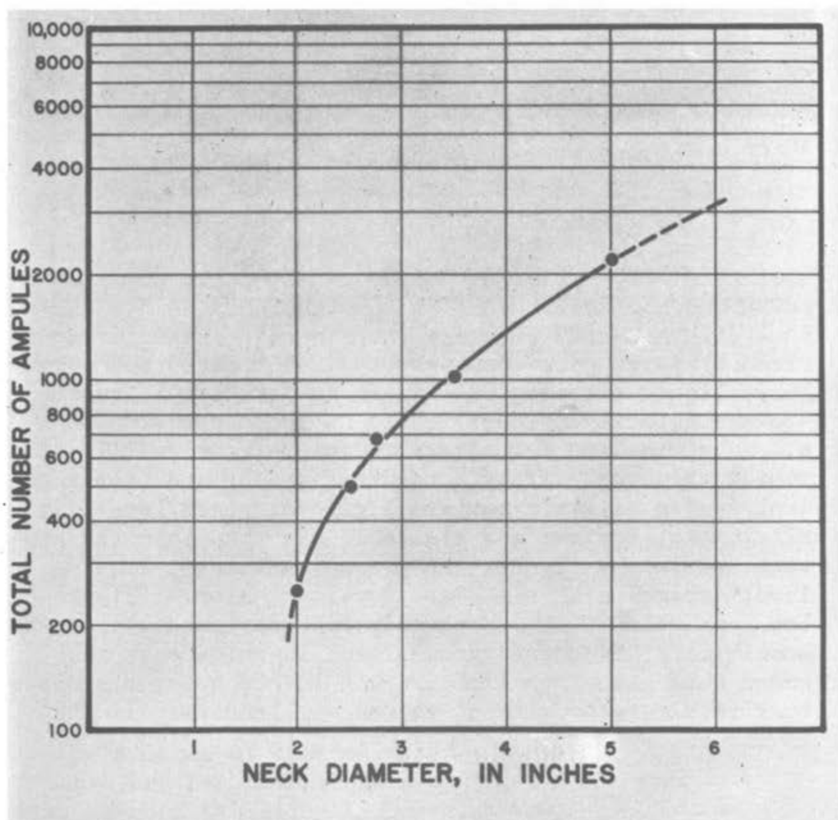


FIGURE 2 Total number of ampules vs. neck diameter.

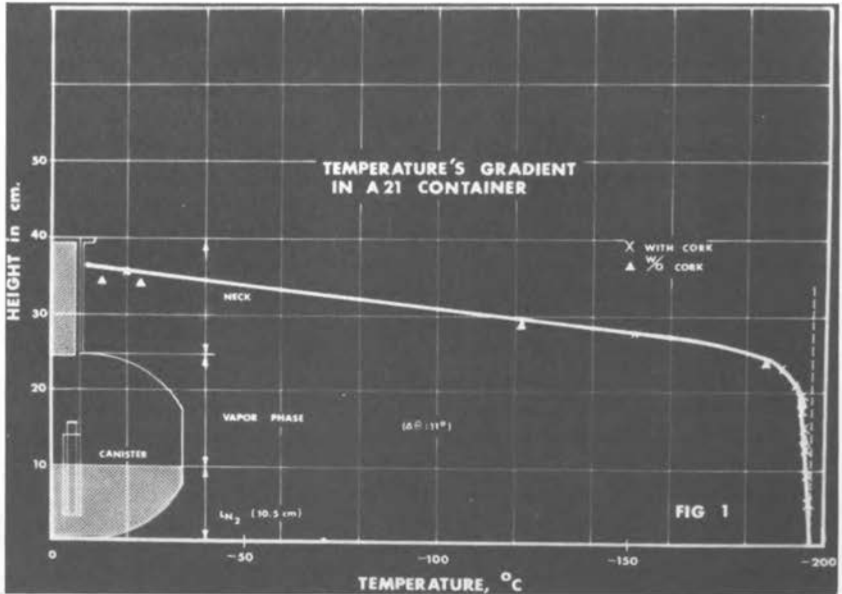


FIGURE 3 Temperature gradient, A-21.

refrigerators, either a foam or vacuum-jacketed line being the most common.

Bulk capacity cryogenic refrigerators are characterized by large neck openings (15 in. or more) and very large liquid nitrogen reservoirs to permit bulk semen-storage capacity. Their liquid nitrogen consumption allows operations for several weeks between refills of fluid. However, safety considerations demand that a control system maintain maximum liquid nitrogen levels at all times. Systems now available are tied into the storage and supply circuit and provide automatic liquid-level control with high- and low-level alarms (Figure 6). Low-level alarms are absolutely necessary because of the possibility of sudden vacuum loss, in which case the semen bank has approximately 24 h before a superinsulated bulk refrigerator without vacuum will consume all its liquid nitrogen.*

*MVE A-9000 w/o vacuum loses 100 liter/day.

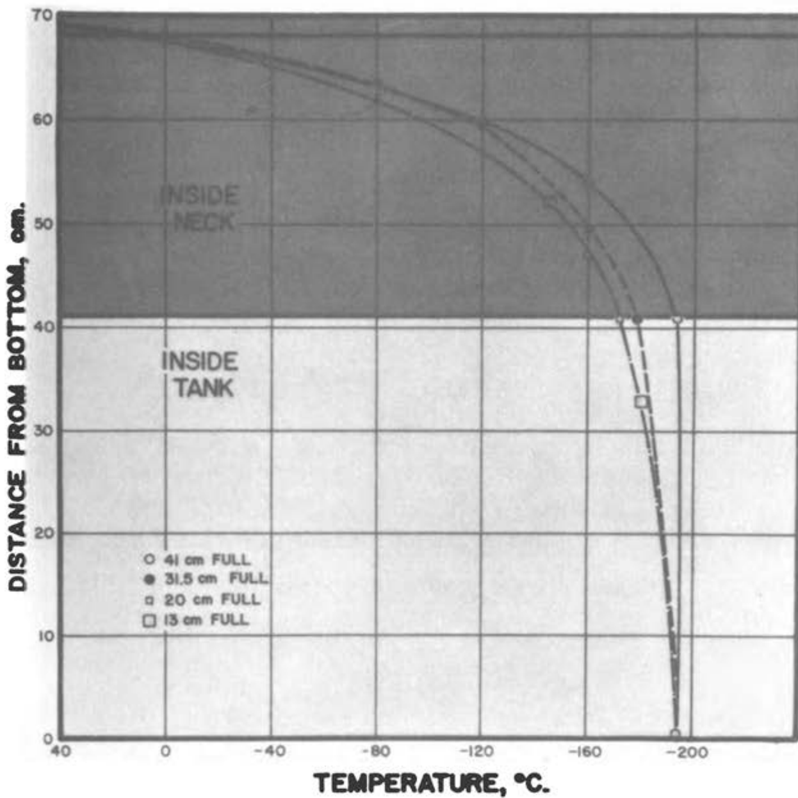


FIGURE 4 Temperature gradient, CF-400 (open).

A comprehensive inventory control system is necessary in any banking operation. Sealed glass ampules on marked canes are an example of a proven system in bull semen banking (Figure 7).

Distribution of semen is made with portable shipping containers and stored in portable refrigerators. Dewars of this type need only be refilled with liquid nitrogen once in 8 to 16 wk (Figure 8).

The science of thermodynamic efficiency in cryogenic refrigerators has changed by a factor of 10 in as many years. Minnesota Valley Engineering can trace the history of technological improvements over the years in containers offering similar refrigerant and product storage

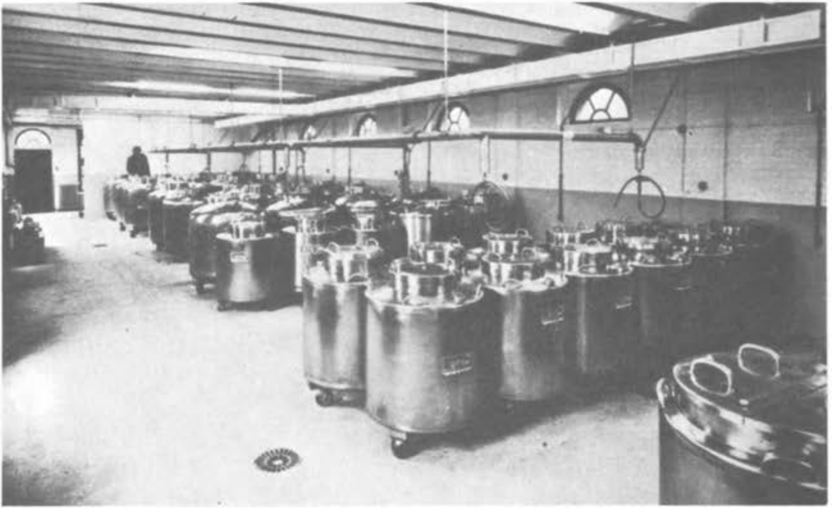


FIGURE 5 Semen bank in operation.

FIGURE 6 VPS-3500 with liquid level controller.

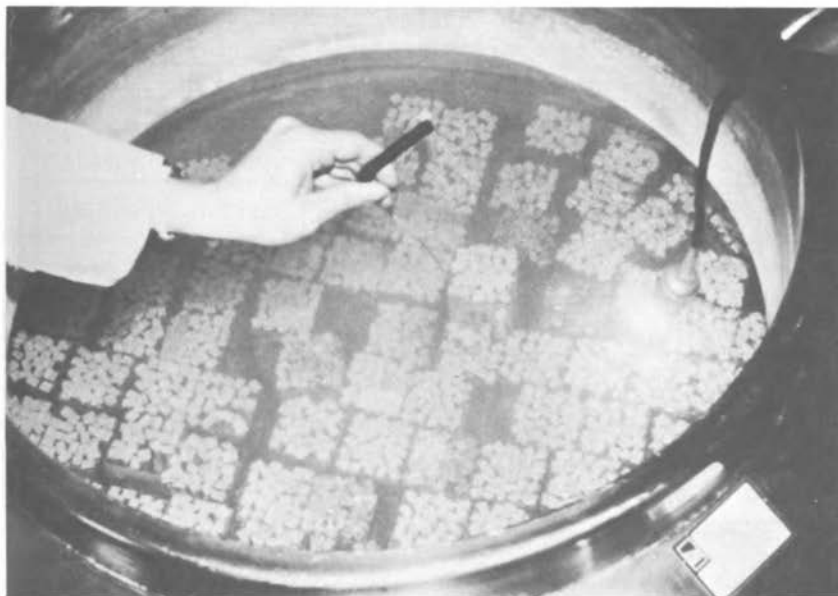


FIGURE 7 A-8000 with glass ampules on canes.

FIGURE 8 Apollo SX-34 portable refrigerator

capacities to illustrate this evolution. Decreases in nitrogen consumption results in comparably lower expenses and maintenance costs (Figure 9).

Along with the advances in refrigeration efficiency, better refrigerators have lowered the operating costs of storing semen at liquid nitrogen temperatures (Figure 10).

THE CURRENT SITUATION

Complete systems can be supplied at this time to provide liquid nitrogen storage and transfer equipment, liquid-level control and alarm circuitry, bank refrigerators, sub-bank refrigerators, and distribution containers for shipment or storage. The sizing of vessels and piping is

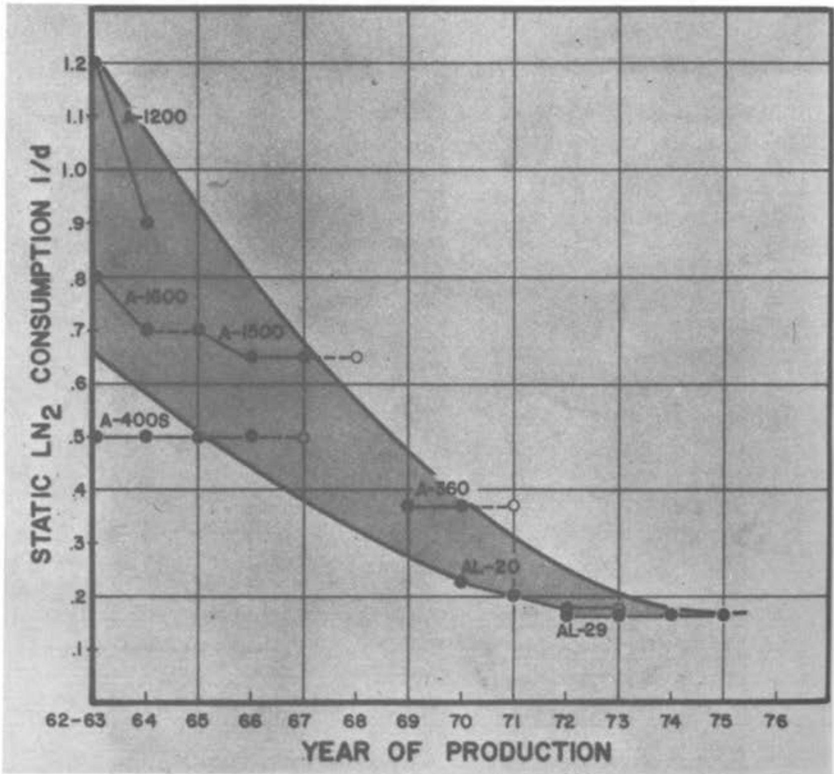


FIGURE 9 Static LN_2 consumption vs. year of production.

REFRIGERATOR MODEL	(1975) LIST PRICE	SEMEN STORAGE CAPACITY		OPERATING COST $/YEAR/DOSE	
		MINITUBE STRAW	1ml amp 6/cane	MINITUB	1ml ampule
AL-20	282.00	1920	252	.059	.450
AL-29	345.00	1920	252	.065	.495
APOLLO SX-34	382.00	3630	684	.038	.202
A-7000	1155.00	90,000	6600	.012	.164
VPS-3500	3700.00	290,000	24,450	.011	.130
LZ-100	7600.00	440,000	49,500	.008	.071

FIGURE 10 Economic analysis of semen storage.

FIGURE 11 Semen retrieval from A-29.

FIGURE 12 Transfer to Mini-Cryo.

well known in the bull semen industry, and optimum systems can be assembled for human semen banks with consideration given to the inherent differences.

The logistics of handling human semen, as well as its value, represent peculiar characteristics in semen banking. Distribution of small quantities of semen is probably going to be done often. For this application the Mini-Cryo has been developed by Cryo Diffusion of Lery, France, for shipping small doses in vapor phase (Figures 11 and 12).

It should also be emphasized that safety considerations are very important; a level control and alarm system should be a fundamental component rather than an accessory item in the bank facility. An additional component that is in developmental stages is a maximum temperature indicator designed for cryogenic storage, and this will certainly have many possibilities when perfected.

HISTORY OF ARTIFICIAL INSEMINATION AND THE DEVELOPMENT OF HUMAN SEMEN BANKING

J. K. Sherman

ARTIFICIAL INSEMINATION

The introduction of semen or spermatozoa into the genital tract of a female, for the purpose of progeny production, by means other than that used in natural coitus, is referred to as artificial insemination or AI.

The principle of AI is part of Hebrew heritage, written into the ancient Talmud in the third century (Hag. 15a). It evolved from a hypothetical case presented to a Hebrew sage regarding the adultery status of a virgin who was impregnated by semen ejaculated earlier into water in which she innocently bathed. It was the opinion of the wise man, in this the first literary reference to either human or animal artificial insemination, that this unnatural event, without coitus, did not constitute an act of adultery. It was not a Jew but an Arab, however, who was the first practitioner of this art with animals, not humans, much later, in the fourteenth century. Under the cover of silence and darkness, the Arab in 1322 induced the ejaculation of a rival's prize stallion into a cloth. He then introduced the semen-laden cloth into his mare's vagina with subsequent successful pregnancy and foaling. This was the shady beginning for what has developed into a respectable international multimillion dollar breeding industry.

In 1949 the accidental discovery of glycerol as a cryoprotective agent was the key to the future development of banks for animal, primarily bovine, semen. Cryobanking has permitted frozen storage of semen from thousands of prize sires for the genetic improvement of millions of animals annually within a country and of thousands through shipment of frozen semen by common carriers between countries.

John Hunter, a Scottish surgeon, is credited with being the original practitioner of AI with humans, in

England, sometime between 1776 and 1779. Ten births from AI were reported in France in a paper by Girault in 1833. It was not until 1866, however, that the practice was introduced into the United States, through a description of the procedure in a publication by Marion Sims. Documented cases of insemination in all countries up to the latter part of the nineteenth century were with husband semen (AIH), with few if any known attempts made with donor semen (AID). It was the clandestine clinical practice of Robert Dickerson, however, who used donor semen in cases of male sterility, which initiated AID in this country in 1890. Since that time there has been gradual evolution of acceptance and use of AID throughout the world, especially in the United States.

Today the overwhelming majority of inseminations are performed with donor semen, a therapeutic medical practice for infertility, which is approved by the American Medical Association. An estimated 10,000 to 100,000 births per year results from AID, a figure that reflects but a surprisingly small percentage of use by couples with infertile male partners. It is said that this performance, which is far removed from universal acceptance, reflects the same approach many patients and some doctors had to contraception about 50 yr ago. The negative reaction of many people to AID is still largely emotional or religious, in spite of its documented beneficial effects on family life.

DEVELOPMENT OF HUMAN SEMEN BANKING

The history of frozen human semen is intimately related to the initiation and development of the field of cryobiology. Human spermatozoa were among the first cells to be observed as to effects of freezing. The events in the establishment of cryobanks closely parallel the sequence, if not the degree, of those with bull semen, which provided such a stimulus to the growth of low-temperature biology. Highlights of these events are summarized in Tables 1 and 2.

Spallanzani in 1776 was perhaps the first to report observations on the effects of freezing temperatures on human spermatozoa, and Montegazza in 1866 was the first to suggest banks for frozen human semen. During the period of 1938 through 1945, it was observed that a small number of human spermatozoa could survive freezing and storage at temperatures as low as -269°C. The possibility of preservation by freezing of significant numbers for prolonged periods subsequently arose from the successful use of

TABLE 1 Development of Human Semen Bryobanking (1776-1964)

Date	Contributor	Contribution
1776	Spallanzani	First low-temperature observations
1886	Mantegazza	First suggestion of frozen semen bank
1938	Jahnel	-269°C. survival; storage at -79°C.
1940	Shettles	Individual variation, aging, and thawing
1942	Hoagland and Pincus	Vitrification principle; foam freezing
1945	Parkes	Survival better in greater volumes
1949	Polge, Smith, and Parkes	Glycerol as cryoprotective agent
1953-55	Sherman	Freezing rates; glycerol; preservation
1953-55	Bunge, Keettel, and Sherman	First progeny from stored spermatozoa; dry ice method
1954-59	Keettel *et al.*	Sixteen births with stored spermatozoa
1958-59	Sawada *et al.*	Six births with stored spermatozoa
1962-63	Sherman	Survival factors; banking applications; nitrogen-vapor technique
1964	Perloff, Steinberger, and Sherman	Four births with nitrogen-vapor technique

TABLE 2 Development of Human Semen Bryobanking (1964-present)

Date	Contributor	Contribution
1964	Sherman	DMSO unsuitable (toxic) as cryoprotectant
1968	Iizuka *et al.*	More births from semen with egg yolk extender
1968	Ackerman	Metabolic effects: temperature shock, freeze-thawing
1969	Matheson *et al.*; Trelford and Mueller	Births from semen stored in plastic straws
1969	Fjallbrant and Ackmerman	Fertility after 3-yr storage; mucus test
1968-70	Ackerman and Sod-Moriah	Stability of DNA, hyaluronidase level and citric acid in frozen storage
1970	Sherman	Normal progeny from semen stored over 10 yr
1972-73	Sherman	Motility and Y chromosome fluorescence unaltered after 13 yr of frozen storage
1974	Barkay, Zuckerman, and Heiman	Pellet freezing of diluted semen: births
1976	Sherman	About 1,500 births worldwide; fewer abnormalities and abortions than in normal population

glycerol as a cryoprotective agent, first with fowl and then with mammalian spermatozoa, starting in 1949.

The emphasis of attempts at cryopreservation, though, was placed upon semen of farm animals, with the coincident neglect of research on developing techniques for man. It was not until 1953 and 1954 that reports of successful research with human semen at "dry ice" temperatures, along with the first demonstration that frozen-thawed human spermatozoa were capable of fertilization and induction of normal embryonic development, were made. About 25 births resulting from frozen human semen were reported in the next 9 yr. During the period of 1953 to 1963 various factors of cryosurvival were evaluated in the development of a liquid-nitrogen-vapor technique for the cryopreservation of human semen and other cells. The method was successful in clinical application with the reported births of four healthy children in 1964. The state of the art at this time was expressed as follows, "We now have available a simple efficient and clinically proved method for frozen-storage of human spermatozoa, suitable for immediate but planned application."

Today, as in 1964 and in 1973 when the topic was reviewed, one has reason to ask why there has not been considerably more activity in human semen banking, especially in view of research that confirmed principles and extended clinical findings (Tables 1 and 2). There were improvements since 1964 both in frozen-storage methodology and in medical acceptance, as well as in the accompanying increased clinical use of donor insemination with fresh semen. The advantages of semen banking for applications in infertility and population control have been reviewed again here. Their benefits to mankind have not been realized to an appreciable degree, mainly because of the following reasons:

1. Conditions in medical practice, especially the requisite demand for clinical application of frozen-stored semen and the economic balance of time and effort in infertility cases, even with fresh semen, still are not conducive to a favorable rate of development.
2. Commercial banks have not developed as well as anticipated in the predicted dependent relationship between increased demand for semen by men who will undergo vasectomy and the success of commercial banking. Most men have shown little interest or motivation in maintaining their seed by prevasectomy collections of their ejaculates for frozen storage.
3. Most important to the restriction of progress is

that both the lay and medical publics are not convinced that frozen storage of human spermatozoa, with subsequent AI either in infertility or population control, is a safe and efficacious clinical procedure. Unsubstantiated and unscientific published warning and expressed fears by certain medical groups have raised doubts concerning the genetic stability of frozen-stored spermatozoa with possible induced production of malformed progeny. Aspects of this problem, especially in manifestations of aging *in vitro,* have been discussed earlier in the volume.

Current tabulation of available results of AI with frozen-stored human semen show about 1,500 births with fewer abnormalities and spontaneous abortions than in the normal population (Table 3). With rare exceptions, however, storage time was no more than a year. In spite of the appreciable evidence against cryo-induced genetic mutations during short-term storage of human semen and long-term storage of bull semen and other mammalian cells, it is essential to have data on fertility and births with human semen stored for much longer periods. Future development of banking human semen will depend heavily upon the establishment of an absence of such possible adverse aging effects during prolonged storage for periods of 5 to 10 yr.

TABLE 3 Births From Frozen-Stored Human Semen[a]

No. of normal children born	1,464
No. of abnormal children born	11
No. of spontaneous abortions	113
No. of pregnancies still unreported as births	71
Longest period of cryopreservation of fertility	
Asia (Iizuka; Japan)	13 mo
Europe (Lebech; Denmark	5 yr
United States (Sherman)	10 yr
Before international shipment (Sherman, U.S.)	6 yr

[a]Pregnancies for births reported followed, on the average, a protocol of 2 inseminations of 0.5 to 1 ml of semen in each of 4 cycles, aside from results of one clinician who routinely inseminates 14 times per cycle.

There is little doubt that the use of frozen-stored semen will continue, but progress toward realizing its potential will be extremely slow unless it receives authoritative approval. In the interim, guidelines and controls, especially in ethical and safe methods and practices of commercial banks, are needed both to protect the public and to give proper medical status to this important area of development in modern medicine. As I suggested in the Tissue Bank Symposium in 1975, peer group input on standards for such guidelines or regulations is of critical importance to realize this goal.

BIBLIOGRAPHY

Behrman, S. J. 1975. Artificial Insemination. In: Behrman, S. J., and Kistner, R. W. *Progress in Infertility*. Boston, Little Brown. p. 779.

Sherman, J. K. 1973. Synopsis of the use of frozen human semen since 1964: State of the art of human semen banking. Fertil. Steril. *24*:397.

Sherman, J. K. 1976. Banks for frozen human semen: Current status and prospects. In this volume.

A PLAN FOR THE CONTROLLED GROWTH AND DEVELOPMENT OF TISSUE BANKS*

K. W. Sell and G. E. Friedlaender

The desire to transfer healthy tissues from a donor for the purpose of replacing or replenishing diseased, injured, or insufficient tissues in a recipient has existed for hundreds of years. Transplants of bone and transfusions of blood between animals led to successful human allografts during the nineteenth century, and this approach has become a routine in modern medicine. Artificial insemination likewise has a long and similar history. Of all potentially useful tissue allografts, however, blood and blood products have received the closest scrutiny, resulting in comprehensive guidelines, regulations, licensing, and accreditation. The evolution of controls and constraints within the field of blood banking over the past 30 yr should serve as an important example for other areas of human transplantation. Guidelines and regulations serve to protect the tissue recipient as well as the individuals involved in donor selection, tissue procurement, processing, distribution, and patient care. Standards and quality are assured. It would be a mistake, however, for these same regulations to stifle basic science and clinically related research concerned with improved technological development and patient care.

It should become readily apparent that the most intelligent guidelines, ensuring high standards and quality but avoiding unreasonable and repressive constraints, can best

*This work was supported by the Naval Medical Research and Development Command, Work Unit No. MPN06.01.0003AAJ2. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the U.S. Navy Department or the naval service at large.

be developed by the peer group, that is those technicians, scientists, and clinicians involved in the varied aspects of the tissue's banking and clinical application. It was in this manner that the field of blood banking first developed guidelines that could, whenever necessary, be adopted as regulations and enforced by the federal government (FDA) and that led to a basis for accreditation and licensure.

Today a variety of viable and nonviable cells, tissues, and organs have demonstrated potential and/or realized clinical efficacy. Certainly the problems of tissue banking extend far beyond blood and semen and so must the development of reasonable guidelines. The use of skin allografts and xenografts has become standard for the treatment of major burns; over 200,000 bone grafts are performed in this country each year with an increasing proportion involving preserved allografts; and the widely publicized and accepted transplantation of corneas and kidneys also require attention.

None of these other tissues are presently governed by guidelines or regulations. There are no standards for their selection, procurement, or processing, and no control over their clinical application. In the case of kidney transplants, the federal government may pay as much as $3,000 to $5,000 to acquire an organ for a patient, but not a single guideline or regulation exists for donor selection, organ perfusion, or any other aspect of the transplant process.

The Navy has been involved in tissue banking since 1950 and recognizes the importance of allografts in the field of reconstructive and reparative surgery. It was for this reason that the Naval Medical Research Institute jointly cosponsored the second day of this conference along with the Bureau of Biologics.

It has also become apparent to those of us involved in research and clinical application of various tissue allografts that many of the problems related to the banking of one particular tissue may have important applications or implications for other transplantable tissues. In addition, those individuals responsible for technological advances include those involved in all levels from donor selection and tissue procurement to preservation to clinical care. In order to develop the most widely based and responsive peer group for discussion and definition of guidelines, it is necessary to include input from all of these individuals. It is equally important that peers involved with any one transplantable tissue work closely

with those involved with all other efficacious grafts. This will ensure the broadest input concerning common problems and hopefully common solutions, and it will also avoid guidelines in one field that may unnecessarily slow technological progress in another.

An appropriate forum for this type of peer group interaction may be the American Association of Tissue Banks. Within this framework, individuals involved in all phases of tissue banking and transplantation may interact and develop the guidelines required for the safe and efficient continued growth in this field. These guidelines may then, when necessary, be used by the federal government as a basis for regulations, and accreditation by the AATB may serve as a basis for licensure. Hopefully what took over 30 yr for the blood bank can be efficiently shortened for the remaining areas of tissue banking and transplantation and yet preserve the most important element of peer group input.

DISCUSSION

J. K. SHERMAN: It is particularly significant that the concept of peer group as the source for the derivation and input of guidelines and controls for cryobanking of human semen introduced in our last year (Sherman, Graham, and Walters, 1976) was so enthusiastically endorsed by Dr. Sell. His experience and contributions in tissue banking, along with the proposed establishment of the American Assocation of Tissue Banks, should prove valuable in our attempts at organizing a peer group of researchers and practitioners in frozen semen banking. The aim would be to develop a close working relationship of this peer group with the FDA in the evaluation of standards, guidelines, and controls, as needed, in this sensitive area of medicine.

MORRIS SCHAEFFER: About voluntary versus regulatory approaches to the maintenance of standards: As one with long experience in the development of a clinical laboratory improvement program by regulation and inspection, I have come to the following conclusion. Voluntary methods make good laboratories better. They do nothing for the bad laboratories. To emphasize Dr. Feldschuh's remarks, so long as the activity is small and there is little profit, the groups control

themselves well. As soon as there is money to be made, the entrepreneurs get in to make it, by hook or crook. Then regulation becomes necessary.

GARY FRIEDLANDER: The development of guidelines and/or regulations for semen (and tissue) banking should rely heavily upon peer group input. This peer group must include individuals knowledgeable about all transplantable tissues for at least two reasons. Clinical and basic research related to donor selection, tissue procurement, processing, distribution, and evaluation of results represent common concerns and have produced data that often is of relevance to all tissue "bankers" and "users." In addition, a peer group developing guidelines or regulations in one specific field should be aware of the potential implications these statements will have on the basic science and clinical research programs associated with other transplantable tissues.

RUNE ELIASSON: In my opinion there is a need for regulations related to: (a) *Donor selection*. Minimal requirements for the selection of donors should include a physical examination. (b) *Selection of couples*. Should one accept couples when it is obvious that the man cannot be the father because of a condition such as hypogonadism? With the increase in biological education, can we foresee that the children will find out that the father has a condition such as Kleinfelter's syndrome or severe testicular atrophy? (c) *Follow-up*. At the present time those in favor of AID are gynecologists, while those in opposition to it or who question the "beneficial effects" are psychiatrists. I regard it unfortunate if one physician is responsible for the selection of couples and follow-up. This should be a joint project to guarantee objectivity.

BIBLIOGRAPHY

Sherman, J. K. 1976. Clinical use of frozen human semen. Transplant. Bull. In Press.

Sherman, J. K., Graham, E. J., and Walters, L. 1976. Banking of frozen spermatozoa and embryos. Cryobiology *13*:11.

ARTIFICIAL INSEMINATION IN CLINICAL PRACTICE

Joseph Feldschuh

The change in abortion laws in the United States has resulted in a sharp decrease in available infants for adoption. Male infertility, where the female is normal, is a potentially treatable condition by the use of anonymous donor semen. At the present time, an estimated 50,000 infants are conceived by anonymous donor insemination. Only approximately 1,500 are known to be from the use of frozen semen. The remainder, therefore, are primarily from fresh human semen.

Sources for this donor semen vary depending on locale. In many instances, a gynecologist will have a small pool of two or three donors who must be available during office hours to provide a fresh specimen. Donor matching under these circumstances is often related to race. The use of fresh semen necessitates a donor who can be available at the precise day and hour when the recipient woman is to be inseminated. Because of this logistics problem, it is difficult to develop a stable population of donors who have been evaluated both from the medical-genetic point of view and who have reasonably consistent semen of good quality. In New York State there is a law requiring semen culture 1 wk prior to use of a fresh specimen to rule out gonorrhea. In actual clinical practices, fresh semen donors rarely have cultures performed, even in New York State where it is mandated by law. Where frozen semen is used, the actual specimen may be cultured. In addition to possible transmission of gonorrhea or syphilis, there is the problem of confidentiality.

In most cases, the physician performing the insemination knows both the donor and the recipient. The identity of the donor may also be known to the physician's staff. These problems do not occur where the physician selecting donors at a sperm bank is separate from the physician

performing the insemination. Adopted children have identity problems that vary from minimal to serious ones requiring psychotherapy. When anonymous donor insemination is used these identity traumas need never occur. Children born this way are natural children of their mother, who, of course, goes through a full pregnancy and childbirth. Under these circumstances, no one need ever know that this particular child is not the natural child of a particular set of parents. Confidentiality is therefore very important to protect parents and child.

The advent of sperm banking has for the first time permitted the development of a broad profile of donors. Where hundreds of donors are available, it is possible to obtain a relatively close match. It is important that there be recognition of the "recipient father's" problem. It has been my observation that there is much less of an identity problem on the part of the potential father when he feels that the donor is very similar to him. Interestingly enough, though, occasionally parents will request a slight improvement over the father in some physical characteristic, such as where there is short stature. Of course any genetic or undesirable traits are eliminated when a donor is selected. The sperm bank can then be used when both parents carry sickle-cell trait or Tay-Sachs. Blood types can also be matched when there is a large number of donors.

At the present time it is possible to ship semen via air freight to any area in the United States. It thus becomes possible to provide the resources of a central sperm bank to a broad area of the country. At the present time the use of sperm banks for donor insemination has been limited. There has been the additional problem that sperm banks have not been able to maintain themselves financially without outside assistance.

THE CONJOINT ARTIFICIAL INSEMINATION TECHNIQUE

Leon Zussman

I wish to describe the practice of the "Conjoint Artificial Insemination" technique to introduce humanistic methods and mitigate the cold and impersonal connotation of using frozen sperm for human reproduction. This is a major problem.

Ten percent of all males are sterile. There are still questions concerning the legal status of babies born through artificial insemination. The State of New York licenses the practice of artificial insemination, nevertheless the legal requirement for culturing semen specimens is 95 percent ignored. Only 15 percent of the donor candidates are accepted and are paid $20 for an acceptable specimen (100 million cells). The charge to the recipient is $35 plus shipping costs.

The scientific and technological advances made in preserving the integrity of human spermatozoa in the frozen state are remarkable. However, the real resistance to its acceptance by the public is emotional. The "Conjoint Artificial Insemination" technique facilitates clinical practice by introducing humanistic methods whereby we hope to mitigate the sometimes cold and impersonal connotation of using frozen sperm for human reproduction. In this method, the recipient is not merely the woman, but the couple. The guilt, the fear, the ego depreciation that a man feels when he cannot impregnate his wife could be devastating. To help overcome this feeling, he is encouraged to be an active participant in the process of artificial insemination.

The husband should be told that without his help and cooperation this baby could not be created. He is instructed to take the basal body temperature of his wife daily and to bring her to the office just before ovulation. The husband is urged to be present during the insemination and

assist in the procedure. If he is adept, he is asked to draw up the semen in the syringe and inject it into the cervix. Of course, the doctor is present and carefully supervises every move. At every step the husband is made to feel that he is in the driver's seat, and this is to be his baby: ethically, morally, legally, socially, and economically, if not biologically.

The "Conjoint Artificial Insemination" technique is a logical corollary of the conjoint examination that has been utilized in the treatment of couples with sexual dysfunction at the Human Sexuality Center at the Long Island Jewish-Hillside Medical Center. Modifications of this method are used prenatally, premaritally, and during consultations for contraception, abortion, or menopause.

The guiding principle is that the couple is the patient, not one individual. Couples have been overwhelmingly enthusiastic with this approach.

The advances made by scientists in the preservation of human semen are outstanding. Their utilization by a couple who wants a baby can be accelerated when those concerned adopt a warm, humanistic attitude, and by encouraging active participation by the husband.

MICROBIAL CONTAMINATION OF HUMAN SEMEN

Philip D. Noguchi, Joseph E. Kiss, and
John C. Petricciani

INTRODUCTION

This paper will introduce briefly the broad subject of microbial contamination of human semen, with particular emphasis on bacteria. The medical implications of such contamination also will be discussed.

In order for bacteria to survive and grow they must be provided with a nutritive medium. Semen offers such a milieu in that it contains a variety of energy sources, as well as other substances necessary for the support of spermatozoa. Table 1 lists the most important biochemical constituents of human semen and suggests that it is not only sufficient to maintain spermatozoa, but it should be able to support the growth of bacteria as well. And indeed, a variety of microorganisms have been isolated successfully from human semen. These organisms include chlamydia,[2] mycoplasmas,[3] viruses such as cytomegalovirus (CMV)[4] and herpes simplex virus type II,[5] and hepatitis antigen B.[6] In addition, it has been demonstrated recently that the gonococcus, long thought to be a rather fragile organism, can be cultured from human semen kept at room temperature for up to 8 days and can be recovered from semen frozen at -196°C.[7]

Because of these reports and our interest in semen as a biological product, we recently initiated a small study to confirm for ourselves, the presence of various contaminating microorganisms in human semen.

METHODS AND MATERIALS

Table 2 outlines our general procedure for semen collection and processing. Volunteers were recruited from among the

TABLE 1 Some Representative Biochemical Constituents of Human Semen[a]

Sugars	*Concentration*
Fructose	225 mg %
Inositol	50 mg %
Nitrogenous bases	
Spermine	50-350 mg %
Vitamins	
Vitamin B_{12}	300-600μμg/ml
Amino acids	
Glycine	59 mg %
Serine	112 mg %
Metal ions	
Zn^{++}	14 mg %
Mg^{++}	14 mg %

[a]Mann, 1964.[1]

TABLE 2 Semen Collection and Processing

1. Wash hands and penis with soap and water X2
2. Rinse with isopropyl alcohol X2
3. Masturbate
4. Collect semen in sterile glass jar
5. Semen liquification (1/2-1 h)
6. Microbial isolations:
    a. GC (0.2 ml)
    b. other bacteria (0.2 ml)
    c. mycoplasmas (0.4 ml)
    d. hepatitis B Ag and Ab (0.2 ml)
    e. viruses (cell culture) (0.2 ml)

male patients of a private physician. Donors were instructed to wash hands and penis thoroughly with soap twice and then rinse with isopropyl alcohol twice. Semen was collectted by masturbation into sterile glass containers. After the semen had liquified, portions of the ejaculate were tested for gonococcus,[8] other bacteria,[9] mycoplasmas,[10] hepatitis antigen B, and antibody[11] and viruses.[12]

RESULTS

Table 3 summarizes our findings. A total of 26 donors gave 36 specimens. Of 29 specimens tested for bacteria other than gonococcus, 26 were positive, chiefly skin flora such as *Staphylococcus epidermidis* or enterococcus. From our 26 donors, 1 had *Neisseria gonorrheae* in his semen, while another had a nonpathogenic neisseria present. Our mycoplasma isolations are currently in progress, and we cannot report on them. One of six specimens tested for hepatitis B antigen was positive; and finally, 2 donors proved to have cytomegalovirus in their semen.

CONCLUSIONS

The finding that nearly all samples were contaminated with skin flora illustrates the inherent difficulty in maintaining bacterial sterility of semen collected by masturbation. The presence of hepatitis antigen in one sample was somewhat surprising since that patient's serum was negative for antigen. This may represent a situation similar to

TABLE 3 Summary of Findings

Results:

1. 26 donors/36 specimens
2. Bacteria: 26/29 specimens
3. *Neisseria gonorrheae:* 1/26 donors
4. Nonpathogenic neisseria: 1/26 donors
5. Mycoplasmas: in progress
6. Hepatitis antigen B: 1/6 specimens
7. Cytomegalovirus (CMV): 2/26 donors

that described for CMV where the concentration of virus was highest in semen when compared with blood or urine.[4] The finding that 2 of 25 donors had CMV in their semen and were asymptomatic at the time of testing is consistent with already published data.[4] The isolation of *N. gonorrheae* was from a patient with a urethral exudate containing gram-negative diplococci. The growth of classical gonococci from that specimen was, therefore, not surprising. The other neisseria isolate, however, was from an asymptomatic patient. At first, the growth of the organism on Thayer-Martin medium and the characteristic gram-negative diplococcal morphology led us to believe that we had uncovered a case of asymptomatic gonorrhea. However, because of the patient's history of having had only one episode of oral sex 6 mo prior to testing, we did additional tests. The organism was oxidase positive, but it fermented maltose, which the pathogenic organism *N. gonorrheae* does not. *N. meningiditis* does ferment maltose, but it does not produce yellow colonies as this organism did.

This last finding emphasizes several salient points: (1) asymptomatic donors can harbor "latent" agents, and (2) neisseria isolated from semen are not automatically gonococci. These findings show that a variety of organisms can be cultured from semen samples of a private patient population that included law students, physicians, and other professionals in addition to nonprofessionals. Whether these findings can be extended to the more usual populations of semen donors, such as medical, dental, or graduate students, is at present unknown; but such data should be developed. In a recent article on artificial insemination, it was suggested that since the donor population consisted of medical and dental students in stable marriages, they should be at low risk for venereal disease.[13] Such a statement begs the question: How do you know if you don't look?

The implications of the above findings warrant further discussion. The hazards, if any, of skin flora in semen used for artificial insemination (AI) are largely unknown. One could speculate on both sides of the question; but what is needed far more than speculation is data on the frequency of such contaminants in actual AI semen specimens and their relationship to disease. Even if found to be innocuous, however, it is reasonable to attempt to obtain and use semen that is free of avoidable extraneous agents including skin bacteria.

The potential dangers of nonpathogenic neisseria in

semen are also not known, but of interest is a recent report that showed that both pathogenic and nonpathogenic neisseria can adhere to sperm *in vitro*.[14] Whether this adherence might alter sperm fertility or provide a means of entry for neisseria into the femal genital tract is speculative, but studies should be made to answer these questions.

The finding of *Neisseria gonorrheae* in semen presents a more substantive problem. There is already one published case report of gonorrhea transmitted to the husband of a woman who had received AI; the donor proved to have gonorrhea.[15] Perhaps a more distressing problem is that gonorrhea can be asymptomatic not only in females but in males as well.[16] The potential for inadvertently increasing the silent reservoir of gonococcal carriers by use of semen from asymptomatic males is obvious. In addition, asymptomatic gonorrhea in women can ironically lead to infertility from fibrosis of the fallopian tubes, while both men and women with asymptomatic genital gonorrhea may develop gonococcal arthritis.[16]

From our discussion of the frequency of microbial contamination of semen and its possible consequences, it might seem desirable to screen every sample of semen for microbial contaminants. While considerations for freedom from adventitious agents are of obvious importance, it is also of very real significance to recognize that the size of any given semen sample places practical constraints on just how much testing can be done without jeopardizing the clinical value of the sample. For example, in the studies we are now conducting from 0.2 ml to 0.4 ml of semen is used per microbial isolation for a total of 1.2 ml per sample. To suggest that a substantial portion of an ejaculate be tested for a number of organisms in addition to the usual tests for physical parameters would now be impractical; and yet, the potential dangers of gonococcus, mycoplasmas, and viruses in semen certainly cannot be ignored. Clearly, further effort is needed to minimize the amount of semen required for the various isolations. For example, specific fluorescent antisera to various organisms could be used to screen smears made of semen samples. Another possibility is the development of sensitive radioimmunoassays for various organisms.

As with many scientific endeavors, if one looks for something, very often it will be found. In this case, microbial contamination of semen was found. To take the further step of clarifying its significance and eliminating or reducing its incidence is much more difficult. We are

now only beginning to raise our foot in the first step along that road.

DISCUSSION

A. BENDICH: Are any semen specimens ever *absolutely* microbe-free? Can 10 bacterial cells in an ejaculate be detected, for example? Is the urethra ever *absolutely* bacteria- or microbe-free?

PHILIP NOGUCHI: Three of 29 specimens were sterile at least by our tests. I don't know the lower limit of sensitivity for our bacterial tests. Semen was inoculated into transport medium to amplify any bacteria present, so the actual number of original bacteria present is unknown. We have not looked at urethral swabs for bacteria or other microorganisms.

RONALD MAYNER: I would like to comment on the isolation studies described this morning by Dr. Noguchi. In screening the specimens in WI-38 cell cultures, the appearance of clones of epithelial cells was noted, also yeast and fungal contaminants were not uncommon. Of the two donors in which virus was isolated, one donor was repeatedly found to have CMV in semen specimens over a 2-mo period. During this time approximately 4 $\log_{10}$ foci-forming units/ml were recovered. On the other hand, the second donor had one positive CMV isolation in which only *one* focus/0.2 ml semen was observed. This isolate was identified as CMV by neutralization, fluorescent antibody, and acridine orange. The data raises some questions about the sensitivity of present viral isolation techniques to screen semen for CMV.

NOGUCHI: Recently an asymptomatic hepatitis B antigen carrier gave birth to two consecutive infants who died with fulminant hepatitis (P. A. Shurin *et al.*, N. Engl. J. *293*:5, 1975). Thus, hepatitis B antigen in semen could conceivably eventually affect the fetus, as CMV possibly can.

RICHARD DIGIOIA: Are semen samples being cultured for *Chlamydia*? Considering the role of *Chlamydia trachomatis* in NSU and trachoma, should this not be routine?

NOGUCHI: I agree that *Chlamydia trachomatis* is an important pathogen. It may be a cause of nongonococcal urethritis (K. Holmes *et al.*, N. Engl. J. Med. *292:*1199, 1975) and its prevalence as a venereal disease is increasing worldwide (J. T. Grayston and S. Wang, J. Infect. Dis. *132:*87, 1975). It is also the leading cause of blindness in countries other than the United States, although its prevalence is decreasing (J. Infect. Dis. *132:*87, 1975). We are not presently testing semen for *Chlamydia,* but we plan to do so in the near future.

JAMES HILL: From your talk we realize that you are concerned about the introduction of known pathogenic sexually transmitted bacteria such as gonococci into recipients of semen from donors and you express some concern as to possible unknown harmful effects of some of the skin contaminants.

Since you are still in the early stages of studying the microbial ecology of donor semen samples and trying to identify possible problem areas, I would like to mention the importance of the group B beta-hemolytic streptococci, which may have been included in your skin contaminant category, and encourage you to give this organism special attention in your studies of human semen samples.

This decade has seen the emergence of the group B streptococci as a very important cause of serious neonatal disease, which has been the source of alarm in many hospitals, particularly those with large number of deliveries. Approximately 3 infections per 1,000 deliveries occur in some of the large hospital studies. The disease exists as "early onset" and "late onset" disease, and the mortality of early onset disease is about 50 percent. Most early onset disease is caused by serotype III, one of the five recognized serotypes, and it seems to possess a special virulence factor. Early onset disease results from intrapartum transmission of the organism from the maternal genital tract. It is estimated that 25 to 30 percent of pregnant women come to term carrying group B streptococci, and approximately one-third of those are type III. From unpublished studies by Carol J. Baker and others, colonization appears to be sexually acquired but not sexually transmitted in the same sense as those organisms that we commonly regard as sexually transmitted (i.e., mycoplasma and

gonococci). Females with some sexual experience do show significantly higher colonization, but this does not correlate with promiscuity as with mycoplasma infection and gonorrhea.

Studies by Franciosi have shown the same group B serotype in the urethras of 45 percent of male partners of women from whom vaginal isolates were obtained. Dr. Baker in other unpublished studies found isolation rates of 50 and 20 percent, respectively, from females and males in a VD clinic study. Studies in England have given similar results. This may not be highly relevant, as your donor population may be at lower risk. The elimination of group B streptococci from semen samples would not, of course, guarantee that colonization may not occur either from the sexual partner or from some other source that may come into play, but it seems warranted to make every effort economically and logistically feasible not to introduce a dangerous neonatal pathogen such as type III group B streptococcus into an artificial insemination recipient.

Also, recent data indicate that 50 percent of NGU may be due to *Chlamydia*. These should also be monitored in semen samples.

REFERENCES

1. Mann, T. 1964. *The Biochemistry of Semen and the Male Reproductive Tract*. New York: John Wiley & Sons.
2. Holmes, King. Unpublished observations.
3. DeLourois, J., Blades, M., Harrison, R. F. *et al.* 1974. Frequency of mycoplasma in fertile and infertile couples. Lancet *1073*:i.
4. Lang, D. J., and Kummer, J. F. 1975. Cytomegalovirus in semen: Observations in selected populations. J. Infect. Dis. *132*:472.
5. DeTure, F. A., Drylie, D. M., Kaufman, H., and Centifanto, Y. 1975. Human male reproductive organs harbor herpes simplex virus type 2. Summary of a presentation at the Southeast Section of the American Urological Association, as reported in *Infectious Diseases*, Vol. *1*, Sept. 1975.
6. Heathecote, J., Cameron, C. H., and Dane, D. S. 1974. Hepatitis-B antigen in saliva and semen. Lancet *71*:i.
7. Sherman, J. K., and Rosenfeld, J. 1975. Importance

of frozen-stored human semen in the spread of gonorrhea. Fertil. Steril. *26:*1043.

8. Kellog, D. G. Jr. Neisseria gonorrhoeae. In: E. N. Lennette, E. H. Spaulding, and J. P. Truant, eds. *Manual for Clinical Microbiology,* 2d ed. American Society for Microbiology. p. 124.
9. Isenberg, H. D., Washington, J. A., Balows, A., and Sonnenwirth, A. C. Collection, handling and processing of specimens. In: E. N. Lennette, E. H. Spaulding, and J. P. Truant, eds. *Manual for Clinical Microbiology,* 2d ed. American Society for Microbiology. p. 59.
10. Barile, M. 1973. Mycoplasma contamination of cell cultures: Incidence, source, prevention and problems of elimination. In: P. F. Kruse and M. K. Patterson, eds. *Tissue Culture, Methods and Applications.* New York: Academic Press. p. 729.
11. Hepatitis B surface antigen ^{125}I (human) AUSAB antibody to hepatitis B surface antigen ^{125}I (human) AUSRIA II-125. Diagnostic procedures, Abbott Laboratories, Diagnostics Division, North Chicago, Ill.
12. Frank, A. L., Bissell, J. A. *et al.* Rhesus leucocyte-associated herpes virus. I. Isolation and characterization of a new herpes virus recovered from rhesus leucocytes. J. Infect. Dis. *128:*618.
13. Strickler, R. C., Keller, D. W., and Warren, J. C. 1975. Artificial insemination with fresh donor semen. N. Engl. J. Med. *293:*848.
14. James-Holmquist, A. N., Swanson, J., Buchanan, T. M. *et al.* 1974. Differential attachment by piliated and non-pilated *Neisseria gonorrheae* to human sperm. Infect. Immun. *9:*897.
15. Fuimara, N. J. 1972. Transmission of gonorrhea by artificial insemination. Bri. J. Vener. Dis. *48:*308.
16. Handsfield, H. H., Lipman, T. O., Harnisch, J. P. *et al.* 1974. Asymptomatic gonorrhea in man. N. Engl. J. Med. *270:*117.

BIBLIOGRAPHY

Feigin, R. D. 1976. The perinatal group B streptococcal problem. More questions than answers. N. Engl. J. Med. *294:*106.

Baker, C. J., Kasper, D. Cl., and Davis, C. E. 1976. Immunochemical characterization of the "native" type III polysaccharide of group B streptococcus. J. Exp. Med. *143:*258.

Baker, C. J., Barrett, F. F., Gordon, R. C., and Yow, M. D. 1973. Supurative meningitis due to streptococci of Lancefield group B. A study of 33 infants. J. Pediatr. *82:*724.

Baker, C. J., and Barrett, F. F. 1973. Transmission of group B streptococci among parturient women and their neonates. J. Pediatr. *83:*919.

Baker, C. J., and Barrett, F. F. 1974. Group B streptococcal infections in infants. The importance of various serotypes. JAMA *230:*1158.

Franciosi, R. A., Knostman, J. D., and Zimmerman, R. A. 1973. Group B streptococcal neonatal and infant infections. J. Pediatr. *82:*707.

MYCOPLASMA CONTAMINATION OF HUMAN SPERMATOZOA

Michael F. Barile

The purpose of this paper is to briefly discuss (a) the properties used to identify and classify mycoplasmas, (b) some of the pertinent mycoplasma-host cell interactions, (c) mycoplasma colonization of the human genital mucosal tissues, and (d) mycoplasma contamination and/or infection of human seminal tissues. The reviews and other references cited were selected by the author.

HISTORICAL ASPECTS

The first mycoplasma, designated later as *Mycoplasma mycoides*, was isolated in 1898 and shown to be the cause of contagious pleuropneumonia of cattle, an economically important veterinary disease.[1] The organism was filterable and was first thought to be a virus. Subsequently, it was grown on agar medium-producing microscopic colonies with characteristic "fried egg" appearance. During the 1930's, a number of organisms were isolated that produced similar microscopic colonies, and these became known as the pleuropneumonialike group of organisms, or PPLO.[2]

CLASSIFICATION OF MYCOPLASMAS

In 1956, Edward and Freundt[3] proposed a system of classification that was universally accepted, and PPLO were designated as mycoplasmas. In 1967, the mycoplasmas were raised to class status, Mollicutes, which separated them taxonomically from the bacteria.[4] Presently, there are one order (Mycoplasmatales), three families (Mycoplasmataceae, Acholeplasmataceae, Spiroplasmataceae), six genera (*Mycoplasma, Ureaplasma, Acholeplasma, Spiroplasma,*

Thermoplasma, and *Anaeroplasma*), and at least 60 distinct species.[5] The *Mycoplasma*, *Ureaplasma*,[6] and *Acholeplasma*[7] species are agents of man and animals. The *Spiroplasma* are pathogens of plants and insects; the *Anaeroplasma*[7] are strict anaerobes found only in the rumen of ruminants; and the *Thermaplasma*, which prefer to grow at 56°C and at pH 3, have only been recovered from hot-burning coal piles.[7] There are 50 species of *Mycoplasma*,[6] 6 species of *Acholeplasma (A. axanthum, A. equifoetale, A. granularum, A. laidlawii, A. modicum,* and *A. oculi)*; 2 species of *Anaeroplasma (A. bactoclasticum* and *A. abactoclasticum)*; and 1 species each of *Ureaplasma (U. urealyticum), Spiroplasma (S. citri),* and *Thermaplasma (T. acidophilum)*. In addition, there are at least 12 distinct human serotypes of *Ureaplasma urealyticum*.

MYCOPLASMA SPECIES OF MAN

Of the 10 human species recognized, the only proven pathogen is *M. pneumoniae*, the cause of cold-agglutinin positive, primary atypical pneumonia.[6,8] However, *M. pneumoniae* has been associated with other disorders, seen either alone or as a complicating feature of primary atypical pneumonia, including bullous myringitis, acute pancreatitis, and various neurologic diseases, such as meningitis, meningoencephalitis, and cerebellar ataxia. In addition, *M. pneumoniae* has been isolated from tissues of the aborted human fetus.

Because *Mycoplasma hominis*, *M. fermentans*, and *Ureaplasma urealyticum* are frequently isolated from the normal genital mucosal tissues of healthy, asymptomatic subjects, these mycoplasmas are generally considered to be a part of the normal genital microbial flora of men and women.[6] However, there is evidence to suggest that there may be difference in strain virulence, and on occasion, *M. hominis* and *Ureaplasma urealyticum* have been isolated from patients with various genital diseases. These findings have stimulated study, and there has been debate on the possible role of mycoplasmas in the biology of some human genital disorders.[9-11] For example, mycoplasmas have been isolated, occasionally in pure cultures, from tubo-ovarian abscesses,[12] from the blood of patients with postpartum fever,[13] from amniotic fluids,[14] and from the aborted human fetus.[27] Some of the patients with spontaneous abortions and postpartum fever developed increased antibody titers to the genital mycoplasmas isolated.[15-17,19] Human genital

mycoplasmas have also been isolated from infants with fatal congenital pneumonias.[14,24,27] Mycoplasmas and especially *U. urealyticum* have been associated with fetal waste disease,[14,15,18,24] low birth weights of newborns,[20-22] infertility of men and women,[23,24] and chorioamnionitis.[14,15,24,25] In addition, mycoplasmas have been associated with various reproductive disorders of animals, including cattle[26] and mice.[42]

The remaining six *Mycoplasma* species are commonly isolated from the human oral and/or genital mucosal tissues and have not been associated with any disease. These include *Mycoplasma orale, M. buccale, M. faucium, M. salivarium, M. primatum* and *M. lipophilum*.[6,8]

IDENTIFICATION OF MYCOPLASMAS

Mycoplasmas are identified on the basis of their cellular and colonial morphology and, in particular, their size, filterability, and pleomorphism.[1] Unlike bacteria, they lack a cell wall and are bounded by a three-layered unit cell membrane. Their size ranges from 200 to 1,000 µm, and a small portion, about 1 percent of each culture is filterable. Mycoplasmas have both DNA and RNA, GC base ratios from 20 to 40 percent, genome sizes from 4.5×10^8 to 10×10^8 daltons, can code for 600 to 1,000 proteins, and represent the smallest free-living prokaryote. Colonies are microscopic, ranging in diameter from about 20µ for the tiny T strains (ureaplasmas) to 500µ for some of the *Mycoplasma* and *Acholeplasma* species. All mycoplasmas are resistant to penicillin and to other antibiotics that inhibit cell wall synthesis. Most mycoplasmas are resistant to streptomycin, and most are susceptible to the broad-spectrum antibiotics.[28] However, mycoplasmas can develop antibiotic resistance. The salient properties and the procedures recommended for characterizing new species of mycoplasmas have been proposed by the Subcommittee on Taxonomy of Mycoplasmatales.[29]

DIFFERENTIATING MYCOPLASMAS FROM OTHER PROKARYOTES

Mycoplasmas are differentiated from other microorganisms by their growth on cell-free medium, absence of a cell wall, and independence of host cell nucleic acids for multiplication.[5] Mycoplasmas can synthesize proteins and obtain energy from their own enzymes. They contain both

DNA and RNA, and some require cholesterol for growth. Although they are filterable, mycoplasmas can be visualized by optical microscope at 1,500 magnifications. They are inhibited by certain antibiotics and are neutralized by specific antiserum. There are exceptions, however, for example some strains of *Mycoplasma hyorhinis* cannot grow on broth or agar media, and currently can only be detected by cell culture procedures.[30,31]

MYCOPLASMA-HOST CELL INTERACTIONS

The pathogenicity of mycoplasmas is dependent, in part, upon their interactions with host cells.[1] For example, *Mycoplasma pneumoniae*, the human pathogen, can produce hemolysis, hemadsorption, hemagglutination, and is probably responsible for causing both the antigenic changes in the red blood cell membrane (development of antigen-I negative cells) and the production of cold agglutinins, which develop during primary atypical pneumonia. Because *M. pneumoniae* initiates infection by attaching to the bronchial ciliated epithelial cells,[32] it may also be responsible for the development of lung tissue autoantibodies, as well as brain tissue autoantibodies, which are also produced during a mycoplasma pneumonia. Primary atypical pneumonia would probably be regarded as an autoimmune disease if the cause of this infectious disease had not been shown to be a mycoplasma.[33]

MYCOPLASMA SPERMADSORPTION

A number of pathogenic mycoplasmas can adsorb to various tissue cells, including the human spermatozoa.[34,35] Spermadsorption is dependent, in part, on the species and age of the mycoplasma culture; the reaction temperature; and upon the motility, viability, and host source of the spermatozoa.[34] Taylor-Robinson and Manchee[34] examined colonies of five pathogenic hemadsorbing and two nonpathogenic nonhemadsorbing mycoplasmas for spermadsorption. Both human and bovine spermatozoa and erythrocytes absorbed to colonies of *M. pneumoniae*, *M. gallisepticum*, and *M. pulmonis*. Only the bovine spermatozoa absorbed to colonies of *M. bovigenitalium*, a bovine genital mycoplasma that has been associated with infertility of cattle. None of the sperm tested adsorbed to colonies of *Mycoplasma hominis* and *M. fermentans*, the two nonhemadsorbing human genital

mycoplasmas. However, some but not all of the human genital serotypes of *Ureaplasma urealyticum* can hemadsorb and spermadsorb.

DIFFERENTIAL SPECIATION OF GENITAL MYCOPLASMAS

The first human mycoplasma was isolated in 1937 by Dienes and Edsall[12] from a patient with an abscessed Bartholin gland. Since them, mycoplasmas have been shown to be common inhabitants of the normal oropharyngeal and genital mucosal tissues of men and women. More recently, a number of reports have provided some measure of evidence linking the genital mycoplasmas and especially *Ureaplasma urealyticum* with various human genital diseases. *Mycoplasma hominis* and *Ureaplasma urealyticum* are readily differentiated[36] on the basis of colony size and morphology; growth requirements; hydrolysis of either arginine or urea for energy; susceptibility to either erythromycin, lincomycin, or thallium acetate; and by immunologic procedures using species specific antisera.

MYCOPLASMA COLONIZATION OF GENITAL TISSUES

Mycoplasma colonization of the human mucosal genital tissues occurs at birth.[10] The mycoplasmas are probably acquired from the birth canal because infants that are delivered by Caesarian section have a lower prevalence of colonization.[20] Infant girls are more frequently colonized than infant boys. Sequential studies during the first year of life show a progressive decrease in the prevalence of colonization. Whereas most prepubertal boys are free of genital mycoplasmas, many adults are colonized with either ureaplasmas and/or *Mycoplasma hominis*.[9]

EFFECT OF SEXUAL ACTIVITY ON COLONIZATION

Colonization is directly related to sexual activity.[37,38] Men with little or no sexual contact are virtually free of genital mycoplasmas. Among sexually active men, prevalence increases with the number of sexual partners. Moreover, McCormick and colleagues[9] showed that the use of condoms can lower the frequency of colonization among sexually active males, indicating that the genital mycoplasmas are sexually transmitted. The ureaplasmas colonize men more

frequently than does *M. hominis*. Women are colonized more frequently than men.[9,39]

EFFECT OF NGU ON COLONIZATION

Although ureaplasmas have been associated with nongonococcal urethritis, NGU, their role in this disease is still unclear. There are as many well-controlled studies that suggest a causative role for the ureaplasmas as there are studies that do not support their involvement. Evidence or support for involvement is based on the increased prevalence of genital ureaplasmas among patients with NGU. A tabulation of results from many studies supporting a role for the ureaplasmas[9] shows that 1,368 of 2,106 (65 percent) patients and 263 of 1,052 (25 percent) control subjects examined had positive urethral cultures for ureaplasmas. Unfortunately, ureaplasma serotyping was rarely done. Moreover, the definition of a control group is a very important but a rather difficult problem to resolve, because mycoplasma colonization among normal, asymptomatic male subjects is dependent on a number of factors, including age, sexual activity, the use of condoms, and circumcision. McCormack *et al.*[39] also tabulated the results from many studies showing no differences in the prevalence of ureaplasma colonization among males with and without NGU; 284 of 518 (55 percent) NGU patients, and 177 of 388 (46 percent) control subjects examined were positive for urethral ureaplasmas. Again, ureaplasma serotyping was rarely done, and, in fact, there are very few studies showing serologic responses to specific ureaplasma serotypes. One such study was reported in 1973 by Purcell and colleagues.[40] They tested paired sera from 20 patients with a panel of 6 of the 12 known human serotypes and reported no significant rises in neutralizing antibody. On the other hand, some workers have challenged the usefulness of serologic studies, arguing that NGU is a superficial mucosal infection restricted to the urethral canal without systemic invasion and that a superficial, localized, epithelial infection may not provide sufficient antigenic stimulation or insult to the patient to produce an appreciable antibody response. However, these studies show that the urethral canal of man is frequently inhabited by mycoplasmas, and, therefore, the seminal fluids are subject to contamination.

RECOVERY OF MYCOPLASMAS FROM SEMINAL FLUIDS

A number of studies have shown that the seminal fluids of man[23] and animals[26] can contain mycoplasmas. Gnarpe and Friberg[23] reported that the seminal fluids and the cervical secretions of infertile couples are frequently positive for ureaplasmas. In these studies, ureaplasmas were isolated from the seminal fluids of 47 of 55 (85 percent) infertile men and from 6 of 23 (26 percent) fertile men. *M. hominis* was also isolated from 3 of the infertile men. They also reported that the ureaplasmas are found in close association with or attached to the spermatozoa.

In another study, Carpi *et al.*[15] examined a patient who became pregnant by artificial insemination and developed amnionitis involving twins. Mycoplasmas were isolated from one of the infants who was born dead. As a precaution, the husband had no intercourse with his wife during the pregnancy and was negative for genital mycoplasmas, while the semen donor was positive.

In animal studies, Taylor-Robinson and colleagues[41] isolated ureaplasmas from the semen of 28 of 34 (82 percent) bulls examined. Although the seminal fluids were positive, the testicular tissues, the vas deferens, and scraping of urethral mucosa of 2 slaughtered bulls tested were negative. It appeared that the mycoplasmas were confined to the preputial cavity and gained access to semen during ejaculation. Washings from the preputial cavities of 10 bulls each contained at least 10^5 ureaplasmas per ml. All of the ureaplasma-positive bovine semen samples had a high fertilizing capacity, indicating that the presence of mycoplasma alone was not sufficient evidence to suggest a causative role for infertility. Nonetheless, these findings show that seminal fluids are subject to mycoplasma contamination.

CONCLUSIONS AND SUMMARY

The prevalence of mycoplasma colonization of the genital mucosal tissue of men is dependent on several factors, including the age of the subject. Prepubertal boys are virtually free, whereas most adults are colonized. Mycoplasma colonization is directly related to sexual activity. Higher rates of colonization were also found in patients with NGU, prostatitis, and in infertile men. Higher rates were found among black men and among men who were not

circumcized. In addition, mycoplasma colonization of the adult male urethra can persist for many years.[38] Isolation rates also depended on the specimen tested. The urethral scrapings are generally the preferred specimen. Therefore, mycoplasma are frequently present in the genital mucosal tissues of normal and infected men, and, accordingly, semen is subject to mycoplasma contamination.

REFERENCES

1. Barile, M. F. 1973. Mycoplasma contamination of cell cultures: Mycoplasma-virus-cell culture interactions. In: J. Fogh, ed. *Contamination of Cell cultures.* New York: Academic Press. pp. 131-172.
2. Sabin, A. B. 1941. The filterable microorganisms of the pleurapneumonia group. Bacteriol. Rev. *5:*1.
3. Edward, D. G., and Freundt, E. A. 1956. The classification and nomenclature of organisms of the pleuropneumonia group. J. Gen. Microbiol. *14:*197.
4. Edward, D. G., and Freundt, E. A. 1969. Classification of the mycoplasmatales. In: L. Hayflick, ed. *The Mycoplasmatales and the L-phase of Bacteria.* New York: Appleton-Century-Crofts. pp. 147-200.
5. Tully, J. G., and Razin, S. In Press. The Mollicutes (mycoplasmas). In: *Handbook of Microbiology.* Cleveland: Chemical Rubber Co.
6. Tully, J. G., and Razin, S. In Press. Mycoplasmas and ureaplasmas. In: *Handbook of Microbiology.* Cleveland: Chemical Rubber Co.
7. Tully, J. G., and Razin, S. In Press. Acholeplasmas, spiroplasmas, thermaplasmas and anaeroplasmas. In: *Handbook of Microbiology.* Cleveland: Chemical Rubber Co.
8. The WHO/FAO Programme on Comparative Mycoplasmology. 1975. Vet. Rec. *94:*457-461.
9. McCormack, W. M., Braun, P., Lee, Y-H., Klein, J. O., and Kass, E. H. 1973. The genital mycoplasmas. N. Engl. J. Med. *288:*78-89.
10. Lee, Y-H., McCormack, W. M., Marcy, S. M., and Klein, J. O. 1974. The genital mycoplasmas, their role in disorders of reproduction and in pediatric infections. Pediatr. Clin. North Am. *21:*457-466.
11. Lee, Y-H., and McCormack, W. M. 1974. Clinical implication of the genital mycoplasmas in obstetrics and gynecology. J. Reprod. Med. *13:*123-127.

12. Dienes, L., and Edsall, G. 1937. Observations on the L-organisms of Kleineberger. Proc. Soc. Exp. Med. *36:*740.
13. Tully, J. G., and Smith, L. G. 1968. Post-partum septicemia with *Mycoplasma hominis*. JAMA *204:*827-828.
14. Brunell, P. A., Dische, R. M., and Walker, M. B. 1969. Mycoplasma amnionitis and respiratory distress syndrome. JAMA *207:*2097-2099.
15. Caspi, E., Herczeg, E., Solomon, F., and Sompolinsky, D. 1971. Amnionitis and T strain mycoplasmemia. Am. J. Obstet. Gynecol. *111:*1102-1106.
16. Harwick, H. J., Purcell, R. H., Iuppa, J. B., and Fekety, F. R. 1970. *Mycoplasma hominis* and abortion. J. Infect. Dis. *121:*260-268.
17. McCormack, W. M., Lee, H-H., Lin, J-S., and Rankin, J. S. 1973. Genital mycoplasmas in post-partum fever. J. Infect. Dis. *127:*193-196.
18. Driscoll, S. G., Kundsin, R. B., Horne, H. W., and Scott, J. M. 1969. Infection and first trimester losses. Possible role of mycoplasmas. Fertil. Steril. *20:*1017-1019.
19. Sompolinsky, D., Solomon, F., Leiba, A., Caspi, E., Lewinsohn, G., and Almog, C. 1971. Puerperal sepsis due to T-strain mycoplasma. Israel J. Med. Sci. *7:*745-748.
20. Klein, J. O., Buckland, D., Finland, M. 1969. Colonization of newborn infants by Mycoplasmas. N. Engl. J. Med. *280:*1025-1030.
21. Braun, P., Lee, Y-H., Klein, J. O., Marcy, S. M., Klein, T. A., Charles, D., Levy, P., and Kass, E. H. 1971. Birth weight and genital mycoplasmas in pregnancy. N. Engl. J. Med. *284:*167-171.
22. DiMusto, J. C., Bohjalian, O., and Millar, M. 1973. *Mycoplasma hominis* Type 1: Infection and pregnancy. Obstet. Gynecol. *41:*33-37.
23. Gnarpe, H., and Friberg, J. 1972. Mycoplasmas and human reproductive failures. Am. J. Obstet. Gynecol. *114:*727-731.
24. Kundsin, R. B., Driscoll, S. G., and Ming, P-M, L. 1967. Strain of mycoplasma sssociated with human reproductive failure. *Science 157:*1573-1574.
25. Shurin, P. A., Alpert, S., Rosner, B., Driscoll, S. G., Lee, Y-H., McCormack, W. M., Santamarino, B. A. G., and Kass, E. H. 1975. Chorioamnionitis and colonization of the newborn infant with genital mycoplasmas. N. Engl. J. Med. *293:*5-8.
26. Langford, E. V. 1975. Mycoplasma recovered from

bovine male and female genitalia and aborted foeti. Am. Assoc. Vet. Lab. Diag. Proc., Portland. pp. 221-231.

27. Romano, N., Romano, F., and Carollo, F. 1971. T strains of mycoplasma in broncho-pneumonic lungs of an aborted fetus. N. Engl. J. Med. *285:*950-952.

28. Braun, P., Klein, J. O., and Kass, E. H. 1970. Susceptibility of genital mycoplasmas to antimicrobiologic agents. Appl. Microbiol. *19:*62.

29. Subcommittee on Taxonomy of Mycoplasmatales. 1972. Proposal for minimum standards for descriptions of new species of Mycoplasmatales, class Mollicutes. Int. J. Syst. Bacteriol. *22:*184.

30. Hopps, H. E., Meyer, B. C., Barile, M. F., and DelGiudice, R. A. 1973. Problems concerning"non-Cultivable"mycoplasma contaminants in tissue cultures. N.Y. Acad. Sci. *225:*265-276.

31. Barile, M. F. In press. Mycoplasma contamination of cell cultures: A status report. In: R. Acton, ed. *Cell Culture and Its Applications*. Birmingham, Ala.:, Univ. Alabama Press.

32. Collier, A. M. 1972. Pathogenesis of *M. pneumonias* infection as studied in the human foetal trachea in organ culture. In: *CIBA Foundation Symposium on Pathogenic Mycoplasma*. Amsterdam: Associated Scientific Publishers. pp. 320-328.

33. Thomas, L. 1968. Mycoplasmas as pathogens. Yale J. Biol. Med. *40:*444-448.

34. Taylor-Robinson, D., and Manchee, R. J. 1967. Spermadsorption and spermagglutination by mycoplasmas. Nature *215:*484-487.

35. Gnarpe, H., and Friberg, J. 1973. T-mycoplasmas as possible cause for reproductive failure. Nature *242:*120.

36. Razin, S. 1972. Mycoplasmatales. In: *Handbook of Microbiology*. Cleveland: Chemical Rubber Co. pp. 105-130.

37. McCormack, W. M., Lee, Y-H., and Zinner, S. H. 1973. Sexual experience and urethral colonization with genital mycoplasmas. Ann. Intern. Med. *78:*696-698.

38. Holmes, M. J., Furr, P. M., and Taylor-Robinson, D. 1974. The persistence of mycoplasmas in the urogenital tract of men in the Antartic. J. Hyg. Camb. *72:*355-363.

39. McCormack, W. M., Rankin, J. S., and Lee, Y-H. 1972. Localization of genital mycoplasmas in women. Am. J. Obstet. Gynecol. *112:*920-923.

40. Hill, D. A., Philip, R. N., Greaves, A. S., and Purcell, R. H. 1973. Mycoplasmas recovered from patients with urethritis. Br. J. Vener. Dis. *49:*524-530.
41. Taylor-Robinson, D., Thomas, M., and Dawson, P. L. 1969. The isolation of T-mycoplasmas from the urogenital tract of bulls. J. Med. Microbiol. *2:*527-533.
42. Taylor-Robinson, D., Rassner, C., Furr, P. M., Humber, D. P., and Barnes, R. D. 1974. The effect of *Mycoplasma pulmonis* on the materno-foetal relationship in mice. In: J. M. Bove and J. F. Duplan, eds. *Les Mycoplasmes.* Colloques INSERM *33:*325-330.

VIRUSES IN HUMAN SEMEN*

David J. Lang

In 1972 I reviewed the medical literature pertaining to the presence of viruses in human semen and found little other than speculation. Transovarian passage of viruses has been described in various mammals, insects, helminths, and birds.[1,2] The association of viruses with sperm in mammals had been studied primarily by veterinarians.[3-5] Branny and Zembala[6] studied semen samples obtained from 194 bulls at artificial insemination centers in Poland. Thirty-three specimens (17 percent) yielded cytopathogenic agents, including entero, reo, and papova viruses. These authors demonstrated a relationship between the presence of viruses and "low semen quality" (not further specifically described).

Martini and Schmidt[7] noted in 1968 that in a case of Marburg virus infection of man (acquired from vervet monkey tissue cultures) viral antigens could be demonstrated by immunofluorescence in semen. Orchitis may accompany the replication of viruses during acute infections,[8] and mumps virus has been recovered from testicular biopsy specimens.[9] Although the agent of mumps may be shed in urine for days after resolution of the acute infection,[10] it is unlikely that this virus persists in the male genital tract after the subsidance of clinical disease for sufficient time to permit the reestablishment of normal sexual activity. Transmission of viruses with semen is rendered less likely in association with acute self-limited illnesses, as functional sexual activity is in

*This work was supported in part by Public Health Service Grant 5 R01 HD04183-05 from the National Institute of Child Health Development

most instances sharply reduced if not precluded. In contrast, virus contaminants of semen associated with asymptomatic persistence and/or latency could be transmitted horizontally to the sexual partner and perhaps in some cases passed with semen vertically to offspring. Those viruses of man that have to date been demonstrated unequivocally in semen are cytomegalovirus (CMV) and hepatitis B virus (more accurately, the surface antigens of hepatitis B-HB_sAg). Both CMV and hepatitis B are agents capable of establishing persistent infections.

In 1964, while I was a post doctoral fellow in the Research Division of Infectious Diseases at the Boston Children's Hospital, my attention was attracted by CMV, a species-specific member of the herpes virus group with the capacity to infect and damage the human fetus. Thus it was of considerable interest to me when Dr. Thomas Weller--a pioneer in the study of CMV--told me that a "routine" prenatal pap smear at the Boston Lying-in Hospital had been found to contain inclusion-bearing cells characteristic of CMV cytopathology. The patient moved from Boston, could not be traced, and was lost to follow-up. I was unable to determine the outcome of the pregnancy. Nevertheless, the seeds of an inquiry had been planted. The observation of CMV cytopathology in the cells of the uterine cervix suggested that transmission of this virus might be associated with sexual contact.

Prolonged shedding of CMV in urine had been described. Since inclusion-bearing epithelial cells had been seen lining renal tubules, it was assumed that this virus was carried into the urine from infected kidney cells. It seemed logical that, in the presence of viruria, the urethral surfaces would be contaminated, and thus the ejaculate. However it also seemed possible that in some instances CMV infection might directly involve portions of the male genital tract. It was reasoned that, if CMV could be transmitted in connection with sexual contact, this might in certain cases be relevant to the pathogenesis of congenital CMV infections.

Subsequently the observation was confirmed of CMV infection of the uterine cervix,[11-14] and, although the source and means of transmission of virus was undefined, it was postulated on the basis of epidemiologic observations that CMV might be transmitted in connection with sexual contact.[14]

In 1972 we were enabled to study a 23-yr-old patient recovering from a CMV heterophile-negative mononucleosis. We demonstrated that in this case CMV was present in semen

in titers that far exceeded the quantity of virus in preejaculate urine samples.[15] In several instances the titers of CMV present in semen (greater than 10^7 infectious particles per ml) were higher than we had ever seen previously in a variety of specimens from a wide distribution of patients. In our initial report and in subsequent publications,[16,17] we were able to show that CMV might persist in semen for many months (even beyond 1 yr) in the absence of any symptoms and even when virus could not be recovered from urine, saliva, or blood. CMV was also recovered from the uterine cervix of sexual partners of two men with virus-positive semen.

Electron-micrographs of CMV-infected semen revealed extracellular herpes viruslike particles (Figure 1) and cell-fluid separation studies indicated that most of the infectious virus was present in extracellular fluids. However, residual CMV remained associated with the cellular fraction of semen, even after multiple washes with large volumes of Mann's saline. A preliminary study initiated in collaboration with Drs. Huang and Pagano provided evidence that some CMV in semen may be closely associated with spermatozoa. Employing the technique of *in situ* complementary RNA-DNA hybridization, Huang and Pagano were able to demonstrate the presence of CMV genomic equivalents apparently in or on the sperm heads (Figure 2). These very preliminary studies must be expanded before any conclusions are warranted.

The presence of CMV in semen has now been confirmed in a publication by Manuel and Embil[18] and in unpublished studies of Alexander (personal communication). Alexander (who has kindly permitted me to quote some of his preliminary findings) has recovered CMV in semen from 7 out of 70 males with a history of recurrent genital infection with herpes virus hominis, 4 of 22 young fertile males at a military base, 1 of 8 patients with epidydimitis, and 1 of 55 (older) infertile males. Furthermore, these studies also confirm that CMV may persist in semen (thus far more than 9 mo in one of the cases). Alexander notes that those men with positive semen cultures frequently exhibit the simultaneous presence of CMV in urine and prostatic fluids.

Investigations of several selected populations (Table 1) have revealed the presence of CMV in 2 of 251 men seeking a fertility evaluation, 1 of 10 patients being treated in a venereal disease clinic, and 4 of 37 additional young adults. It is noteworthy that, as seen in Table 1, these 4 cultures were obtained from men with a history of or

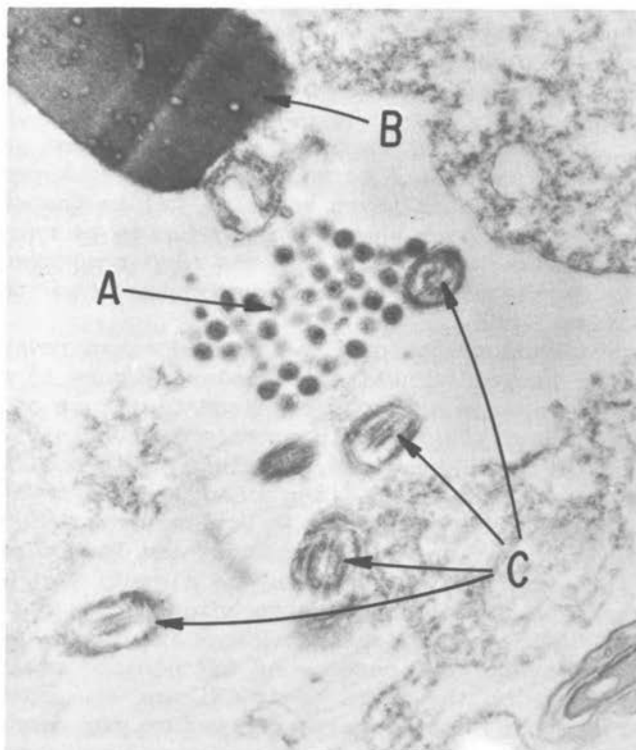


FIGURE 1 Electron micrograph of cytomegalovirus-infected semen (x 41,375). (A) Virus particles; (B) sperm head; (C) section of sperm tails. Reproduced with permission from the *New England Journal of Medicine 291*:121-123, 1974.

close contact with a case of "mononucleosis" (heterophile negative or undefined). Two of these 4 virus-positive men are homosexuals, as was the patient reported by Manuel and Embil.[18] Additional epidemiologic studies seem to be warranted by these initial observations.

Attempts to recover CMV from semen have been hampered by several technical problems. CMV replicates slowly and, initially, in order to detect small quantities of virus the inoculum was incubated with the indicator cell layer overnight. However, the semen samples were frequently (though not always) highly toxic to the cell monolayers.

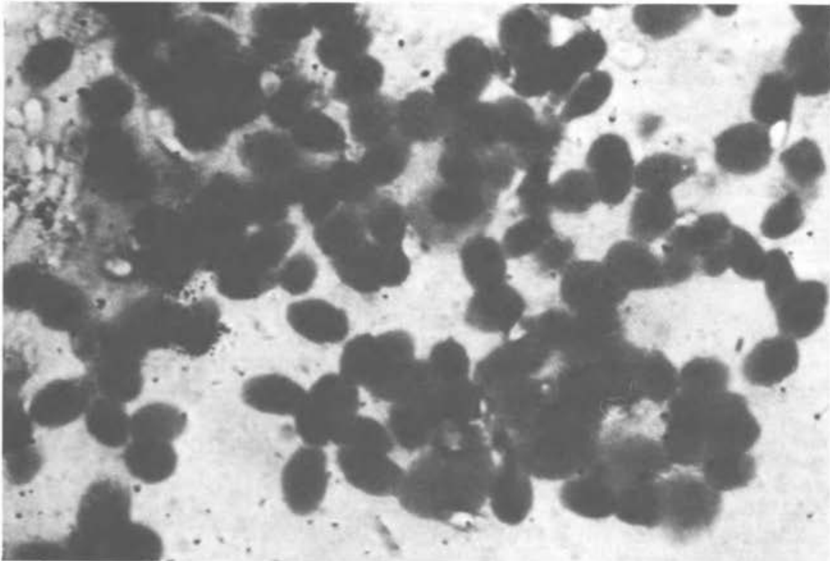


FIGURE 2 Cytohybridization with cytomegalovirus complementary RNA applied to semen. The fresh semen was washed with TBS and fixed with ice-chilled glacial acetic acid and ethanol (1:3) for 10 min, then rewashed with the same fixative. The suspension of cells was spread on the slide, air dried, and alkalinized with 0.07 N NaOH, and cytohybridization was carried out. The exposure time was 3 wk. At a magnification of 1,000x mostly the heads of the spermatozoa are visible. A cluster of several sperm show a concentration of grains. A few grains are scattered throughout the rest of the field. Reproduced with permission from *Viral Immunodiagnosis,* ed. by Edouard Kurstak and Richard Morisset, Academic Press, Inc., 1974. Chapter 16,[11] The Application of RNA-DNA Cytohybridization to Viral Diagnostics, by J. S. Pagano and E. S. Huang, p. 291.

Therefore, the inoculum was introduced in dilutions and the incubation period was reduced to 2-3 h. Most isolations have been achieved by us when the semen samples were diluted prior to their introduction into tissue cultures. We do not know whether the dilution of semen facilitates virus recovery by reducing the concentration of substances toxic to tissue culture cells or virus, or perhaps by lowering the concentration of locally produced immunoglobulins.[19]

TABLE 1 Recovery of CMV from Semen Among Selected Men

Men Surveyed	Positive Total
Index Case (heterophile-negative mononucleosis)	1
Wife with heterophile negative mononucleosis	1
Healthy young adults	0/19
Fertility clinic	2/251
VD clinic	1/10
Healthy young adults with history of mononucleosis (heterophile negative or positive)	

In order to assess the presence and potential diagnostic usefulness of virus-specific immunoglobulins in semen, we have initiated a collaborative study with Dr. Pearay Ogra of specific CMV IgG and IgA present in semen that had been subjected to virus culture procedures. The presence of specific CMV IgG and IgA antibody was assessed by indirect immunofluorescence using samples that had been maintained for a variable time in storage (-70°C). The results, shown in Table 2, were inconclusive. However, it is possible that with different antibody procedures a screening test could be developed that would correlate with, and obviate the need for, more tedious virus isolation procedures.

The role of CMV in semen in the pathogenesis of congenital infections is unknown. It is possible that virus can be carried with spermatozoa to invest the ovum and infect the zygote. It may be that the presence of virus at the time of conception will blight the ovum and lead to enhanced fetal wastage. Berenberg and Nankervis[20] noted that, following the birth of a CMV-infected infant, subsequent pregnancies were complicated by an increased frequency of spontaneous abortions.

It is also possible that the presence of CMV in semen

TABLE 2 The Demonstration in Semen of Infectious Cytomegalovirus (CMV) and CMV Antibody

Number of Samples	Virus Cultures	Samples Positive for CMV Antibody[a]	
		IgG	IgA
10	Positive	4	1
12	Negative	0	2

[a]Determined by indirect immunofluorescence.

will not inhibit conception and may be associated with the vertical transmission of virus. This might provide a means by which congenital CMV infections can occur in the face of maternal immunity.[21]

With my colleagues, James Young and Kwok-Sing Cheung, I have been conducting experiments designed to answer some of these questions. Employing a mouse model, and exploiting the technique of artificial insemination, we have thus far demonstrated that the presence of CMV in the inseminating fluids results in a predictable systemic maternal CMV infection and does not reduce the efficiency of fertilization. The data accumulated to date suggest that the presence of virus in the suspensions of sperm may reduce the number of embryos per litter and yield--for the first time in an experimental system--a congenital murine CMV infection. Whether transmission by this route occurs in the mouse under natural conditions is not yet known.

These observations and the questions they pose underline the need for further understanding of CMV persistence and pathogenetic potential. We should probably not be hastening to test candidate human virus strains for a CMV vaccine.[22-24]

Recent epidemiologic and experimental observations have indicated that nonparenteral spread of hepatitis B occurs.[25-29] Krugman and co-workers have shown that oral transmission of hepatitis B is feasible. As they had in the case of CMV,[30] epidemiologic observations had also indicated that hepatitis B may be sexually transmitted,[31-34] and indications were strong that homosexuals were frequently involved.

Heathcote and co-workers demonstrated the presence of hepatitis B surface antigen in saliva and semen obtained from individuals with positive blood tests for HB_sAg.[35] Samples of semen were antigen-positive, though in very low titer, whether obtained from patients with acute hepatitis B, chronic-persistent and chronic-aggressive antigen-positive hepatitis, or healthy HB_sAg carriers. In all, 18 of 24 patients exhibited positive tests of saliva, while 10 of these 18 manifested antigen in semen. None had an antigen-positive semen in the absence of salivary HB_sAg. Though specific tests were apparently not done, the possible presence of blood in these samples was dismissed by the authors as unlikely.

Wright reported a small outbreak of hepatitis B among patients and staff within a nursing home.[36] Two of the individuals who acquired hepatitis B had prior sexual contact with a symptomatic carrier of HB_sAg, and neither parenteral inoculations nor other exposures could be implicated. Semen from the carrier was positive for HB_sAg, as was saliva, though both were contaminated by trace amounts of blood.

The virus of hepatitis B may or may not appear in semen as a reflection of local production. As pointed out by Heathcote and colleagues, based upon their quantitative observations, it is possible that the presence of HB_sAg in semen reflects a leak from circulating virus.[35] However, whatever the derivation of the virus, it seems possible that its presence in semen can lead to the sexual transmission of hepatitis B.

An epidemiological study by Szmuness and colleagues indicates that, although promiscuity and homosexuality are associated with the acquisition of hepatitis B infection, the results were inconclusive pertaining to the role of vaginal intercourse.[37] As indicated by the authors these uncertain data probably reflect the difficulties in gathering and interpreting information relating to sexual behavior, which is associated with many modes of close personal contact. It may also be that the variable results of some epidemiologic studies of hepatitis B reflect the presence of small amounts of antigen, which contaminate semen and saliva unpredictably.

Epidemiologic evidence is well documented for the venereal transmission of herpes virus hominis type 2.[38-40] Though this virus has been recovered from urethral swabs, prostatic fluid, and vas deferens,[41] there are no reports documenting the presence of herpes virus hominis in semen. We have recovered herpes virus hominis from one sample of

semen obtained from a patient attending the Durham County Venereal Disease Clinic. However, we do not know whether this patient had active lesions of herpes progenitalis at the time the semen sample was collected. It is of interest that Alexander (personal communication) found not a single positive culture for herpes virus hominis among semen samples obtained from 70 men with a history of recurrent genital herpes.

Even in the absence of virus in semen, the lesions of genital herpes provide a source of virus transmission by direct skin and mucous membrane inoculation related to the intense friction and contact associated with coitus. In this case transmission of virus may be expected to occur to and from either sex, whether the contact is heterosexual or homosexual. In contrast, some viruses probably require the direct transmission of a virus-positive body fluid for infection rather than contact with infected integument. We lack evidence for the acquisition of CMV or hepatitis B infections through direct application of virus to unbroken skin, even with pressure and friction. Therefore, the presence of these viruses in the cells and secretions of the uterine cervix might or might not result in the acquisition of infection in connection with vaginal intercourse. In contrast, the presence of virus in semen could plausibly result in transmission of the infection to a virus-negative sexual partner, whether male or female. Thus the venereal transmission of CMV and HB_sAg may be preferentially accomplished by males, which may in part explain the prevalence of hepatitis B among male homosexuals and the demonstration of CMV in the semen of several homosexual men. Similar observations and reasoning originally led Heathcote and associates to study saliva and semen for the presence of HB_sAg.[35] It must be added that homosexuals are said to change sexual partners frequently and thus to increase the risk of acquiring infections that may be transmitted by sexual contact.

Whereas the presence of genital herpes virus hominis suggests strongly the venereal acquisition of this virus by either sex, the finding of CMV or hepatitis B surface antigen in semen suggests the potential for venereal transmission but does not necessarily imply that these virus infections were acquired by sexual contact. Indeed, it is quite possible that the presence of CMV in seminal fluids, or in or on spermatozoa, reflects a phase in the spread and pathogenesis of some or all of these infections. In contrast, it is possible that the presence of CMV in the cells and secretions of the uterine cervix is virtually

always a reflection of the direct (sexual) introduction of virus.

In summary, the presence has been demonstrated in human semen of cytomegalovirus and hepatitis B surface antigen. In the case of CMV the virus may be present in very elevated titer, suggesting that replication of CMV occurs within the male genital tract. The specific location of CMV within the components of semen is as yet undetermined. The possible role of CMV in semen in the genesis of intrauterine infections must be explored. The impact of CMV infection upon the male and female genital tract also remains uncertain. It is clear that considerable epidemiologic and experimental work is warranted.

The epidemiology of hepatitis B virus infection bears some similarity to that of CMV. HB_sAg has been demonstrated in semen, and the presence of surface antigen is generally believed to correlate with the presence of infectious virus. However, quantitative data suggest that the virus may not be replicating within the male genital tract and may be present as a reflection of the occasional contamination of semen with blood or urine.

It is suggested that prospective studies should be undertaken of semen to be employed for human artificial insemination. It may be that, based upon the admittedly incomplete information in our possession, we would be justified to restrict semen donors to those otherwise acceptable individuals who give a negative history for herpes progenitalis, are demonstrated hepatitis B surface antigen (?antibody) negative (in blood), and are CMV-negative by virus culture (or urine and semen).

Future studies of viruses in human semen should concentrate attention on those infectious agents characterized by persistence or latency and by an association with subtle or negligible immediately apparent dysfunction of infected cells. The consideration of viruses associated with germ cells will lead to a further exploration of the uncertain boundary line between certain transmissible and heritable conditions.

REFERENCES

1. Mims, C. A. 1968. Pathogenesis of viral infections of the fetus. Prog. Med. Virol. *10:*194-237.
2. Cottral, G. E. 1952. Endogenous viruses in the egg. Ann. N.Y. Acad. Sci. *55:*221-235.

3. Stein, C. D., and Mott, L. O. 1942. Studies on congenital transmission of equine infectious anemia. Vet. Med. *37:*370-377.
4. Spradbrow, P. B. 1968. The isolation of infectious bovine rhinotrachetis virus from bovine semen. Aust. Vet. J. *44:*410-412.
5. Cottral, G. E., Gailiunas, P., and Cox, B. F. 1968. Foot-and-mouth disease virus in semen of bulls and its transmission by artificial insemination. Arch. Gesamte Virusforsch. *23:*362-377.
6. Branny, J., and Zembala, M. 1971. Some characteristics of viruses isolated from bull semen and their possible pathogenicity. Br. Vet. J. *127:*88-92.
7. Martini, G. A., and Schmidt, H. A. 1968. Spermatogene Ubertragung des "Virus Marburg" (Erreger der "Marburger Affenkrankheit"). Klin. Wochenschr. *46:*398-400.
8. Riggs, S., and Sanford, J. P. 1962. Viral orchitis. N. Engl. J. Med. *266:*990-993.
9. Bjorvatn, Bjarne 1973. Mumps virus recovered from testicles by fine-needle aspiration biopsy in cases of mumps orchitis. Scand. J. Infect. Dis. *5:*3-5.
10. Utz, J. P., and Szwed, C. F. 1962. Mumps III. Comparison of methods for detection of viruria. Exp. Biol. Med. *110:*841-844.
11. Diosi, P., Babusceac, L., Nevinglovschi, O. *et al.* 1967. Cytomegalovirus infection associated with pregnancy. Lancet *2:*1063-1066.
12. Alexander, E. R., and Wentworth, B. B. 1968. A prospective perinatal study of cytomegalovirus infection. Presented at the Ninety-Sixth Annual Meeting of the American Public Health Association, Detroit, Mich., November 10-15.
13. Numazaki, Y., Yano, N., Morizuka, T. *et al.* 1970. Primary infection with human cytomegalovirus: Virus isolation from healthy infants and pregnant women. Am. J. Epidemiol. *91:*410-417.
14. Montgomery, R., Youngblood, L., and Medearis, D. N. Jr. 1972. Recovery of cytomegalovirus from the cervix in pregnancy. Pediatrics *49:*524-531.
15. Lang, David J., and Kummer, John F. 1972. Demonstration of cytomegalovirus in semen. N. Engl. J. Med. *287:*756-758.
16. Lang, D. J., Kummer, J. F., and Hartley, D. P. 1974. Cytomegalovirus in semen: Persistence and demonstration in extracellular fluids. N. Engl. J. Med. *291:*121-123.
17. Lang, D. J., and Kummer, J. F. 1975. Cytomegalovirus

in semen: Observations in selected populations. J. Infect. Dis. *132:*472-473.

18. Manuel, F. R., and Embil, J. A. 1975. Virusemenia after cytomegalovirus mononucleosis. Can. Med. Assoc. J. *112:*604.
19. Rumke, P. 1974. The origin of immunoglobulins in semen. Clin. Exp. Immunol. *17:*287-297.
20. Berenberg, W., and Nankervis, G. 1970. Long-term follow-up of cytomegalic inclusion disease of infancy. Pediatrics *46:*403-410.
21. Stagno, S., Reynolds, D. W., Lakeman, A., Charamella, L. J. *et al.* 1973. Congenital cytomegalovirus infection: Consecutive occurrence due to viruses with similar antigenic compositions. Pediatrics *52:*788-794.
22. Elek, S. D., and Stern, H. 1974. Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. Lancet *1:*1-5.
23. Editorial. 1974. Vaccine against cytomegalovirus. Med. J. Aust. *1:*463-464.
24. Hanshaw, J. B. 1974. A cytomegalovirus vaccine? Am. J. Dis. Child. *128:*141-142.
25. Krugman, S., Giles, J. P., and Hammond, J. 1967. Infectious hepatitis: Evidence for two distinctive clinical, epidemiological and immunological types of infection. JAMA *200:*365-373.
26. Prince, A. M., Hargrove, R. L., Szmuness, W. *et al.* 1970. Immunologic distinction between infectious and serum hepatitis. N. Engl. J. Med. *282:*987-991.
27. Szmuness, W., and Prince, A. M. 1971. Epidemiologic patterns of viral hepatitis in eastern Europe in light of recent findings concerning the serum hepatitis antigen. J. Infect. Dis. *123:*200-212.
28. London, W. T., Sutnick, A. I., and Blumberg, B. S. 1969. Australia antigen and acute viral hepatitis. Ann. Intern. Med. *70:*55-59.
29. Villarejos, V. M., Visona, K. A., Gutierrez, A. *et al.* 1974. Role of saliva, urine and feces in the transmission of type B hepatitis. N. Engl. J. Med. *291:*1375-1378.
30. Jordan, M. C., Rousseau, W. E., Noble, G. R., *et al.* 1973. Association of cervical cytomegaloviruses with venereal disease. N. Engl. J. Med. *288:*932-934.
31. Hersh, T., Melnick, J. L., Goyal, R. K., *et al.* 1971. Nonparenteral transmission of viral hepatitis type B (Australia antigen-associated serum hepatitis). N. Engl. J. Med. *285:*1363-1364.

32. Heathcote, J., and Sherlock, S. 1973. Spread of acute type B hepatitis in London. Lancet *1:*1468-1470.
33. Jeffries, D. J., James, W. H., Jefferiss, F. J. G. *et al.* 1973. Australia (hepatitis-associated) antigen in patients attending a venereal disease clinic. Br. Med. J. *2:*455-456.
34. Fulford, K. W. M., Dane, D. S., Catterall, R. D., *et al.* 1973. Australia antigen and antibody among patients attending a clinic for sexually transmitted diseases. Lancet *1:*1470-1473.
35. Heathcote, J., Cameron, C. H., and Dane, D. S. 1974. Hepatitis B antigen in saliva and semen. Lancet *1:*71-73.
36. Wright, R. A. 1975. Hepatitis B and the HB_sAg carrier. An outbreak related to sexual contact. JAMA *232:*717-721.
37. Szmuness, W., Much, I., Prince, A. M. *et al.* 1975. On the role of sexual behavior in the spread of hepatitis B infections. Ann. Intern. Med. *83:*489-495.
38. Parker, J. D., and Banatvala, J. E. 1957. Herpes genitalis: Clinical and virological studies. Br. J. Vener. Dis. *43:*212.
39. Rawls, W. E., Tompkins, W. A. F., Feguerva, M. E. *et al.* 1968. Herpesvirus type 2: Association with carcinoma of the cervix. Science *161:*1255.
40. Nahmias, A. J., Dowdle, W. R., Naib, Z. M. *et al.* 1969. Genital infections with type 2 herpesvirus hominis. A commonly occurring venereal disease. Br. J. Vener. Dis. *45:*294.
41. Deardourff, S. L., DeTure, F. A., Drylie, D. M. *et al.* 1974. Association between herpes hominis type 2 and the male genitourinary tract. J. Virol. *112:*126-127.

MOTILITY AND VIABILITY OF HUMAN SPERMATOZOA

Rune Eliasson

In most reports on human semen qualities emphasis is given the number of spermatozoa per ml. This is unfortunate, since from clinical and scientific points of view the total number of spermatozoa is a more accurate measure of the sperm output from the testes. On the other hand, it must be stressed that the quality of the spermatozoa is more crucial than the quantity in relation to fertility. Functional properties that are or can be of importance for the fertilizing ability of human spermatozoa are motility, viability, resistance to various stress factors (e.g. cold shock), structural stability of the chromatin and membranes, metabolism, uptake of compounds from the seminal plasma, and/or release of compounds from the spermatozoa.[9] This presentation will deal with the evaluation of motility and viability and the relation between these parameters and male fertility.

To avoid confusion it is necessary to use a terminology that is clearly understood. In research on the relation between semen quality and human fertility this has not always been the case. The terminology is therefore presented in Table 1.

MOTILITY

The motility of the spermatozoa is easy to observe but difficult to express in absolute and objective units of measurement. This presentation will in substance deal with the main principles in assessing motility with a subjective microscopic rating. There are many reports about methods for objective estimation of sperm motility, but none has been more generally accepted for routine evaluation of human semen. Suitable references for the more

TABLE 1 Nomenclature Related to Male Fertility

No children	
Not tried	Fertility unknown
Tried > 12 mo	Infertile
One or more children	Father
Recently made a woman pregnant[a]	Fertile
Clinical and laboratory examinations prove that the man cannot get children of his own	Sterile

[a]Recently = within 3 mo unless there has been a recent illness or other factors that will seriously call for a modification.

sophisticated methods are, for example, Barták,[1] Castenholtz,[3] Dubois *et al.*,[6] van Duijn *et al.*,[7] Gassner,[12] Jecht and Russo,[13] Katz and Dott,[16] and Troll and Goldzieher.[21] (1962).

The method published by Dubois *et al.*[6] and Jouannet *et al.*[15] seems to be most promising for rapid and accurate measurements of the motility characteristics of human spermatozoa in undiluted semen. It is not yet available for general use, but a brief presentation will be included.

The motility of the spermatozoa in the seminal plasma should be evaluated from the following aspects.

1. The mean progressive motility, usually assessed as none, poor, medium, good, and very good.
2. The percentage of motile cells.
3. The decline in motility over a given time period, usually 4 to 6 h.

In studies related to fertility, it is frequently important to determine the motility also in "normal" cervical mucus as well as in the cervical mucus from the female partner of a given man.

Before the motility is assessed, the semen should be liquefied and well mixed. A drop is placed on a clean microscopic glass, and a coverslip is placed on top of it. The thickness of the fluid layer should be approximately

0.05 to 0.1 mm, i.e., not so thick that the spermatozoa regularly can move out of the focus and not so thin that their movements are interfered with. The motility is best observed with a phase contrast microscope and at a magnification of about 400x. The evaluation should be based on at least 10 randomly selected fields, and care should be taken not to include areas near the edges of the coverslip.

The *mean progressive motility* can be graded in scores, e.g., 0 = no, 1 = poor, 2 = medium, 3 = good, and 4 = very good progressive motility. From studies by Jouannet *et al.*[15] and others, one can recommend that "good progressive motility" should correspond to a mean velocity of approximately 0.05 to 0.08 mm/s. In almost all semen samples, one can observe a great heterogenicity in the progressive motility pattern. The subjective assessment of the mean progressive motility therefore requires considerable experience to be reliable and reproducible. In case the progressive motility of the motile spermatozoa falls into clearly defined groups, e.g., the majority of the spermatozoa have a progressive motility score of 1 but there are a significant proportion of cells having a progressive motility score of 3, this should be noted in the protocol. However, the accuracy of the subjective method is not sufficient to motivate a subdivision into more than the five groups given above.

The relation between progressive motility score and male fertility is presented in Table 2. The results in the table are obtained from a study of men selected in such a way that there is no reason to believe that they are not fulfilling the criteria for a "fertile" man as defined in Table 1. The study has been supported by the World Health Organization, and a full report will be published elsewhere. From Table 2 it can be noted that only 1 out of the 50 men had semen samples with a mean progressive motility score of 2 at the initial examination (fractions of scores are used, since all values are based on the examination of two semen samples delivered with an interval of 1 or 2 wks).

The mean progressive motility declines with time, and it is of interest that the two men whose semen had nonprogressive spermatozoa at the end of the observation period had suffered from prostatitis in the past. They had neither symptons nor any objective signs of a prostatic infection/inflammation at the time of examination.

The *percentage of motile spermatozoa* can be estimated by counting the number of motile and nonmotile spermatozoa

TABLE 2 Frequency Distribution of Mean Progressive Motility Scores in Semen Samples from 50 Fertile Men about 20 to 40 Min after Ejaculation (1st Obs.) and 4 H Later (2d Obs.)[a]

Mean Motility Score	Distribution in Percent	
	1st Obs., %	2d Obs., %
0	0	4
0.5	0	0
1.0	0	12
1.5	0	14
2.0	2	22
2.5	10	24
3.0	86	24
3.5	2	0
4.0	0	0

[a]Mean motility score is based on evaluation of two semen samples. 0 = no progressive motility, 1 = poor, 2 = medium, 3 = good, and 4 = very good progressive motility.

in at least 10 randomly selected microscopic fields. At higher sperm concentration this is, however, not feasible, and one must then make a general estimation. This includes naturally a higher degree of unreliability but, despite this experienced observers have a surprisingly good agreement in their evaluations.[6,11,15]

The distribution of semen samples from the 50 fertile men with regard to the percentage of motile spermatozoa is presented in Table 3. At the first observation 95 percent of the samples had 50 percent or more motile spermatozoa. At the same time it is important to realize that, also among these specially selected men, there were a few who, 3 to 4 mo after their wives had conceived, produced semen with less than 40 percent motile spermatozoa. The results presented in Table 3 correspond to those published earlier.[8]

The method recently developed by Dubois *et al.*[6] seems to be a major break through with regard to rapid, objective, and accurate measurements of sperm motility. The principle is that one part of a monochromatic laser beam passes a cell

TABLE 3 Frequency Distribution of Percentage of Motile and Dead Spermatozoa in Semen Samples from 50 Fertile Men (Two Specimens from Each Man) about 20 to 40 Min after Ejaculation (1st Obs.) and 4 H Later (2d Obs.)

	Distribution in Percent			
	Motile Spermatozoa		Dead Spermatozoa	
Percentage	1st Obs.	2d Obs.	1st Obs.	2d Obs.
0-10	0	0	0	0
11-20	0	4	2	0
21-30	2	4	25	14
31-40	1	14	51	38
41-50	18	46	16	32
51-60	41	32	4	8
61-70	38	0	0	8
71-	0	0	0	0

containing the semen (17 µl), and the spermatozoa cause a scattering of the light. Light scattered in a given angle is collected by a photomultiplier. By a system of mirrors, the photomultiplier also receives a part of the light that has not passed through the semen sample. The moving spermatozoa give rise to a Doppler effect, and the scattered light intensity is received by the photomultiplier as a spectrum of frequencies, the characteristics of which depend on the sperm motility. The frequency spectrum is analyzed and transformed into a graph on an X-Y recorder (Figures 1 and 2).

The parameters that can be assessed and graphically recorded within 5 min are the percentage motile spermatozoa, the velocity distribution, and the characteristic (mean) velocity. In addition, one can determine the total number of spermatozoa by measuring the light absorbed by the sample. The accuracy of the technique has been analyzed by comparing the results with those obtained from repeated measurements of the percentage motile spermatozoa and their velocity as recorded by film (64

frames/s) and play-back frame by frame (Figure 3). For further information see Jouannet *et al.*[15]

The importance of the prostatic fluid for the motility of human spermatozoa has been demonstrated by Lindholmer.[18] The motility of the spermatozoa can be increased by addition of albumin,[10] caffeine,[14,20] and kallikrein,[20] but the significance of these *in vitro* effects for the fertilizing ability of the spermatozoa remains to be demonstrated.

Spermatozoa with good motility in the seminal plasma can have a decreased capacity to penetrate the cervical mucus, and in some investigations it is therefore important to include this parameter in the evaluation of sperm motility. It is conceivable that drugs will be able to influence this parameter without significantly altering the motility pattern in the seminal plasma. For reviews see Kremer[17] and Ulstein.[22,23]

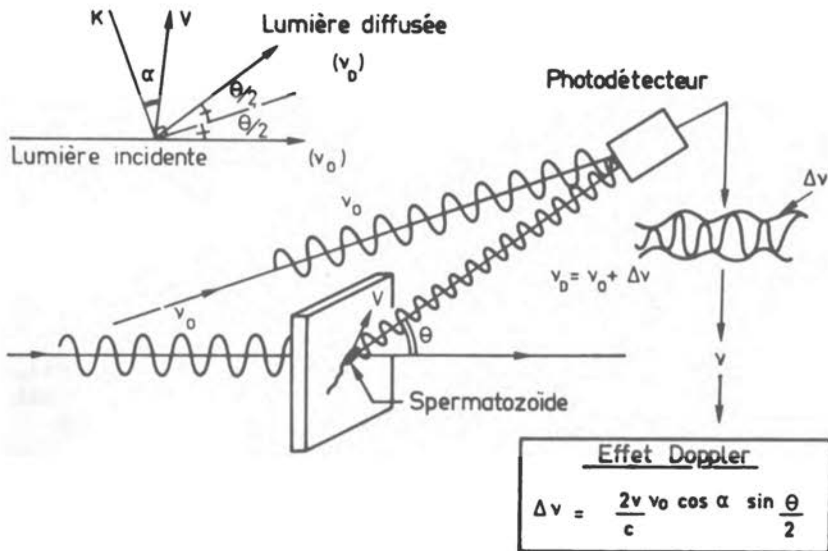


FIGURE 1 Principle for determining motility of spermatozoa with the light-scattering technique. The light scattered at the angle θ has wave lengths different from the incoming light due to the Doppler effect caused by moving spermatozoa. The frequency spectrum of the scattered light is recorded as illustrated in Figure 2.[6]

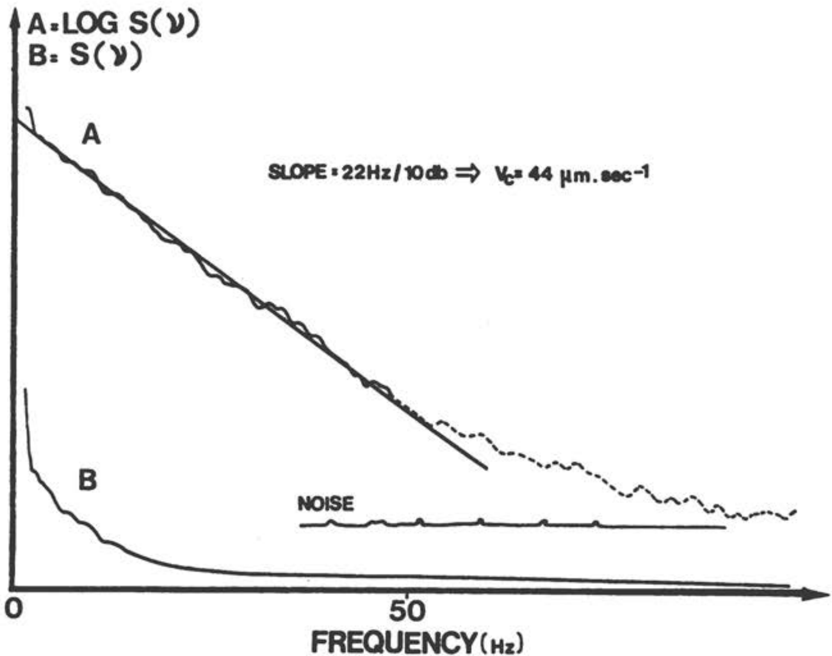


FIGURE 2 Frequency spectrum of light scattered by motile spermatozoa with a characteristic velocity of 44 µm/s. (A) A recording with semilogarithmic and (B) with linear coordinates. Noise = light scattered by the seminal plasma.[15]

VIABILITY

A nonmotile spermatozoon is not necessarily a dead cell, and for this reason it is of importance to determine not only the percentage of nonmotile spermatozoa but also that of dead cells. Assessment of the percentage of live spermatozoa is in most instances also a valuable check on the accuracy of the evaluation of the percentage of motile cells. Several supravital staining methods for spermatozoa have been proposed, but only a few are applicable for human spermatozoa.

Eosin Y is the commonly used supravital stain. It is, however, toxic for washed human spermatozoa even in a concentration of 0.1 percent, and the technique can therefore only be used if the suspending medium is seminal plasma or contains albumin (2 to 4 percent). Eosin is

bound to albumin or other proteins in the seminal plasma, and the capacity to bind the stain may vary from sample to sample. Too high concentrations of eosin (e.g. 5 to 10 percent) can therefore kill the spermatozoa in some semen samples.

Three methods can be recommended. In the first one, equal volumes of eosin 0.5 percent in saline or isotonic buffer and semen are mixed during 30 to 60 s. One drop of the mixture is placed on a clean microscopic glass, covered with a thin glass slip, and the specimen is evaluated with an ordinary microscope (magnification 400 to 600x). Motile spermatozoa are unstained. The dead spermatozoa are stained weakly red and are immotile. Some spermatozoa are immotile but have intact membranes and are therefore unstained. The specimens cannot be stored for later evaluation, and it is sometimes difficult to differentiate between stained and unstained cells.

To store the specimens for later or repeated analyses, one can make smears of semen mixed with an equal volume of

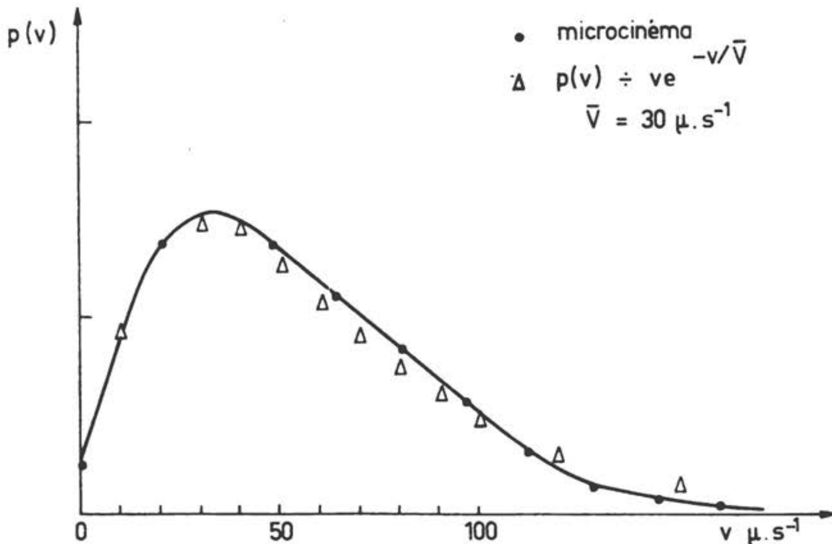


FIGURE 3 The correlation between velocity distribution [p(v)] vs. sperm velocity (v) measured from (a) recordings with high speed film (64 frames/s) and analysis of slow motion play-back (•——•), (b) the analysis of the frequency spectrum of the scattered light (Δ).[6]

eosin Y (0.5 percent in phosphate buffer, pH 7.5). The air-dried smear must, however, be examined with an anopthral (negative phase) contrast microscope (1,200x). With this technique the background is brown, live spermatozoa blueish, and dead ones yellow.[11]

Blom[2] introduced the eosin-nigrosin technique to differentiate between live and dead bull spermatozoa. This method has been widely used also for human spermatozoa but for these specimens the accuracy is not satisfactory. The concentration of nigrosin and also other factors (e.g. the protein concentration in the seminal plasma) can influence the results.

A modification of Blom's technique has recently been reported by Dougherty *et al.*[4] One drop of semen is mixed with two drops of 5 percent aqueous eosin Y for 15 s and then three drops of 10 percent aqueous nigrosin are added. A portion of the mixture is smeared on glass slides and dried on a warming plate. The slides can be evaluated with an ordinary light microscope (1,000x). The results have been compared with those obtained with the method by Eliasson and Treichl.[11] The eosin-nigrosin solution gave slightly (but highly significant) higher percentages of dead spermatozoa than that with eosin alone. However, if the eosin concentration in the method by Dougherty *et al.* was lowered to 1 percent the results with the two methods were similar (Eliasson, unpublished).

From studies on fertile men (Table 3, and Eliasson,[8] it can be concluded that only exceptionally will a semen sample from a fertile man contain more than 50 percent dead spermatozoa 40 min after the ejaculation or more than 60 percent 4 h later.

CONCLUSION

Evaluation of the motility and viability of human spermatozoa is usually carried out with the objective to assess fertility. Despite the imperfections of the techniques used, there is a general opinion that poor motility is a sign of decreased fertility. On the other hand, it must be emphasized that good motility in the seminal plasma is not a predictor of fertilizing ability. A number of factors in the semen, cervical mucus, endometrial fluids, and diluting fluids (used for *in vitro* experiments and inseminations) will influence the motility and viability of the spermatozoa.[18,19]

Objective research on the relation between motility and viability of human spermatozoa and the fertilizing capacity of these cells involves many and very difficult problems. Nevertheless, advances in this field will be made only if some basic requirements are fulfilled. The motility must be measured with regard to both quality (speed, type of progressive motility) and quantity (percent moving spermatozoa) and with due attention to the time factor and the fact that the individual spermatozoa show wide variability in their swimming rate. In addition, the fertility of the semen or fertilizing ability of a given sperm population must be defined properly.

DISCUSSION

F. I. ELLIOTT: You stated that the light-scattering technique is used where semen plasma is the medium. Can or could it be used in other media, such as in diluted frozen-thawed semen?

RUNE ELIASSON: Yes, the light-scattering technique can be used with seminal plasma.

REFERENCES

1. Barták, V. 1971. Zur Problematik der objektiven Bestimung der Spermatozoen-Motilität. Andrologie *3*(3):99-107.
2. Blom, E. 1950. A one-minute live-dead sperm stain by means of eosin-nigrosin. Fertil. Steril. *1*:176.
3. Castenholz, A. 1974. Photokymographische Registriermethode zur Darstellung und Analyse der Spermatozoenbewegungen. Andrologia *6*(2):155-168.
4. Dougherty, K. A., Emilson, L. B. V., Cockett, A. T. K., and Urry, R. L. 1975. A comparison of subjective measurements of human sperm motility and viability with two live-dead staining techniques. Fertil. Steril *26*(7):700-703.
5. Dubois, M., Jouannet, P., Berge, P., and David, G. 1974. Spermatozoa motility in human cervical mucus. Nature *252*(5485):711-713.
6. Dubois, M., Jouannet, P., Berge, P., Volochine, B., Serres, C., and David, G. 1975. Méthode et appareillage de mesure objective de la mobilité des spermatozoides humains. Ann. Phys. Biol. Méd. *9*(1):19-41.

7. van Duijn, C., van Voorst, C., and Freund, M. 1971. Movement characteristics of human spermatozoa analyzed from kinemicrographs. Eur. J. Obstet. Gynecol. *4*:121-135.
8. Eliasson, R. 1975. Analysis of semen. In: Behrman and Kistner, eds. *Progress in Infertility,* Vol. 2. pp. 691-713.
9. Eliasson, R., Johnsen, Ø., and Lindholmer, C. 1974. Effects of seminal plasma on some functional properties of human spermatozoa. In: R. E. Mancini and L. Martini, eds. *Male Fertility and Sterility. Proceedings of the Serono Symposia,* Vol. 5. pp. 107-122.
10. Eliasson, R., and Lindholmer, C. 1975. Human seminal plasma and sperm survival and transport. In: *The Biology of Spermatozoa.* INSERM Int. Symp. Nouzilly 1973. Basel: Karger, pp. 83-92.
11. Eliasson, R., and Treichl, L. 1971. Supravital staining of human spermatozoa. Fertil. Steril. *22*:134-137.
12. Gassner, F. X., Goldzieher, J. W., Masken, J. F., and Hopwood, M. L. 1959. The objective measurement of sperm motility. Fertil. Steril. *10*(5):488-501.
13. Jecht, E. W., and Russo, J. J. 1973. A system for the quantitative analysis of human sperm motility. Andrologie *5*(3):215-221.
14. Johnsen, Ø., Eliasson, R., and Abdel Kader, M. M. 1974. Effects of caffeine on the motility and metabolism of human spermatozoa. Andrologia *6*(1):53-58.
15. Jouannet, P., Volochine, B., Deguent, P., Serres, C., and David, G. 1976. Light scattering determination of various characteristic parameters of spermatozoa motility in a serie of human sperm. Andrologia. In press.
16. Katz, D. F., and Dott, H. M. 1975. Methods of measuring swimming speed of spermatozoa. J. Reprod. Fertil. *45*:263-272.
17. Kremer, J. 1968. The in vitro spermatozoal penetration test in fertility investigations. Ph.D. Dissertation, University of Groeningen, The Netherlands. Groeningen: van Denderer.
18. Lindholmer, C. 1974. The importance of seminal plasma for human sperm motility. Biol. Reprod. *10*:533-542.
19. Nelson, L. 1975. Spermatozoan motility. In: *Handbook of Physiology,* Section 7: Endocrinology. Volume V: D. W. Hamilton and R. O. Greep, eds. *Male Reproductive System.* Washington, D. C.: American Physiological Society.

20. Schill, W. B. 1975. Caffeine- and kallikrein-induced stimulation of human sperm motility: A comparative study. Andrologia *7*(3):229-236.
21. Troll, J., and Goldzieher, J. W. 1962. Quantitative evaluation of the motility of spermatozoa. Fertil. Steril. *13*(1):72-83.
22. Ulstein, M. 1972. Sperm penetration of cervical mucus as criterion of male fertility. Acta Obstet. Gynecol. Scand. *51:*335.
23. Ulstein, M. 1973. Fertility of donors at heterologous insemination. Acta Obstet. Gynecol. Scand. *52:*97.

PHYSIOMORPHOLOGICAL PARAMETERS OF HUMAN SPERMATOZOA

E. S. E. Hafez

Spermatozoa normally constitute no more than 5 percent of the volume of the human ejaculate. The normal concentration of spermatozoa in semen varies between 40 and 125 million ml. Unlike other mammalian species, human spermatozoa show a greater variability in head size and shape, and are characterized by the presence of conspicuous head vacuoles and the prevalence of nuclei with incomplete chromatin condensation.

The following discussion deals with modern methods to examine physiomorphological parameters of spermatozoa, structural and ultrastructural characteristics of human spermatozoa, common and uncommon morphological anomalies, and physiochemical characteristics of spermatozoa in relation to male fertility and infertility.

TECHNIQUES TO STUDY SPERM PHYSIOMORPHOLOGY

Several techniques have been utilized to examine sperm characteristics not commonly observed by light microscopy. Transmission[7,18,21,22] and scanning electron microscopy[8,9] have been used more extensively than immunoelectron microscopy. Ultrastructural techniques are invaluable to provide basic information in andrology and of clinical value in several types of male infertility.[13,14,19,20]

Several methods have been used for scanning electron microscopy of human spermatozoa. A simple technique for clinical use follows.

Semen sample is centrifuged in physiologic saline for 2 to 4 min at 350 rpm and washed twice with distilled water for 2 min each. One drop of cell suspension is placed on a cover glass and air-dried, or fixed in 0.5 percent or 2 freshly prepared solution of glutaraldehyde

in Sorensen's phosphate buffer, pH 7.4. Several specimens from each sample are coated with gold-palladium using the HUMMER DC sputtering system. A negative potential is applied to the gold cathode, which is enclosed in the process chamber at a pressure of 150-200 millitorr. This process is advantageous to vacuum evaporation for the preparation of SEM specimens. The specimens are observed in a scanning electron microscope equipped with a 200 μm aperture and operated with an accelerating voltage of 25kV.

X- and Y-containing spermatozoa do not seem to vary in shape and dimension. Several unsuccessful attempts have been made to control sex ratio by separating X- and Y-spermatozoa by electrophoresis, countercurrent centrifugation, or density-gradient sedimentation, but no conclusive results have been obtained. The long arm of the Y-chromosome in somatic cells exhibits an intense fluorescence with certain fluorescent compounds, e.g., quinacrine mustard and quinacrine hydrochloride. The same technique was used to identify Y- and X-spermatozoa in humans, which can be readily distinguished by the presence in the former of a discrete fluorescent spot.

Plasmalemma of spermatozoa plays a functional role in several phenomena, such as sperm motility, capacitation, and fertilization. Electroanalytical techniques are used to evaluate the changes induced on the cell surface of intact and metabolically active spermatozoa by treatment with neuraminidase and human follicular fluid.[25] Other techniques are designed to uncover differences in the glycolytic metabolism of spermatozoa from fertile and infertile men.[23] Coulter Counters are used to measure the equivalent volume of spermatozoa.[5]

Several immunological techniques have been used to detect sperm agglutination, e.g., cytotoxicity, immobilization, and immunofluorescence. These techniques reveal antibodies against various antigens localized on the surface of or inside the spermatozoa. In a simple and sensitive microagglutination procedure, 200 to 300 sera may be tested simultaneously with a single donor sperm sample, and the type of agglutination identified.[11] The immunofluorescence technique with fixed spermatozoa appears to involve antigens in the structures under the cell membrane of the spermatozoon, whereas the other methods probably demonstrate reactions with antigens in the cell membrane.[15]

Immunocytochemical techniques are useful for studying sperm enzymes and the sperm membrane. Flechon *et al.*[9] have developed an elegant immunocytochemical technique.

Smears of washed spermatozoa are treated by an indirect immunocytochemical technique. The first antiserum used is prepared in rabbits against ovine (or bovine) hyaluronidase. A sheep antiserum against rabbit globulin, labeled with fluorescene or peroxidase, is used as the second reagent.

SPERMATOZOA WITH NORMAL MORPHOLOGY

It is difficult to define normal and abnormal spermatozoa. Several criteria have been used to identify a normal and fertile ejaculate, and several descriptions have been used to define morphologically normal spermatozoa. More is known about "sperm with normal morphology" than "normal sperm." Spermatozoa of normal morphology of the head, acrosome, midpiece, and tail are more likely to be functionally normal with ability to penetrate cervical mucus and achieve fertilization of the egg.

Human semen is unique when compared with other mammalian semen. For example, there is a high percentage of morphologically abnormal spermatozoa in specimens from donors of high fertility. Also, the standard hydroplane-shaped sperm head is not uniform in size or shape. Because of this lack of uniformity in human spermatozoa, several classification schemes for morphologic characteristics have been described.[10,13]

Human spermatozoa with normal morphology are characterized by flattened and ovoid heads that appear pointed at the posterior end and become somewhat round and flat anteriorly (Figure 1). Spermatozoa of fertile men show remarkable heterogeneity in the size and shape of the head and size of mitochondria. A simple and inconspicuous acrosome covers the anterior two-thirds to three-fourths of the nucleus. The two segments of the postacrosomal sheath are demarcated by a shallow circumferential groove, the anterior segment being more regular than that of the posterior one. There are remarkable individual variations in the shape of this groove.

There are considerable variations in the morphology and thickness of the spindle-shaped and narrow neck. Occasionally the head is surrounded by a circumferential band at the head-neck junction. This structure, from studies on thin-section electron microscopy and freeze-etching, has been referred to as striated band, basal belt, or posterior ring. In some spermatozoa the neck appears as a thickening or as a slight constriction connecting the

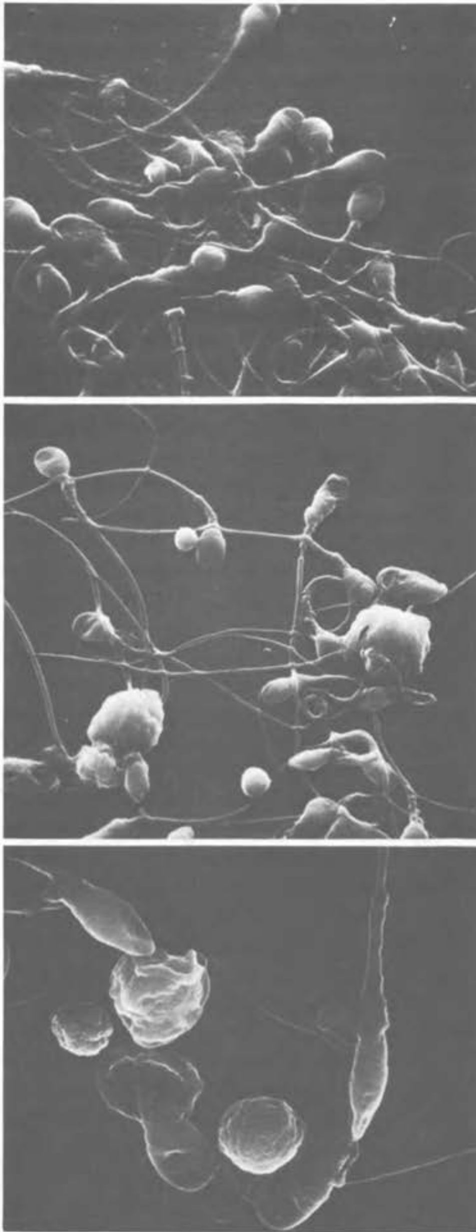


FIGURE 1 Scanning electron micrographs of normal spermatozoa from men of known fertility. Note variability is size and shape of sperm head.

midpiece. The neck cytoplasm may extend anteriorly to the posterior ring and posteriorly into the anterior part of the middle piece. The cytoplasm of the neck extends forward to surround the basal portion of the sperm head.

The ultrastructure of the spermatozoon adapts it to its propulsive forward motility with rotation around its longitudinal axis. The common pattern of sperm motility is an undulating movement from side to side, superimposed on a twisting component.[24] The close apposition of the mitochondria to the anterior end of the contractile portion of the tail ensures a short diffusion path for ATP and other metabolites involved in contraction. The transverse furrow observed on the head of the human spermatozoon under scanning microscopy seems to correspond to the light zone observed under light microscopy and at the infolding of the plasma membrane in the gap between the acrosome and postnuclear cap.[12,18]

The physiological significance of the plasmalemma of spermatozoa has been studied during *in vitro* and *in vivo* fertilization using transmission electron microscopy. The plasmalemma overlying the postacrosomal sheath is the part of the sperm head that first contacts the oocyte surface and fuses with its membrane.[1,2,29-31]

SPERM ANOMALIES

The most commonly abnormal forms are tapered-headed, giant-headed, pin-headed, double- or quadruple-headed, dumbbell-headed, or frazzled-headed spermatozoa with coiled tails, bent tails, or two tails.[17] Other morphological anomalies of the acrosome and midpiece are noted by scanning and transmission electron microscopy (Figures 2 and 3).

Several types of immature germ cells have been noted in semen, e.g., spermatids, spermatocytes, and even spermatogonia. A count of more than 10^6 spermatids/ml is indicative of a serious spermatogenic derangement and may result in male infertility.[13] The presence of large numbers of cytoplasmic droplets on the spermatozoa indicates epididymal dysfunction or that the patient was abstinent for too short a period of time. The latter may be determined by asking the patient to be abstinent for a longer period of time and repeating his semen analysis.

Morphological anomalies in spermatozoa may be a result of biochemical abnormalities. The recurving and coiling of the tails seems to arise during transport through the excurrent ducts, probably as a result of

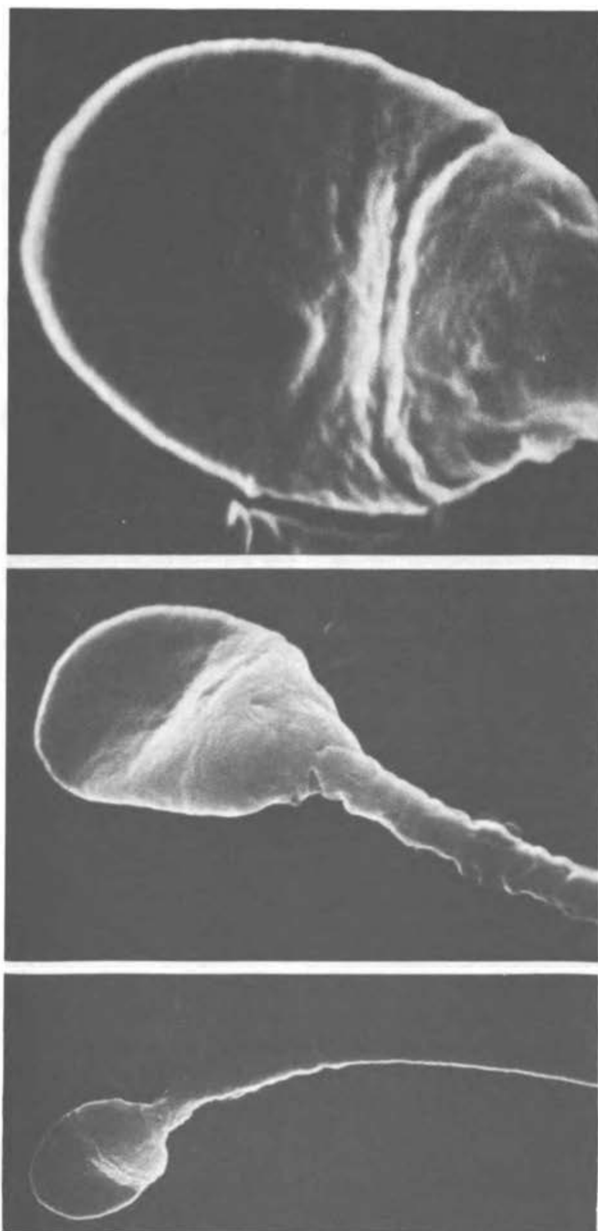


FIGURE 2 Scanning electron micrographs of abnormal spermatozoa from men of known fertility. Note abnormal acrosome and mid-pieces.

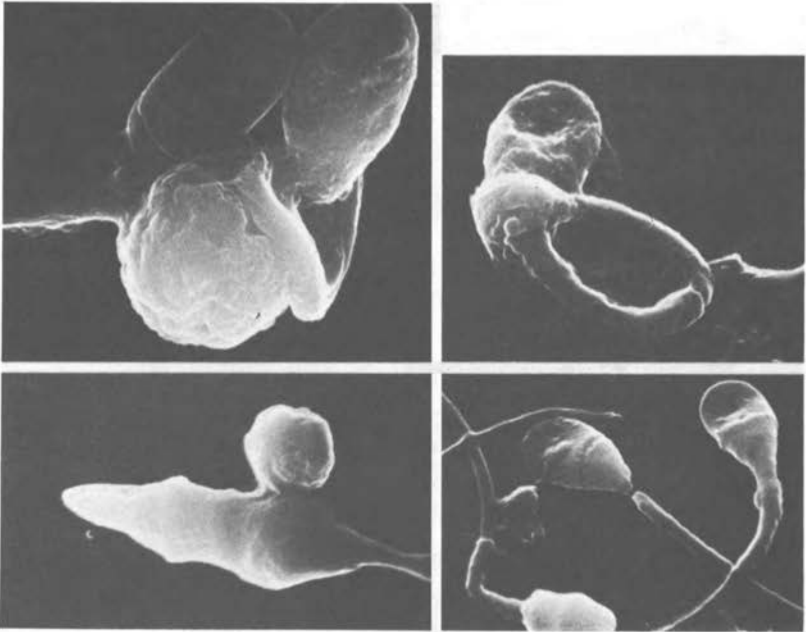


FIGURE 3 Scanning electron micrographs of abnormal spermatozoa from infertile men attending clinic. Note abnormal acrosome and midpiece.

physiological changes in concentrations.[3,4,19] These spermatozoa seem to undergo remarkable degenerative changes. It is possible that the cytoplasmic clumps observed in some abnormal spermatozoa may be due to metabolites involved in mitochondrial metabolism.

Varying degrees of asthenospermia are not uncommon. However, it is rather rare to identify severe astenospermia-necrospermia as a constant feature in a given patient. It is known whether the given spermatozoa are dead or just immotile, and whether the changes are of testicular or epididymal origin.[20] Spermatozoa from patients with astenospermia and/or necrospermia may show coiling or sharp bending of the tail. Such changes may arise during the excurrent passage of the spermatozoa.[20]

Several types of abnormal spermatozoa have been reported.[20,26,28] Pedersen *et al.*[20] reported on two unrelated, infertile patients who produced only spermatozoa with globular heads that lack both an acrosome and a postacrosomal sheath. The unique ultrastructural

characteristics included partial or total absence of nuclear envelope, abnormally arranged mitochondria, and derangement of the axoneme. Occasionally infertile men, with round globular sperm beads, have normal sperm count and sperm motility.

Exclusively round-headed spermatozoa without acrosomes were detected in the ejaculates of infertile men.[16] Abnormal acrosomal development, independent, of spermatid nuclei, occurs during the differentiation phase of spermatogenesis; the acrosome is incorporated into the Sertoli cell after release of the spermatozoon from the germinal epithelium.[16] Four acrosomal anomalies have been recognized: (a) the acrosomal vesicle develops in close contact to the spermatids nucleus but with abnormal development of electron dense material, (b) the acrosome develops independently from the spermatids nucleus, (c) two nuclei of spermatids are connected by one acrosome, and (d) two acrosome are attached to the nuceus of one spermatid.[16]

PHYSIOCHEMICAL PARAMETERS

The pattern of sperm motility is closely related to the exogenous and endogenous respiration rates. There are several types of motility, but two types are common: (a) directional progressive motility, where the spermatozoon actually moves forward; and (b) vibratory motility, where the spermatozoon moves its tail but does not progress in a forward direction. The motility pattern is different in the epididymal as compared to ejaculated spermatozoa, and motility also varies in different fractions of the ejaculate. Since motility rating is higher in the first fraction of the ejaculate,[6] the sperm-rich first half of the ejaculate is used for artificial insemination. The patterns of sperm motility change as spermatozoa encounter the changing ionic milieu and biophysical states of the epididymal fluid; seminal plasma; cervical mucus; and endometrial, oviductal, and peritoneal fluids. Sperm velocity increased in the presence of oviductal or follicular fluid. Improvement of sperm motility can be produced by admixture of kallikrein or kinins to semen samples, whereas systemic administration of kallikrein for several months to men with oligospermia improved sperm motility and increased the number of sperm in the ejaculate.[27]

The vaginal environment is hostile to sperm survival. Sperm transport through the cervix is affected by contractile

activity of vagina and cervix, properties of cervical mucus, rate of liquifaction of coagulum, directional motility of sperm, and possibly orgasm. Sperm penetration in cervical mucus depends on its biophysical and biochemical characteristics, pH, degree of cellularity, and presence of immunoglobulins in the mucus; proteolytic enzymes in spermatozoa; and concentration of prostaglandin and related substances in seminal plasma. Several techniques have been used to study sperm distribution in the female reproductive tract utilizing laparotomy, laparoscopy, salpingectomy with segmental flushing, or fractional postcoital tests. During their transport in the female tract, spermatozoa are separated from seminal plasma and resuspended in endometrial and oviductal fluid.

Sperm transport to the site of fertilization is affected by endocrine, hereditary, immunological, and psychological factors. Before being able to penetrate the zona pellucida, the spermatozoon undergoes final maturation in the female reproductive tract (capacitation). Although this penomenon has been established for a variety of experimental animals, it is not confirmed in man. This phenomenon is followed by the "acrosome reaction," which involve multiple fusions between the plasma and outer acrosomal membranes, with subsequent vesiculation. The acrosome contains several enzymes that play a major role in the fertilization process.

Glycolysis is the major energy producing pathway to spermatozoa. The metabolic pattern of ejaculated spermatozoa may be of diagnostic value for certain types of male infertility. Spermatozoa from oligoasthenospermic men produce four times more lactate and CO_2 than spermatozoa from normal men, and 6.4 times more pyruvate. Spermatozoa from asthenospermic men form similar amounts of lactate and pyruvate as from normal men.[23]

There are regional differences in the biochemical properties of the sperm membrane. Hyaluronidase is mainly localized in the acrosomal contents, whereas acrosin is mainly bound to the inner acrosomal membrane and equatorial segment. The sperm midpiece possesses a cytoplasmic region and a lipid-rich mitochondrial sheath and contains the enzymes and the cytochrome oxidase system utilized for carbohydrate metabolism and respiration. The glycosphingolipids of spermatozoa, concentrated in the outer cell membrane, seem to play a role in the interaction of the cell with its environment. After ejaculation, several components of the accessory fluids adhere to the spermatozoa, most of which are removed during uterine transport.

Maturational interaction of spermatozoa with the genital tract fluids of the female and shedding of the acrosome involve dramatic changes in the characteristics of the surface membranes.

REFERENCES

1. Barros, C., and Franklin, L. E. 1968. Behavior of the gametes membranes during sperm entry in the mammalian egg. J. Cell Biol. *37:*13.
2. Bedford, J. M. 1970. Morphological aspects of sperm capacitation in mammals. In: G. Raspe, ed. *Mechanisms Involved in Conception.* Vieweg: Pergamon Press. p. 15.
3. Blom, E., and Birch-Andersen, A. 1966. The ultrastructure of a new sterilizing defect (The dog defect) in the bull sperm tail. In: A Ingelman-Sundberg and B. Westin, eds. Proc. V Int. Congr. Fertil. Steril. Ser. *109:*602.
4. Blom, E., and Birch-Andersen, A. 1965. The ultrastructure of bull sperm. II. The sperm head. Nor. Vet.-Med. *17:*193.
5. Brotherton, J. Sperm counting and sizing with a Coulter counter. In: P. O. Hubinont and M, L'Hermite, eds. *Sperm Action.* New York: Karger-Basel. pp. 36-41.
6. Eliasson, R., and Lindholder, C. H. 1972. Distribution and properties of spermatozoa in different fractions of split-ejaculates. Fertil. Steril. *23:*252.
7. Fawcett, D. W., and Phillips, D. M. 1969. Recent observation on the ultrastructure and development of mammalian spermatozoon. In: B. Baccetti, ed. *Comparative Spermatology.* Rome: Academia Nazionale Dei Lincei.
8. Flechon, J. E. 1971. La membrane du spermatozoide de lapin-repartition et nature des glycoproteins de la surface cellulaire. J. Micros. *11:*53.
9. Flechon, J. E. 1975. Surface coat of spermatozoa. In: E. S. E. Hafez, ed. *Scanning Electron Microscopical Atlas of Mammalian Reproduction.* New York: Harper & Row.
10. Freund, M. 1968. Semen analysis. In: J. S. Behrman and R. W. Kistner, eds. *Progress in Infertility.* Boston: Little.
11. Friberg, J. 1974. Acta obstetricia et gynecologica. Scand. Suppl. *36:*76.

12. Fujita, T., Miyoshi, M., and Tokunaga, J. 1970. Scanning and transmission electron microscopy of human ejaculate spermatozoa with special reference to their abnormal forms. Z. Zellforsch. *105:*403.
13. Hafez, E. S. E. 1976. *Human Semen and Fertility Regulation in the Male.* St. Louis: Mosby. 670 pp.
14. Hafez, E. S. E. 1977. *Techniques of Human Andrology.* Amsterdam: Elsevier.
15. Hansen, K. B. 1974. A comparative study of various methods for the detection of spermatozoal antibodies. Acta Obstet. Cynecol. Scand. *53:*69.
16. Holstein, A. F., Schirren, C., and Schirren, C. G. 1973. Human spermatids and spermatozoa lacking acrosomes. J. Reprod. Fertil. *35:*489.
17. MacLeod, J. 1973. The parameters of male fertility. Hosp. Prac. *8:*43.
18. Pedersen, H. 1969. Ultrastructure of the ejaculated human sperm. Z. Zellforsch. *94:*542.
19. Pedersen, H., and Lebech, P. E. 1971. Ultrastructural changes in the human spermatozoon after freezing for artificial insemination. Fertil. Steril. *22:*125.
20. Pedersen, H., Rebbe, H., and Hammen, R. 1971. Human sperm fine structure in a case of severe asthenospermia-necrospermia. Fertil. Steril *22:*156.
21. Pedersen, H. 1972. The postacrosomal region of the spermatozoa of man and *Macaca arctoides.* J. Ultrastruct. Res. *40:*366.
22. Pedersen, H. 1974. The human spermatozoa. An electron microscopical study including comparative details of Macaca arctoides spermatozoa. *Costers Bogtrykkera,* Copenhagen.
23. Pedron, N., Giner, J., Hicks, J. J., and Rosado, A. 1975. Comparative glycolytic metabolism of sperm from normal asthenospermic and oligoasthenospermic men. Fertil. Steril. *26:*309.
24. Phillips, D. M. 1972. Comparative analysis of mammalian sperm motility. J. Cell Biol. *53:*561.
25. Rosado, A., Velazquez, A., and Lara-Ricalde, L. 1973. Cell polarography. II. Effect of neuraminidase and follicular fluid upon the surface characteristics of human spermatozoa. Fertil. Steril. *24:*349.
26. Ross, A., Christie, S., and Kerr, M. G. 1971. An electron microscope study of a tail abnormality in spermatozoa from a subfertile man. J. Reprod. Fertil. *24:*99.

27. Schill, W. W., Wallner, O., Palm, S., and Fritz, H. 1976. Stimulation of sperm motility and migration by kinins. In: E. S. E. Hafez, ed. *Human Semen and Fertility Regulation in the Male*. St. Louis: Mosby.
28. Schirren, C. G., Holstein, A. F., and Schirren, C. 1971. Über die Morphogense rundkopfiger spermatozen des Menschen. Andrologia *3*:117.
29. Stefanini, M., Oura, C., and Zamboni, L. 1969. Ultrastructure of fertilization in the penetration of sperm into the ovum. J. Submicr. Osc. Cytol. *1*:1.
30. Yanigamachi, R., and Noda, Y. D. 1970a. Ultrastructural changes in the hamster sperm head during fertilization. J. Ultrastruct. Res. *31*:465.
31. Yanigamachi, R., and Noda, Y. 1970b. Electron microscope studies of sperm incorporation into the golden hamster egg. Am. J. Anat. *128*:429.

SOME METHODS OF FREEZING AND EVALUATING HUMAN SPERMATOZOA

E. F. Graham and B. G. Crabo

INTRODUCTION

The advantages and disadvantages of artificial insemination in humans, with the use of frozen spermatozoa, have been discussed extensively.[9,10,35,47,50,53,81,84-86,97] In general, the use of frozen semen has several advantages for medical applications. The preservation of semen by freezing makes possible the protection of spermatozoa from those males who will be engaged in occupations that may affect their future fertility. Spermatozoa preserved by freezing also provide the possibility for future parenthood of those males who plan to be vasectomized. Banks of frozen semen also provide the opportunity for legitimate parenthood of women who are married to oligospermic males with low fertility.

The primary disadvantage of frozen spermatozoa is at this time reduced fertility. This may be due to deficiencies in the techniques of preservation.

Several hundred human offspring have resulted from the use of frozen semen.[10,14-16,24-27,33,48-51,56,64,71-73,81,87,88,93,101] A diversity of procedures for preservation of spermatozoa by freezing and factors affecting their recovery after thawing have been reported. Due to the variety of techniques employed and the limited numbers of inseminations, an optimal procedure cannot be described. It is unlikely that this problem will be overcome very soon. Many of the pregnancies reported are clinical observations rather than detailed comparative studies. Where attempts were made to compare techniques with different laboratory assays, it was generally found that the fertilizing capacity of frozen semen is less than that of fresh semen. Little or no relationship exists between laboratory assay techniques employed and fertility,

indicating that assay techniques are not sensitive enough to detect differences or that human sperm cells are relatively unaffected over a large range of physical and chemical environments. The only hope for improving the techniques of freezing human semen is for the scientist to implement improved laboratory techniques for measuring cell damage.

The purpose of this paper is to review the steps in freezing spermatozoa, to relate the data collected on humans to what is known of animal species, and to suggest additional test assays.

STEPS IN CRYOPRESERVATION OF SEMEN

Semen Collection

There is little discrepancy in the literature concerning the preferred mode of semen collection, which is masturbation, on site, immediately before employing further preservation steps. The importance of standardization, however, has been suggested.

Liquification and Pre-Dilution Treatment

Upon ejaculation, human spermatozoa are emitted in a mass of gelatinous material from the bulbo urethral glands. Through the action of amylase, the entire ejaculate liquifies. This usually occurs spontaneously over a 20-30-min period. The importance of detrimental effects of this process on the end results of cryopreservation has not been elaborated.

In bull and boar spermatozoa it has been found that holding spermatozoa in their own plasma for a period of time enhances cryopreservation of the cell[39,66] due to the absorption of proteinacious material on the cell. On the other hand, the gelatinous material from the bulbo urethral glands of the boar is strained off prior to holding or dilution. These procedures may also be of benefit for cryopreservation of human spermatozoa.

Buffer

Some fertility data have been reported when no buffer system or dilution was used.[23-26,57,63,64,77,79,80,88,100]

Others reported fertility data when buffer systems consisting of sodium citrate, glucose, and egg yolk,[10-12,14,15] or the same with added glycine[49,72] were used. In comparative studies, greater recovery of motile spermatozoa was reported in Norman-Johnson (NJ) or NJ modified buffer.[37,68] The optimal composition of the buffer system was not determined, but the pH of 7.4 adjusted with sodium bicarbonate[56] was reported to be important.

Buffer systems for animal spermatozoa were the first prerequisite to commercial application and study. The importance of egg yolk as an additive to phosphate and citrate buffers was shown as early as 1939. Since that time, egg yolk has been reported to be a major cryoprotectant to spermatozoa.[60] The cryoprotecting properties of egg yolk have been ascribed to phospholipids and lipoproteins. Although citrate has been the preferred buffer for cattle semen, buffers of lower ionic strength appear essential for freeze preservation of spermatozoa of other species such as boar, ram, goat, and turkey. Lately, zwitter ionic buffers, primarily TES, HEPES, and TRIS have been shown to be universal buffers for freezing of spermatozoa of most species.[22,30,40]

Dilution

The effect of dilution on the freezing of human spermatozoa has been studied, dilution being the primary method for increasing inseminations per ejaculate.[37] Most researchers have used a 1:0, 1:1, or 1:2 dilution ratio. Little information on the optimum number of spermatozoa to facilitate pregnancy is available at this time. However, Colton and Farris[28] state that an excess of 20×10^6 motile spermatozoa are required to ensure pregnancy.

In cattle, dilution ratios of 1:50 and 1:100 are common with the need to inseminate approximately 10×10^6 spermatozoa, while in swine 2.0 to 2.5×10^9 spermatozoa are required with at least 50 ml of fluid. For successful insemination of sheep and goats, 250 to 300×10^6 live spermatozoa are required. There have been no reports of successful freezing of animal spermatozoa in the absence of buffer or dilution or by adding glycerol directly to the semen.

Protective Mechanisms

In general, the classical penetrating cryoprotective agent, glycerol, has been used. The recovery of live spermatozoa after freezing in various concentrations of glycerol has been reported by several investigators. Optimum concentrations were 7.0 percent,[49] 7.5 percent,[56,63,72] 7.7 percent,[72] 8.3 percent,[68] and 10.0 percent.[64,79] Other researchers[35] compared 0, 5, 10, and 15 percent glycerol v/v with recovery rates of 13, 72, 46, and 15 percent respectively. Karow[52] compared glycerol with dimethyl sulfoxide (DMSO) and reported that 5-10 percent of either compound was protective. DMSO was reported to be equal to glycerol as a cryoprotectant at 10 percent concentration of either,[83] while one study[100] showed that glycerol was superior to DMSO as a cryoprotectant for human spermatozoa.

In domestic animals, it has been shown that the greatest protection of spermatozoa during the freezing process is achieved with added lipoproteins, primarily from egg yolk,[60] and that glycerol has an additive effect. Researchers studying human spermatozoa suggest that proteins of milk and egg yolk are beneficial in the buffer system for freeze preservation,[82] while others reported that egg yolk provided no added value in freezing human spermatozoa.[68]

Gradually adding cryoprotectants such as glycerol and DMSO at a low temperature, to prevent rapid changes in osmotic pressure, is advantageous for several species of spermatozoa. The same appears to be true for human spermatozoa.[34,52,56,72] Perloff *et al.*[64] suggested that when glycerol is added directly to neat semen, it be added slowly and mixed well. Others[79,96] found no difference due to the method of adding the cryoprotectant to the semen.

For cattle spermatozoa, glycerol has been the penetrating cryoprotectant of choice in the range of 3.5 to 7.0 percent v/v final dilution. Concentrations of glycerol above 2 percent have been reported to disrupt fertility in both swine and turkeys.[18,21] The cryoprotectant of choice for turkey spermatozoa is either ethylene glycol or DMSO.[21]

Regardless of species, glycerol can be added slowly at 5°C. Turkey, ram, and bull semen can be added directly to the glycerolated buffer at 37°C with no apparent effect on fertility. It must, however, be added at a lower temperature (5 to 10°C) to goat or boar spermatozoa. Although for most species, a penetrating cryoprotectant increases recovery, it is not essential for satisfactory freezing of boar, ram, goat, or bull spermatozoa, provided a proper buffer system and freeze procedure are employed.[31,41,44]

Cooling

Cold shock, a phenomenon caused by cooling too rapidly to 5°C, has been shown to occur in most mammalian spermatozoa. In general, studies with human spermatozoa suggest slow cooling at about 1°C/min for room temperature to 5°C.[6,15,56] Slower cooling rates (3 to 4 h) over this temperature range were also suggested,[35-37] while some studies showed no difference due to rate of cooling to 5°C.[78,79]

Time Lapse between Cooling and Freezing

In other mammalian spermatozoa, a time lapse between the addition of glycerol to the semen and freezing is considered beneficial. This is the procedure used for most animal species. In general 1 to 2 h is considered optimal. For human spermatozoa, Matheson *et al.*[56] suggest 15 min. Fernandez-Cano *et al.*[33] showed no difference between 1 and 4 h, but showed that post-thaw recovery of spermatozoa, frozen after holding for 16 to 20 h was inferior. Long equilibration time of 6 to 18 h has also been suggested.[36]

Freeze Rates

After years of research, the optimum freeze rate for spermatozoa from domestic animals is not known. This is true also for human spermatozoa. The primary reason for the lack of defined optimum freeze rates is that cell recovery at any specific rate varies due to variability among ejaculates in sensitivity to buffers, to cryoprotectant, and to concentration of cryoprotectant. Also, measurement is difficult due to different geometries of the sample and the influence of the heat of fusion. Thermocouples placed in various positions in samples during freezing by various methods show different freeze rates unless the sample is frozen in a very thin film. For human spermatozoa, cryopreservation rates from very slow to very fast are suggested. Some researchers recommend rates of 1°C/min,[34,36,72,77] some recommend 5 to 10°C/min,[15,33,56,74] while others recommend 16 to 25°C/min,[26,64,68,79,80,88,96] and some reported no real difference due to freeze rate after the temperature reached -20 to -30°C. The conclusions reached were primarily based upon motility ratings.[15,56,74] Comparisons of 0.5°C/min vs. 10°C/min[33]

showed a greater recovery rate for the faster freezing rate.[26,67,77,88]

Type of Storage Container

In general, human semen has been frozen in labeled glass ampules. Others have suggested freezing spermatozoa in straws[56] or pellets.[65] However, cross-contamination with bacteria was found in semen frozen in the pellet form and exposed directly to liquid nitrogen. Early research[62] indicated that freezing in a thin film resulted in poorer recovery than semen frozen in bulk. However, when freezing in a thin film, the proper freezing rate is critical.

For practical use, a container for storage of frozen spermatozoa should be as small as possible to facilitate controlled freeze rate and savings in storage space. It should be large enough, however, to contain sufficient volume and sperm numbers for a single insemination and have a shape suitable for adequate labeling.

Thawing or Warming Rates

Few actual measurements have been conducted on the effect of thawing rates on recovery of spermatozoa from the human, but some literature indicates that a slower warming rate in a 5°C bath or air thawing is superior to faster warming rates in a 37°C water bath.[56,74] Others report a greater recovery after a faster warming rate.[79,80,88,96]

Long-Term Storage and Temperature

Most studies conducted on long-term storage of animal spermatozoa indicate no decrease in recovery of spermatozoa or fertilizing capacity due to time of storage, providing the samples are stored at -196°C. For human spermatozoa, no decrease in motility was observed over short-to-long periods of time[34,37,57,62,80] or even up to 10-yr storage.[87] Semen stored at -79°C was significantly different when stored from 1 to 6 mo. Motility studies[23] of semen frozen without extender but with 10 percent glycerol added directly to the neat semen showed that cells had a better survival after being stored at -85°C than at -70°C, with greater losses in recovery of live cells when stored at -66°C and -50°C. No differences in motility were found after stor-

age at -196°C and thawing after 1, 2, or 3 mo.[24] Recovery of human spermatozoa specimens, initially stored at -100°C and gradually increased in temperature to -50°C at +0.28°C/day, decreased in a linear fashion. The gradual rise in temperature, rather than the original temperature, was the cause of loss in motility. Also, less damage to cells occurred when increasing the temperature from -100°C to -75°C than from -75°C to -50°C.[4]

Test Assay Methods

The largest problem associated with developing methods for freeze preservation is establishing a test assay that is highly related to fertility. Apparently, no such assay is presently available, but several test assay methods for human spermatozoa have been suggested and described. Ackerman[1] showed no loss in motility or increase in dead cells using eosin staining after cooling from 35 to 5°C, but showed a large decrease in sugar utilization after freezing to -80°C as compared to the control maintained at 35°C. He also reported a negative regression of glycolysis or lactic acid production on temperature when cooling from 22 to 0°C at 20°C/min or cooling from 0 to -196°C at 200°C/min.[2] Cold-shocked semen, cooled from 35 to 0°C in 60 s and from 0 to -184°C in 90 s and incubated anerobically, produced more lactate than specimens not cooled or frozen.[3] A unique assay of cellular changes was employed,[5] measuring L-dopa oxidase activity and melanin reaction of untreated samples of human spermatozoa and of samples cooled to 0°C in 60 s and frozen to -196°C at 200°C/min. Spermatozoa were previously washed in saline and suspended in saline and 7.5 percent v/v glycerol. In all cases reported, both L-dopa oxidase activity and melanin reaction increased due to the treatment. Ackerman studied the citric acid metabolism of human[8] and subprimate[11] spermatozoa under aerobic and anaerobic conditions before and after cooling cells to 0°C, -78°C and -196°C suspended in a yolk-citrate-glycerol buffer. He reported that exposure to 0°C increased citricolysis in the aerobic condition and increased citric acid production in the anaerobic condition. Increased utilization of citric acid was associated with impaired fructolysis. He also reported that cooling cells from 0 to -78°C or -196°C did not alter citric acid metabolism of the cell.

Others have studied the DNA content of spermatozoa

from humans[12] and bulls after exposure to low temperature. Research on human spermatozoa indicated no difference in DNA content between untreated samples and those stored at 6°C for 7 days or at -196°C for 1 to 75 wk. Differences in DNA content were reported for bull spermatozoa stored at subzero temperatures for different periods of time, but quantitative assessments after Feulgen reaction are difficult to reproduce. At this time there are no conclusive data suggesting that the DNA changes in the mature sperm cell under varying cryogenic conditions.

Hyaluronidase is reported to be the enzyme that permits the spermatozoa to disperse or penetrate the cumulus oophorus on the ova.[13,54,94] It is an enzyme associated with spermatozoa concentration,[95] and diffuses from the cell as preformed hyaluronidase,[17] primarily from the head.[55] The release of hyaluronidase from cells washed in saline or in Mann's Ringer solution and resuspended but containing 7.5 percent glycerol was not different from untreated whole semen or semen cooled rapidly to 0°C, or frozen to -196°C.[7] This may have been due to insufficient precautions taken in sample preparations.[41]

Other enzymes associated with the acrosome, which may be associated with sperm penetration of the ovum, include a trypsinlike enzyme.[89,90,98] Whether these enzymes are leaked from the cell under cryogenic treatment has not been reported. Because hyaluronidase, glutamic oxaloacetic transaminase (GOT), lactic dehydrogenase (LDH), and choline esterase are more closely associated with the cell than plasma, they may be useful indicators of cellular damage due to stress. They have been used in this manner in studies of other mammalian sperm.[31,42-44] Other compounds such as potassium and bicarbonate also appear to leak from the cell under stress.[91]

In the past few years a great deal of interest has centered around acrosin, the enzyme that is capable of digesting the zona pellucida of the ovum, creating a pathway to the egg so that fertilization can occur.[66,75,76,92,99] It is logical to expect that inhibition of this enzyme would prevent fertilization. This has been found to be true both *in vivo*[99] and *in vitro*.[92] An assessment of the acrosin content of seminal plasma may provide information on the fertilizing capacity of a semen sample.

Little is known about morphological changes of the human sperm cell after cryogenic treatment, but Pedersen and Lebech[63] showed, through electronmicroscopy, the swelling of the cell or acrosome after treatment, and Friberg and Nilsson[38] showed morphological differences between human

spermatozoa frozen with or without addition of glycerol and with or without a buffer.

Motility estimates are frequently used, but they have not been reported to be correlated with fertility.[16,56,57] An inverse relationship between percentage recovery and initial sperm cell concentration has been shown.[24,68] Respiration and motility were reported to decrease after rapid cooling to +5°C.[74]

Many conceivable laboratory tests have been employed in the evaluation of spermatozoa of domestic animals. These include several metabolic assays, resistance impedence changes, an array of motility estimates, and the study of seminal chemistry.[7] None has been very useful in evaluating the fertilizing capacity of the spermatozoa. Recently spermatozoan acrosomal changes[31,69,70] and enzymatic release from the cell[18,20,29,41,42.59,61] have been shown to be important in establishing techniques for cryopreservation of spermatozoa.

The following study was designed to use a few of the test assays employed with domestic animal spermatozoa to see if they can be used as a guide for cryopreservation of human sperm cells.

MATERIALS AND METHODS

Enzyme assays were completed on five species: bull, boar, turkey, stallion, and man. Minimal and maximal cell damage values of GOT, LDH, hyaluronidase, CH_e, acid phosphatase, and alkaline phosphatase were obtained. Minimal cell damage was defined as the amount of damage that occurred after density-gradient centrifugation. Maximal cell damage was defined as the amount of damage that occurred after plunging the sample directly into liquid nitrogen and followed by standard centrifugation. Damage was assessed by the enzyme content of the extracellular fluid. The buffers used for the extender trials were made as follows:

Extenders Used in Trial I

1. 3 g percent sodium citrate with 20 percent egg yolk.
2. TEST-yolk buffer (see below) with 20 percent egg yolk.[40]
3. Boiled TEST-yolk buffer with 20 percent egg yolk that was prepared by bringing the buffer to the boiling point, removing the precipitate by filtering through gauze, and centrifuging at 12,000 g for 10 min.

4. TEST buffer (see below).
5. Seminal plasma (obtained from a vasectomized donor).

Extenders Used in Trial II

1. Boiled TEST-yolk buffer with 20 percent egg yolk (prepared same as buffer 3 in Extender Trial I).
2. TEST-yolk skim milk buffer with 50 percent TEST, 20 percent egg yolk, and 30 percent skim milk boiled and treated similarly.

Extender Used in Trial III (Freezing Technique Study)

Boiled TEST-yolk prepared according to Trial I number 3 was used. The TEST for each extender is prepared as follows:

TES: *N-tris*(hydroxymethyl)methyl-2-aminoethane sulfonic acid, 48.3 g
TRIS: *Tris*(hydroxymethyl)aminomethane, 11.6 g
Distilled water q.s.
Osmotic pressure = 320 milliosmoles/kg
pH = 7.2

In all studies 1 ml semen was added to 1 ml extender. After cooling, 1 ml extender with glycerol was added for a final dilution of 1:2 and final glycerol concentration of 4 percent. Samples of the extenders without semen were retained for enzyme analysis and data correction so that enzyme activity of the extenders would not bias the final results.

Enzyme Analyses

Prefreeze and post-thaw enzyme samples were prepared by carefully layering the semen on density gradient fluid and removing the cells by centrifugation according to the method of Brown *et al.*[20] Maximum damage samples were obtained by extending semen 1:2 in TEST buffer without yolk or glycerol, plunging in liquid nitrogen, and slow thawing at room temperature (repeated three times). The samples were then centrifuged at 19,500 g for 20 s to remove the cells. All samples were stored in sample cups at -20°C until analyzed.

Glutamic oxaloacetic transaminase (GOT) was analyzed by the method of Morgenstern *et al.*[58] on a Technicon Autoanalyzer. Lactic dehydrogenase (LDH) was analyzed on a

Technicon Fluorometer according to the method of Brooks and Olken.[19] Hyaluronidase (hyase) was analyzed by the method of Guilbault, Kramer, and Hackley[46] as modified for automation by Graham and Zimmerman.[45]

Acrosome Morphology

Prefreeze and post-thaw acrosome morphology samples were prepared and examined according to the method that Saacke[69] used for bull semen.

Motility

Motility was assessed simultaneously by three trained technicians with the use of closed circuit television.

Semen Treatment--Extender Trials I and II

Semen was obtained from three men by the masturbation technique. The specimens were observed under the microscope to assure motile spermatozoa. The semen was allowed to cool to room temperature. After liquification, the samples were pooled, mixed, and diluted 1 ml semen to 1 ml each of the prepared extenders. The tubes of semen were placed in a 25°C water bath and put into a 5°C cooler (0.8°C/min). A portion of semen was used for cell counts, and 0.5 ml semen was added to 1 ml TEST extender[40] for a maximum enzyme release sample. When the semen samples were cooled to 8°C, 1 ml extender with glycerol was added for a final glycerol concentration of 4 percent v/v and final dilution ratio of 1:2. Prefreeze enzyme and acrosome samples were prepared, and semen was sealed in ampules for freezing at 2.5°C/min in a Linde cryogenic freezer. Prefreeze percent motile spermatozoa were assessed by three technicians using a closed-circuit television with the microscope.[44] Post-thaw percent motile spermatozoa were estimated and enzyme release samples were prepared immediately after thawing the samples in a 37°C water bath.

Semen Treatment--Freezing Technique

Semen was pooled and handled as in the extender trial, except boiled TEST-yolk extender was used. The five portions of semen were treated in the following manner:

1. Cooled slowly, sealed in ampules, frozen at 2.5°C/min in a Linde Biological Freezer.
2. Cooled slowly, centrifuged at 325 g for 10 min. The cells were resuspended in the same extender, placed in ampules, and frozen at 2.5°C/min in a Linde Biological Freezer.
3. Cooled rapidly by placing directly into ice water, sealed in ampules, and frozen at 2.5°C/min in a Linde Biological Freezer.
4. Cooled slowly and frozen by dropping 0.05 ml drops directly into liquid nitrogen.
5. Cooled slowly and frozen in 0.07-ml pellets on dry ice for 5 min and then transferred directly into liquid nitrogen.

Estimations of percent motile spermatozoa, acrosome morphology, and enzyme samples were prepared as in the extender trial.

RESULTS AND DISCUSSION

Specific data on the relationship between seminal chemistry and fertility is lacking in the literature. However, some data has been accumulated on enzyme activity and cellular damage. Studies have been reported that indicate that several of the enzymes found in semen are more closely associated with the spermatozoa than seminal plasma. Analysis of seminal plasma samples collected prior to and after bilateral corpus epididymectomy established that enzymes remaining in the cell was correlated ($P < 0.05$) to the fertility.[61] Also, experiements have shown that large differences exist in enzyme activity in the extracellular fluid under minimal and maximal stress conditions.[42,44]

The relationship between enzyme activity and sperm cell concentration is presented in Table 1.

Since highly significant correlations were found between some of the enzymes tested and sperm concentration, enzyme release under minimal and maximal cellular damage conditions was assessed (Tables 2, 3, and 4). Large

TABLE 1 Correlations Between Number of Spermatozoa and Enzyme Activity

	Bull, r	Turkey, r	Stallion r	Boar r	Man, r
GOT (I.U.)	(118) 0.85[a]	(20) 0.96[a]	(17) 0.98[a]	(6) 1.00[a]	(26) 0.95[a]
LDH (I.U.)	(118) 0.82[a]	(20) 0.42	(17) 0.96[a]	(6) 0.96[a]	(26) 0.90[a]
CH_e (I.U.)	(118) 0.51[a]	(20) 0.05	--	(6) 0.72	(26) 0.61
Hyaluronidase (V.S.P.)	(22) 0.77[a]	(23) 0.047	(17) 0.92	--	(26) 0.80[a]
Acid phosphatase K-A units	(118) 0.26[a]	(20) 0.58[a]	(17) 0.98[a]	(98) 0.41[a]	(26) 0.24
Alkaline phosphatase K-A units	(118) 0.86[a]	(20) 0.80[a]	(17) 0.91[a]	(98) 0.53[a]	(26) 0.27

[a]Significant at $P < 0.01$.

TABLE 2 GOT[a] Activity in Extracellular Fluid After Subjecting Semen to Minimal and Maximal Cellular Damage

	No. of Samples	Minimal Damage	S.D.	Maximal Damage	S.D.
Bull	140	158.7	50.6	432.9	63.2
Boar	140	23.8	9.2	507.1	89.4
Turkey	140	28.1	6.6	88.3	14.8
Stallion/ml	59	45.8	28.7	81.3	63.9
Man	26	80.5	10.7	216.3	19.5

[a]GOT activity expressed in I.U./10^9 spermatozoa unless otherwise indicated.

TABLE 3 LDH[a] Activity in Extracellular Fluid After Subjecting Semen to Minimal and Maximal Cellular Damage

	No. of Samples	Minimal Damage	S.D.	Maximal Damage	S.D.
Bull	35	423	135	671	37
Boar	6	29.0	7.9	99.0	20.5
Turkey	18	92.0	26.0	126.0	39.0
Stallion/ml	58	89.1	37.9	105.5	48.9
Man	26	119.1	26.2	828	82.0

[a]LDH Activity expressed in I.U./10^9 spermatozoa unless otherwise indicated.

TABLE 4 Hyaluronidase[a] Activity in Extracellular Fluid After Subjecting Semen to Minimal and Maximal Cellular Damage

	No. of Samples	Minimal Damage	S.D.	Maximal Damage	S.D.
Bull	24	2,172	1,658	10,925	3,499
Boar	17	13.8	30.2	1,764	1,003
Turkey	23	2.58	1.31	2.62	2.77
Stallion	16	85.6	44.0	83.3	45.3
Man	26	533	189	1,264	100

[a]Hyaluronidase activity expressed in U.S.P. hyaluronidase units/ml seminal plasma.

differences in enzyme release were seen between minimal and maximal stress samples, especially GOT. Since the amount of enzyme activity of a sample appears to be an important indicator of sperm cell integrity, semen samples can be readily evaluated by enzyme analysis. If the minimal and maximal damage values (release of enzyme) are obtained, then the cellular damage of any processing step in the preservation of spermatozoa can be monitored.

In addition, the number of dead and/or abnormal cells in the semen to be inseminated can be greatly reduced by drawing semen through a filter. Filtering the extended semen before freezing leaves a stronger population of cells to be frozen. A still greater portion of dead and/or abnormal cells can be removed by filtering semen immediately prior to insemination.[44]

Extender Trial I

Table 5 shows the effect of different extenders on percent motile spermatozoa and on the content of GOT, LDH, and hyaluronidase in extracellular fluid of human semen. Only two split samples were used. No statistical analysis was conducted, but the data indicates that some of the test assays may be applicable.

Motility

Although motility estimates of spermatozoa have not been a highly reliable assay, these data indicate wide differences in recovery rate as affected by buffer system under a set condition of freezing and thawing. Under these conditions, it appears that the TEST buffer maintains greater motility after freezing than does sodium citrate or seminal plasma. Egg yolk provided additional protection in the TEST buffer.

Enzyme Assay

The results of GOT and LDH assays reveal that sufficiently large differences between minimal and maximal release exist and differences in treatment can be analyzed. There appears to be little difference in prefreeze GOT and LDH in buffers containing egg yolk. When no egg yolk is added, the extracellular GOT is highest. Seminal plasma as a buffer initially protects the cell. Both TEST without egg yolk and

TABLE 5 The Effect of Difference Extenders on Percent Motile Spermatozoa, Glutamic Exaloacetic Transaminase (GOT) Lactic Dehydrogenease (LDH), and Hyaluronidase Content of Human Semen[a,b]

	% Motile Spermatozoa			GOT in Extracellular Fluid			LDH in Extracellular Fluid			Hyaluronidase in Extracellular Fluid		
	Pre-freeze	Post-Thaw	% Recovery	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference
Sodium citrate +20% egg yolk	40	5	12.5	93	127	34	403	573	170	964	1,080	116
TEST No egg yolk	32	10	31.3	100	146	46	462	635	173	989	1,017	28
TEST +20% egg yolk	45	20	44.4	94	126	32	443	571	128	1,047	1,054	7
TEST +20% egg yolk--boiled	45	27	60.0	93	116	23	424	517	93	1,045	1,051	6
Human seminal plasma	32	9	28.1	83	119	36	476	579	103	1,124	1,153	29
All treatments	38.8	14.2	31.5	92.6	126.8	34.2	441.6	575.0	133.4	1,033.8	1,071.0	37.2
Total Releasable enzyme[c]					211			833			1,160	

[a]I.U./10^9 spermatozoa and samples were prepared by density gradient method.[20]
[b]Two observations per mean.
[c]Prepared by plunging semen in liquid nitrogen and thawing (three times).

seminal plasma show higher LDH levels than buffers with egg yolk. Post-thaw enzyme levels in the extracellular fluid indicate a greater retention of GOT and LDH when egg yolk has been added to the buffer over TEST buffer without egg yolk. The differences between prefreeze and postfreeze release indicate the effect of freezing. Differences between buffer systems and protective qualities of egg yolk are seen. In these data, the greatest freeze protection was given by heat-treated TEST egg yolk buffer for all enzyme tests.

Extender Trial II

Table 6 shows the effect of adding skim milk to the TEST-yolk buffer on the freezability of human spermatozoa as indicated by GOT, LDH, and hyaluronidase content of the extracellular fluid and motility.

Motility

The motility figures reveal a substantial difference (14 percent) between the recovery obtained with TEST-yolk buffer and TEST-yolk with skim milk. The added protein (see Table 7) may be responsible for the additional protection afforded to sperm cell with the addition of skim milk to the TEST-yolk buffer. However, we now have information that indicates that milk prevents the pH rise that occurs while semen samples are cooled, and this may be the nature of its protection to the sperm cell.

Trial II (Freezing-Technique Study)

Table 8 shows the results of other treatments within the selected heated TEST-yolk buffer. The treatments conducted on a split-sample basis included the effect of slow and fast initial cooling, slow and fast freezing, and the effect of centrifugation.

Motility

The faster cooling rate resulted in lower prefreeze motility than the slow cooling rate. With the exception of the faster (40°C/min and 160°C/min) freeze rates, the percentages

TABLE 6 The Effect of Added Milk to the TEST-Yolk Buffer on the Freezability of Human Spermatozoa[a,b]

	% Motility			GOT in Extracellular Fluid			LDH in Extracellular Fluid			Hyaluronidase in Extracellular Fluid		
	Pre-freeze	Post-Thaw	% Recovery	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference
TEST + 20% egg yolk--boiled	48	31	65	96	121	25	443	569	126	827	902	75
% Lost					54.7			66.3			14.5	
TEST 50% + 20% egg yolk +30% skim milk--boiled	49	38	79	88	106	18	418	517	101	705	747	42
% Lost					47.9			60.2			61.7	
Total releasable enzyme[c]					221			858			1,210	

[a]I.U./10^9 spermatozoa and samples were prepared by density gradient method.[20]
[b]Six observations per mean.
[c]Prepared by plunging semen in liquid nitrogen and thawing (three times).

TABLE 7 Percentage Reactive Protein in Blood Serum and Buffers After Heat Treatment

Heat Treatment	Protein (g %)				
	Canine	Bovine	Porcine	TEST-Yolk	TEST-Yolk 30% Milk
None	5.42	9.35	7.63	2.23	2.38
45°C	5.27	9.10	7.63	2.20	3.41
55°C	4.55	9.10	8.12	2.25	3.39
65°C	4.25	9.07	7.50	2.27	3.80
75°C	2.30	8.80	4.1	2.17	3.31
85°C	2.15	0.73	1.1	1.05	3.29
95°C	1.17	0.70	0.40	0.80	1.88
100°C	1.10	0.75	0.45	0.78	1.86

TABLE 8 The Effect of Different Cooling and Freezing Rates on Percent Motile Spermatozoa, Glutamic Oxaloacetic Transaminase (GOT), Lactic Dehydrogenase (LDH), and Hyaluronidase Content of Human Semen[a,b]

Treatment		% Motile Spermatozoa			GOT in Extracellular Fluid			LDN in Extracellular Fluid			Hyaluronidase in Extracellular Fluid		
Cool Rate	Freeze Rate	Pre-freeze	Post-Thaw	% Recovery	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference	Pre-freeze	Post-Thaw	Difference
1/2°C/min	2°C/min	52[2]	27	51.9	63	100	37	449	588	139	1,044	1,095	51
16°C/min	2°C/min	27	16	59.3	73	103	30	563	664	101	1,109	1,166	57
1/2°C/min (centrifuged)	2°C/min	45	25	55.6	83	114	31	661	740	79	1,107	1,161	54
1/2°C/min	40°C/min	52	32	61.5	63	85	22	452	579	127	1,050	1,090	40
1/2°C/min ~	160°C/min	52	37	71.2	62	93	31	449	577	128	1,046	1,065	19
All treatments		45.6	27.4	59.9	68.8	99.0	30.2	514.8	629.6	114.8	1,071.2	1,115.4	44.2
Total releasable enzyme[c]						162			996			1,247	

[a]I.U./10^9 spermatozoa and samples were prepared by density gradient method.[20]
[b]Two Observations per mean.
[c]Prepared by plunging semen in liquid nitrogen and thawing (three times).

of recovery of spermatozoa after freezing were similar. The faster freeze rates resulted in a higher percentage recovery of motile cells.

Enzyme assay

In both GOT and LDH assay, prefreeze release of enzymes indicates that fast initial cooling caused a greater release than slow cooling and that semen cooled slowly and then centrifuged resulted in an even greater loss. The major difference in post-thaw levels was the effect of centrifugation and fast cooling, resulting in a greater final loss of enzymes from the spermatozoa. There were little differences due to freeze rate (2, 40, 160°C/min), but, regardless of assay, the faster rates appear to have resulted in less cellular damage.

The total releasable amount of enzyme may be used as a guide in establishing the relationship of treatment from total cellular damage. From the data presented, it appears that for human spermatozoa GOT is the most difficult to release under variable treatment, hyaluronidase the easiest, and LDH intermediate. Each appears to be usable as a tool in measuring cellular damage. Hyaluronidase, an enzyme that should be more closely associated with fertility than either GOT or LDH, appears least suitable under the conditions of this study. If, however, hyaluronidase is only associated with the cell and not the plasma, as has been suggested, analysis of this enzyme may prove to be a sensitive assay, which in this study reflects that nearly total cellular damage had occurred prior to obtaining the prefreeze sample for analysis.

Morphological Changes--All Trials

There was little difference in acrosome morphology between treatments or between prefreeze and postfreeze samples. The centrifuged samples, however, showed an increase in roughened acrosomes, possibly due to adhered particles. Agglutination was also apparent after centrifuging.

It appears that the often irregular head shape of the spermatozoon leaves a poor basis for light microscopic acrosome evaluation or that the human acrosome does not

react unfavorably to diluents and freezing like those of certain animal species.[31,69,70]

CONCLUSIONS

For the utilization of enzyme leakage from the cell to be effective as a test assay for cell damage, standardization of technique is imperative.

For meaningful results accurate cell concentration for each sample must be determined for standardization of results. However, inherent resistance to chemical leakage exists between individuals, and this reflects different membrane integrity between individuals. Many factors cause the release of enzymes from the spermatozoa, and these can be used to monitor cellular damage. These factors include: (a) semen handling--time, cold shock, cooling rate, freezing rate, thawing rate, age of spermatozoa, and centrifugation; (b) buffering systems--osmotic pressure, pH, pK, ionic strength, molecular configuration, and ion-binding properties, and (c) cryoprotective agent--type of agent, concentration and method, temperature, and time of addition.

Quantitative evaluation of acrosome morphology with phase contrast microscopy and stained samples does not seem to give as much valuable information in humans as in other mammals. However, quantitative evaluation with the microscope is difficult due to the low number of cells observable per sample.[38]

Severe impediments face the researcher on cryopreservation of human spermatozoa. Some may be alleviated or reduced by standardization, while some appear to be inherent with the species.[24] These impeding factors include: (a) inconsistency in semen quality between donors, (b) little or no clinical background on donor semen, (c) lack of sufficient fertility data of donor semen, (d) lack of large quantities of semen per donor, (e) comparative low concentration of spermatozoa per ml or per ejaculate, (f) lack of adequate numbers of recipients for insemination per donor or for any one sample of semen, and (g) lack of test assay for semen quality and method of assessing cellular damage.

Many of the discrepancies in the literature on suggested techniques for the cryopreservation of human spermatozoa are probably the result of the foregoing. As tech-

niques are standardized and new methods of assessing cellular damage are found, rapid progress will become possible.

REFERENCES

1. Ackerman, D. R. 1967. Fructose utilization of human spermatozoa after cooling and freezing. Int. J. Fertil. *12:*1-4.
2. Ackerman, D. R. 1968. Antibodies of the ABO system and metabolism of human spermatozoa: Metabolic changes induced by cooling and freezing. Nature (Lond.) *21:*1159.
3. Ackerman, D. R. 1968. Effect of cooling and freezing on the aerobic and anaerobic lactic acid production of human semen. Fertil. Steril. *19:*123-128.
4. Ackerman, D. R. 1968. Damage to human spermatozoa during storage at warming temperatures. Int. J. Fertil. *13:*220-225.
5. Ackerman, D. R. 1970. Determination of in vivo alterations in a phenotype of human spermatozoa. Nature (Lond.) *227:*1148-1149.
6. Ackerman, D. R. 1970. Glycerol metabolism in untreated, cold shocked, and frozen human spermatozoa. Cryobiology *7:*145-147.
7. Ackerman, D. R. 1970. Hyaluronidase in human semen and sperm suspensions subjected to temperature shock and to freezing. J. Reprod. Fertil. *23:*521-523.
8. Ackerman, D. R. 1971. Variation due to freezing in the citric acid content of human semen. Fertil. Steril. *22:*58-60.
9. Ackerman, D. R. 1972. Present techniques and possible advances in freezing and storing human spermatozoa. J. Reprod. Med. *8:*188-189.
10. Ackerman, D. R., and Behrman, S. J. 1967. Freezing-preservation of human sperm. Yale Sci. Mag. *41:*6-10.
11. Ackerman, D. R., and Roussel, J. D. 1971. Citric acid, lactic acid and oxygen metabolism of frozen-thawed semen from four subhuman primate species. J. Reprod. Fertil. *27:*441-443.
12. Ackerman, D. R., and Sod-Moriah, U. A. 1968. DNA content of human spermatozoa after storage at low temperatures. J. Reprod. Fertil. *17:*1-7.
13. Austin, C. R. 1948. Function of hyaluronidase in fertilization. Nature (Lond.) *162:*63.
14. Behrman, S. R. 1971. Preservation of human sperm by

liquid nitrogen vapor freezing. In: A. Ingleman-Sundberg and N. O. Lunell, eds. *Current Problems in Fertility*. New York: Plenum Publishing Corp.

15. Behrman, S. J., and Ackerman, D. R. 1969. Freeze preservation of human sperm. Am. J. Obstet. Gynecol. *103:*654-664.
16. Behrman, S. J., and Sawads, Y. 1966. Heterologous and homologous inseminations with human semen frozen and stored in a liquid-nitrogen refrigerator. Fertil. Steril. *17:*457-466.
17. Bergenstal, D. M., and Scott, W. W. 1948. Studies on hyaluronidase, JAMA *137:*1507.
18. Bower, R. E., Jr., Crabo, B. G., Pace, M. M., and Graham, E. F. 1973. Effects of dilution and glycerol on the release of glutamic-oxaloacetic transaminase (GOT) from boar spermatozoa. J. Anim. Sci. *36:*319-324.
19. Brooks, L., and Olken, H. G. 1965. An automated fluorometric method for determination of lactic dehydrogenase in serum. Clin. Chem. *11:*748-762.
20. Brown, K. I., Crabo, B. G., Graham, E. F., and Pace, M. M. 1971. Some factors affecting loss of intracellular enzymes from spermatozoa. Cryobiology *8:*220-224.
21. Brown, K. I., and Graham, E. F. 1971. Effect of some cryophylactic agents on turkey spermatozoa. J. Poult. Sci. *50:*832-835.
22. Brown, K. I., Graham, E. F., and Crabo, B. G. 1972. Effect of some hydrogen-ion buffers on storage and freezing of turkey spermatozoa. J. Poult. Sci. *51:*840-849.
23. Bunge, R. G. 1960. Further observations on freezing human spermatozoa. J. Urol. *83:*192-193.
24. Bunge, R. G., Keettel, W. C., and Sherman, J. K. 1954. Clinical use of frozen semen: Report of 4 cases. Fertil. Steril. *5:*520-529.
25. Bunge, R. G., and Sherman, J. K. 1953. Fertilizing capacity of frozen human spermatozoa. Nature (Lond.) *172:*767-768.
26. Bunge, R. G., and Sherman, J. K. 1954. Frozen human semen. Fertil. Steril. *5:*193-194.
27. Carlborg, L. 1971. Some problems involved in freezing and insemination with human sperm. In: A. Ingleman-Sundberg and N. O. Lunell, eds. *Current Problems in Fertility*. New York: Plenum Publishing Corp.
28. Colton, S. W., and Farris, E. J. 1958. Low temperature preservation of human spermatozoa with practical implications. J. Urol. *80:*44-46.

29. Crabo, B. G., Bower, R. E., Brown, K. I., Graham, E. F., and Pace, M. M. 1971. Extracellular glutamic-oxaloacetic transaminase as a measure on membrane injury in spermatozoa during treatment. In: A. Ingleman-Sundberg and N. O. Lunell, eds. *Current Problems in Fertility*. New York: Plenum Publishing Corp. pp. 33-38.
30. Crabo, B. G., Brown, K. I., and Graham, E. F. 1972. Effect of some buffer on the storage and freezing of boar spermatozoa. J. Anim. Sci. *35:*377-382.
31. Crabo, B. G., and Graham, E. F. 1972. Correlation between some laboratory methods for evaluation of boar semen after freezing. VII Int. Congr. Anim. Reprod. Artif. Insemination, Munich.
32. Eliasson, R. 1971. Standards for investigation of human semen. Andrologie *3:*49-64.
33. Fernandez-Cano, L., Menkin, M. F., Garcia, C-R., and Rock, J. 1964. Refrigerant preservation of human spermatozoa. I. Factors influencing recovery in euspermic semen: Clinical applications. Fertil. Steril. *15:*390-406.
34. Freund, M. 1967. Complete recovery of motile human spermatozoa after dilution, freezing and storage at -196°C for 24 months. Fed. Proc. *26:*335.
35. Freund, M. 1971. Reversibility of vasectomy and the role of the frozen semen bank. Med. Counterpoint, Nov. 1971. pp. 15, 19, 20, 23, and 27.
36. Freund, M., and Wiederman, J. 1963. Controlling and recording rates of freezing and defrosting of human semen. J. Appl. Physiol. *18:*407-409.
37. Freund, M., and Wiederman, J. 1966. Factors affecting the dilution, freezing and storage of human semen. J. Reprod. Fertil. *11:*1-17.
38. Friberg, J., and Nilsson, O. 1971. Motility and morphology of human sperms after freezing in liquid nitrogen. In: A. Ingleman-Sundberg and N. O. Lunell, eds. *Current Problems in Fertility*. New York: Plenum Publishing Corp.
39. Graham, E. F., and Crabo, B. G. 1972. Some factors influencing the freezing of boar semen. VII Int. Congr. Anim. Reprod. Artif. Insemination, Munich.
40. Graham, E. F., Crabo, B. G., and Brown, K. I. 1972. Effect of some zwitter ions on the freezing and storage of spermatozoa. I. Bull. J. Dairy Sci. *55:*372-378.
41. Graham, E. F., Crabo, B. G., and Schmehl, M. K. L. 1973. Utilization of enzyme assay in developing

techniques for freezing semen. VIII Int. Zootec. Congr., Milan.

42. Graham, E. F., and Pace, M. M. 1967. Some biochemical changes in spermatozoa due to freezing. Cryobiology *4:*75-84.
43. Graham, E. F., Rajamannan, A. H. J., Schmehl, M. K. L., Maki-Laurila, M., and Bower, R. E. 1971. Fertility studies with frozen boar spermatozoa. Artif. Insemination Dig. *19:*6-16.
44. Graham, E. F., Schmehl, M. K. L., and Maki-Laurila, M. 1970. Some physical and chemical methods of evaluating semen. Proc. III Tech. Conf. Anim. Reprod. Artif. Insemination, Chicago. pp. 44-48.
45. Graham, E. F., and Zimmerman, K. J. In press. An automated fluorometric method for determination of hyaluronidase.
46. Guilbault, G. G., Kramer, D. N., and Hackley, E. 1967. Fluorometric determination of hyaluronidase and of Cu (11), Fe (11) and cyanide ion inhibitors. Abstr. Biochem. *18:*241-247.
47. Hill, H. 1970. Vasectomy and A.I.H. Lancet *24:*191.
48. Ilzuka, R., and Sawada, Y. 1958. Successful inseminations with frozen pooled human semen. Jap. J. Fertil. Steril. *3:*241-245.
49. Ilzuka, R., Sawada, Y., Kai, H., Nishina, N., and Ohi, M. 1967. Frozen sperm for storage and A.I.D. V World Congr. Int. Fed. Gynecol. Obs., Sydney, Aust.
50. Ilzuka, R., Sawada, Y., Nishina, N., and Ohi, M. 1968. The physical and mental development of children born following artificial insemination. Int. J. Fertil. *13:*24-32.
51. Ilzuka, T. 1965. Cases of pregnancy using frozen sperm. Sanfujinka no Jissai *14:*569-574.
52. Karow, A. M. 1972. The nature and pharmacology of cryoprotectants. J. Reprod. Med. *8:*207.
53. Kline, C. W. 1963. Potential significance of sperm banks. In: H. G. Beigel, ed. *Advances in Sex Research.* New York: Hoeber.
54. Kurzrok, R., Leonard, S. L., and Conrad, H. 1946. Role of hyaluronidase in human infertility. Am. J. Med. *1:*491.
55. Masaki, J., and Hartree, E. F. 1962. Distribution of metabolic activity, phospholipid and hyaluronidase between the heads and tails of bull spermatozoa. Biochem. J. *84:*347.
56. Matheson, G. W., Carlsborg, and Gemzell, C. 1969. Frozen human semen for A.I. Am. J. Obstet. Gynecol. *104:*495-501.

57. Menkin, M. F., Luisis, P., Zaikis, J. P., and Rock, J. 1964. Refrigerant preservation of human spermatozoa. II. Factors influencing recovery in oligo- and euspermic semen. Fertil. Steril. *15:*511-527.

58. Morgenstern, S., Oklander, M., Auerbach, J., Kaufman, J., and Klein, B. 1966. Automated determination of serum glutamic oxaloacetic transaminase. Clin. Chem. *12:*95-111.

59. Nagase, H., Graham, E. F., Niwa, T. 1964. Pelleted semen: The effect of glycerol level and composition of thawing solution of fertility of bovine spermatozoa. Proc. V Int. Congr. Reprod. Artif. Insemination. *4:*404-409.

60. Pace, M. M., and Graham, E. F. 1973. Components in egg yolk which protect bovine spermatozoa during freezing.

61. Pace, M. M., and Graham, E. F. 1970. The release of glutamic oxaloacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. Biol. Reprod. *2:*140-146.

62. Parkes, A. S. 1945. Preservation of human spermatozoa at low temperatures. Br. Med. J. *2:*212-213.

63. Pedersen, H., and Lebech, P. E. 1971. Ultrastructural changes in human spermatozoa after freezing or artificial insemination. Fertil. Steril. *22:*125-133.

64. Perloff, W. H., Steinberger, E., and Sherman, J. K. 1964. Conception with human spermatozoa frozen by nitrogen vapor technic. Fertil. Steril. *15:*501-504.

65. Piasecks-Serafin, M. 1972. The effect of the sediment accumulated in containers under experimental conditions on the infection of semen stored directly in liquid nitrogen (-196°C). Bull. Acad. Pol. Sci. Ser. Biol. *20:*263-268.

66. Polakowski, K. L., McRorir, R. A., and Williams, W. L. 1973. Purification and preliminary characterization of a proteinase from boar sperm. J. Biol. Chem. *248:*8178-8182.

67. Rajamannan, A. H. J., Graham, E. F., and Schmehl, M. K. L. 1971. Effects of holding on bovine semen. Artif. Insemination Dig. *19:*6.

68. Rubin, S. O., Anderson, L., and Bostrom, K. 1969. Deep-freeze preservation of normal and pathologic human semen. Scand. J. Urol. Nephrol. pp. 144-150.

69. Saacke, R. G. 1970. Morphology of the sperm and its relationship to fertility. Proc. III Tech. Conf. Artif. Insemination Reprod. NAAB. pp. 17-30.

70. Saacke, R. G. 1972. Semen quality tests and their

relationship to fertility. Proc. IV Tech. Conf. Artif. Insemination Reprod. NAAB. pp. 22-27.

71. Sawada, Y. 1959. Studies on the freeze preservation of human spermatozoa. Jap. J. Fertil. Steril *4:*1-10.
72. Sawada, Y. 1964. Preservation of human semen by deep freezing. Int. J. Fertil. *9:*525-532.
73. Sawada, Y., and Ackerman, D. R. 1968. Use of frozen human semen. In: S. J. Behrman and R. W. Kistner, eds. *Progress in Infertility*. Boston: Little, Brown & Co. pp. 731-748.
74. Sawada, Y., Ackerman, D., and Behrman, S. J. 1967. Motility and respiration of human spermatozoa after cooling to various low temperatures. Fertil. Steril. *18:*775-781.
75. Schill, W. B. 1973. Acrosin activity in human spermatozoa. Methodological investigations. Arch. Dermatol. Forsch. *246*(3):257-273.
76. Schill, W. B. 1974. Quantitive determination of acrosin activity in human spermatozoa. Fertil. Steril. *25*(8):703-712.
77. Sherman, J. K. 1974. Freezing and freeze-drying of human spermatozoa. Fertil. Steril. *5:*357-371.
78. Sherman, J. K. 1955. Temperature shock in human spermatozoa. Proc. Soc. Exp. Biol. Med. *88:*6-7.
79. Sherman, J. K. 1962. Preservation of bull and human spermatozoa by freezing in liquid nitrogen vapour. Nature *194:*1291-1292.
80. Sherman, J. K. 1963. Improved methods of preservation of human spermatozoa by freeze-drying. Fertil. Steril. *14:*49-64.
81. Sherman, J. K. 1963. Banks for frozen stored human spermatozoa. Proc. XI Int. Congr. Genet. *1:*273.
82. Sherman, J. K. 1963. Questionable protection of intracellular glycerol during freezing and thawing. J. Cell. Comp. Physiol. *61:*67-74.
83. Sherman, J. K. 1964. Dimethyl sulfoxide as a protective agent during freezing and thawing of human spermatozoa. Proc. Soc. Exp. Biol. Med. *117:*261-264.
84. Sherman, J. K. 1964. Low temperature research on spermatozoa and eggs. Cryobiology *1:*103-129.
85. Sherman, J. K. 1964. Research on frozen semen: Past, present and future. Fertil. Steril. *15:*485-499.
86. Sherman, J. K. 1965. Practical applications and technical problems of preserving spermatozoa by freezing. Fed. Proc. *24:*s-288-S-296.
87. Sherman, J. K. 1972. Long-term cryopreservation of

motility and fertility of human spermatozoa. Cryobiology *9:*332.

88. Sherman, J. K., and Bunge, R. G. 1953. Observations on preservation of human spermatozoa at low temperatures. Proc. Soc. Exp. Biol. Med. *82:*686-688.
89. Stambaugh, R., Brackett, B. G., and Mastroianni, L. 1969. Inhibition of in vitro fertilization of rabbit ova by trypsin inhibitors. Biol. Reprod. *1:*223.
90. Stambaugh, R., and Buckley, J. 1969. Identification and subcellular localization of the enzymes affecting penetration of the zona pellucida by rabbit spermatozoa. J. Reprod. Fertil. *19:*423.
91. Stambaugh, R., Noriega, C., and Mastroianni, L. 1969. Biocarbonate ion; the corona cell dispersing factor of rabbit tubal fluid. J. Reprod. Fertil. *18:*51.
92. Stambaugh, R., Brackett, B. G., and Mastroianni, L. 1969. Inhibition of in vitro fertilization of rabbit ova by trypsin inhibitor. Biol. Reprod. *1:*223-227.
93. Steinberger, E., and Perloff, W. 1965. Preliminary experience with human sperm bank. Am. J. Obstet. Gynecol. *92:*577-578.
94. Swyer, G. I. M. 1947a. The hyaluronidase content of semen. Biochem. J. *41:*409.
95. Swyer, G. I. M. 1947b. The release of hyaluronidase from spermatozoa. Biochem. J. *41:*413.
96. Trelford, J. D., and Mueller, F. 1969. Observations and studies on the storage of human sperm. Can. Med. Assn. J. *100:*62-65.
97. Udupa, K. N., and Singh, R. H. 1972. Frozen sperm banks. JAMA *220:*1365.
98. Zaneveld, L. J. D., and Williams, W. L. 1970. A sperm enzyme that disperses the corona radiata and its inhibition by decapacitation factor. Biol. Reprod. *2:*363.
99. Zaneveld, L. J. D., Robertson, R. T., Kessler, M., and Williams, W. L. 1971. Inhibition of fertilization in vivo by pancreatic and seminal plasma trypsin inhibitors. J. Reprod. Fertil. *25:*387-392.
100. Zimmerman, S. J., Maude, M. B., and Idawer, M. M. 1964. Freezing and storage of human semen in 50 healthy medical students: A comparative study of glycerol and dimethylsulfoxide as a preservative. Fertil. Steril. *15:*505-510.
101. Wako, H. 1961. The study on the practical use of human frozen spermatozoa. Jap. J. Fertil. Steril. *6:*66-78.

GENETIC CONSIDERATIONS IN HUMAN SPERM BANKING

Elizabeth M. Earley and John C. Petricciani

The previous discussions have considered a variety of parameters related to the purity, safety, and effectiveness of human semen samples in the context of artificial insemination (AI). Although freedom from microbial contaminants, and assessments to predict the fertility potential of a given sample, are of clear importance, of equal if not greater importance are the genetic aspects of human spermatozoa used in AI. In this paper we will review briefly the major genetic considerations that would appear relevant to AI, point out areas where more work should be done, and suggest possible directions that AI might take based on current and developing biomedical technology.

Perhaps the most immediately apparent potential genetic problem in the use of donor semen in AI is the transmission of a genetic disease. The scope of the potential problem is highlighted by the fact that to date 2,336 Mendelian abnormal phenotypes have been described.[1] Of particular importance is the fact that about half of those (1,218/2,336) are autosomal dominants. In other words, the abnormality could be transmitted by the semen of the donor, and the probability of an affected offspring would be 50 percent. In order to reduce that possibility, information on the genetic background of a donor can be obtained. There are basically two ways in which this can be done: (a) family and medical history and (b) laboratory tests. Each of those approaches is valuable in documenting the genetic status of the donor, and both have been used in clinical genetics for many years. The value of a history is very dependent on the reliability of the subject and the care with which the history is taken. Laboratory tests for specific genetic diseases also have limitations in part because few of them are generally available. In about a dozen cases, tests have

been developed and applied in large genetic screening programs in the United States. For example, in New York State all newborns for the past 2 yr have been screened for seven genetic diseases.[2]

Other laboratory tests that would be of value in defining the genetic characteristics of a donor include chromosomal analysis and the more routine studies, such as blood glucose to rule out diabetes, that are done as part of a general physical examination. Data on the chromosomal constitution of a donor can be of value in identifying intrinsic abnormalities in the somatic cells, such as in individuals with an XYY complement. While cytogenetic analysis of somatic cells provides only indirect evidence of the chromosomal status of the germ cells (spermatozoa), it can increase the level of confidence that a given donor is normal.

During the past several years major advances were made in cytogenetic techniques. These new methods are generally referred to as the banding procedures and allow the identification of each chromosome. Progress has been slower in the analyses of the chromosomes of spermatozoa, because of biological and technical factors. The biological problem is the fact that testicular biopsies are required to obtain the material to be analyzed. The technical reasons are related to the morphological state of the chromosome in first and second meiotic divisions. In the former, crossing over, called chiasma, occurs between homologous chromosomes, and the chromosomes appear fuzzy. In the latter, the chromosomes never really condense because they have already duplicated their DNA and are simply being segregated without further condensation.

Nevertheless, attempts have been made to assess numerical chromosomal abnormalities in human spermatozoa.[3,4] These studies have taken advantage of the differential staining characteristics of three chromosomes: namely, the Y sex chromosome and the two autosomal chromosomes, numbers 1 and 9. The frequencies of spermatozoa with an extra Y, number 1 or number 9 chromosome ranged from 1 to 2 percent. If the remainder of the haploid set of chromosomes in spermatozoa behave similarly, then about 40 percent of all spermatozoa might be aneuploid and could have one extra or one missing chromosome.[3,4] Whether or not it is reasonable to make such an extrapolation is debatable. Nevertheless, this is an area in which much more effort is needed to establish very basic information of direct relevance to the question of the genetic normality of spermatozoa used in AI. It is of interest to point out here that in a recent

study[5] the incidence of chromosome abnormalities in 1,384 aborted fetuses was almost 30 percent--a figure in the same order of magnitude as the extrapolated value for aneuploid spermatozoa. Further, it has been suggested[6] that human fetal wastage may approach 80 percent. If such a high rate actually occurs in nature, and if abnormal spermatozoa account for even 50 percent of such naturally discarded fetuses, then it would seem prudent in the setting of AI to attempt to reduce the number of genetically abnormal spermatozoa to the extent that current screening methodology will allow.

While a significant amount of work remains to be done on very basic genetic problems relating to human spermatozoa, there have been advances in technology that can be applied to AI well in advance of solving those fundamental problems. As in other areas of science, progress in medicine is not uniform and orderly in each of the disciplines. As a result, we may well move into an era where we are able technically to select at least some genetic characteristics long before we know which parameters correlate well with the fertility of spermatozoa. One example of such a technical advance is the recent report[7] describing the isolation of fractions of human semen enriched from 76 to 85 percent in Y sperm. Two subsequent studies failed to confirm those results.[8,9] If the findings are eventually confirmed in other laboratories, then it should be possible to select for male children with a reasonably high probability of success. The general availability and application of this technique, however, could pose significant biosocial problems if males were heavily overselected.

Another technical advance that may have an impact in the future on AI is the recent identification of HLA antigens on human spermatozoa.[10,11] With the use of affinity column chromatography, one could theoretically select out those sperm that had defined HLA characteristics. Such sperm could in turn be used in AI to select for children with specific transplantation antigen characteristics.

One final social problem with a genetic basis should be mentioned. And that is the possibility of brother-sister marriages. If a given AI donor were responsible for many successful pregnancies, then there is a finite probability that two of his children (half-sibs) could mate. While the probability of such an event is low, it apparently almost did occur on one occasion. In that instance the family physician prevented a marriage of two children conceived by AI who had the same biologic father.[12] The questions raised by this example are difficult ones, and

there are quite valid reasons for the various positions that one might take on them. We mention them here only to point out the problems rather than to offer solutions. Should children conceived by AI be informed of their origins? Should a central registry be maintained? Should the number of children by any one donor be limited? Should multiple donations by a given donor be dispensed to geographically distant locations? The answers to these and other related questions may well be different in different parts of the United States, but we must at least begin to consider them as problems that require the attention of society in general, as well as the scientific community.

As we indicated earlier, the purpose of this presentation was to focus attention on the genetic aspects of AI. By suggesting that transmission of a genetic disease is a real possibility, we are also suggesting that genetic screening of donors is both reasonable and desirable. The specific screening that might be applied could conceivably vary from case to case, and uniform guidelines in this regard do not seem to us to be appropriate with the exception that a family and medical history should be taken from every donor. The value of more detailed screening lies in the higher level of confidence that the donor is free of major transmissible genetic disorders. In that sense, genetic screening of donors falls into the category of preventive medicine and is consistent with the well-established principle that in a therapeutic setting we should attempt to reduce the risk of transmitting a disease during the procedure to the extent that our technology will allow.

DISCUSSION

AARON BENDICH: Human semen contains a particulate complex in seminal fluid (inactivated on freeze-thawing) and *within* the sperm nucleus (not inactivated by freezing) that contains a reverse transcriptase, in some ways resembling oncornavirus. Over 30 individuals have been examined, and all were positive.

REFERENCES

1. McKusic, V. A. 1975. *Mendelian Inheritance in Man: Catalogues of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes*. 4th ed. Baltimore: John Hopkins Univ. Press.

2. Culliton, B. J. 1976. Genetic screening: States may be writing the wrong kind of laws. Science *191*:926-929.
3. Pawlowitzki, I. H., and Pearson, P. L. 1972. Chromosomal aneuploidy in human spermatozoa. Humangenetik *16*:119-122.
4. Geraedts, J., and Pearson, P. 1973. Specific staining of the human no. 1 chromosome in spermatozoa. Humangenetik *20*:171-173.
5. Alberman, E., Elliott, M., Creasy, M., and Dhadial, R. 1975. Br. J. Obstet. Gynaecol. *82*:366-373.
6. Roberts, C. J., and Lowe, C. R. 1975. Where have all the conceptions gone? *Lancet* (1 March): 498-499.
7. Ericson, R. J., Langevin, C. N., and Nishino, M. 1973. Isolation of fractions rich in human Y sperm. Nature *246*:421-424.
8. Evans, J. M., Douglas, T. A., and Renton, J. P. 1975. An attempt to separate fractions rich in human Y sperm. Nature *253*:352-354.
9. Ross, A., Robinson, J. A., and Evans, H. J. 1975. Failure to confirm separation of X- and Y-bearing human sperm using BSA gradients. Nature *253*:354-355.
10. Kerek, G., Biberfeld, P., and Afzelius, B. A. 1973. Demonstration of HL-A antigens "species," and "semen"--specific antigens on human spermatozoa. Int. J. Fertil. *18*:145-155.
11. Halim, A., Abassi, K., and Festenstein, H. 1974. The expression of the HL-A antigens on human spermatozoa. Tissue Antigens *4*:1-6.
12. Artificial insemination breeds legal issues. U.S. Med., December 1, 1974.

DNA-GENERATING COMPLEX IN SEMEN

Aaron Bendich

A comprehensive evaluation of the many factors to be considered in the preservation of semen and for subsequent use should include the DNA-generating complexes recently found both in seminal fluid and within sperm heads.[1-3] This enzymatic activity has been found in the ejaculates of each of over 20 individuals examined, as well as in sperm from the vas deferens or epididymis of mouse, rat, and pig, and is undoubtedly a normal constituent. Although the activity can be demonstrated in the sperm of human specimens after freeze-storage and thawing of either the entire ejaculate or a sperm pellet, the activity in stored seminal fluid is not observed unless 50 percent glycerol is added to the fresh sample at the time of freezing to a temperature of at least -20°C. The origin of the activity in the seminal fluids from vasectomized or intact men has not yet been ascertained, and in consequence we do not know whether it has been introduced from external contacts or is an intrinsic, normal component of the seminal fluids. However, we do have experimental evidence that clearly indicates that the complex residing within the sperm head is in intimate association with the sperm chromatin.[4] Thus, an adventitious contamination of the sperm head external membranes need not be invoked to explain its occurrence.

The discovery of this complex in sperm arose from attempts to understand why normal somatic cells undergo a variety of morphological alterations when they are admixed in culture with washed mammalian sperm.[5-7] Uptake of the sperm by the target somatic cells was clearly demonstrated by electron microscopy and autoradiography. Production of fetal antigens was observed in the progeny of the target cells.[5,8] Since the morphological alterations were of a

type seen after carcinogen treatment of the target cells, the possible presence of an oncogenic virus in semen was suggested as an explanation. Indeed, the particulate complex isolated from the sperm head possesses some physicochemical properties that are also seen in RNA-type oncogenic viruses.[1] All that can be said at the present time regarding possible function is that an extrachromosomal DNA-generating complex exists within sperm heads that may play a significant role in embryogenesis. If it does, methods of sperm preservation for purposes of artificial fertilization and normal embryogenesis should take this into account.

REFERENCES

1. Witkin, S. S., Korngold, G. C., and Bendich, A. 1975. Ribonuclease-sensitive DNA-synthesizing complex in human sperm heads and seminal fluid. Proc. Natl. Acad. Sci. *72:*3295-3299.
2. Witkin, S. S., and Bendich, A. 1976. Particles resembling DNA tumor viruses in normal human semen. Abstract, ICN-UCLA Winter Conferences on Molecular and Cellular Biology: Animal Virology, March 14-21. p. 49.
3. Witkin, S. S., and Bendich, A. 1976. Virus-like particles in the sperm of normal men. Sci. Proc. Am. Assoc. Cancer Res. *17:*78.
4. Witkin, S. S., Evenson, D. P., and Bendich, A. In Press. Chromatin organization and the existence of a chromatin-associated DNA-generating system in human sperm. In: *The Molecular Biology of the Mammalian Genetic Apparatus*. Part A. Associated Scientific Publishers.
5. Bendich, A., Borenfreund, E., and Sternberg, S. S. 1974. Penetration of somatic mammalian cells by sperm. Science *183:*857-859.
6. Bendich, A., Borenfreund, E., and Beju, D. 1974. Alteration of mammalian somatic cells following uptake of spermatozoa. Acta Cytol. *18:*544. Abstract.
7. Bendich, A., Borenfreund, E., Witkin, S. S., Beju, D., and Higgins, P. J. 1976. Information transfer and sperm uptake by mammalian somatic cells. Prog. Nucleic Acid Res. Molec. Biol. *17:*43-75.
8. Higgins, P. J., Borenfreund, E., and Bendich, A. 1975. Appearance of fetal antigens in somatic cells after

interaction with heterologous sperm. Nature *257:*488-489.

9. Witkin, S. S., and Bendich, A. DNA synthesizing particles in normal human sperm: Localization and characterization of the endogenous reaction. Submitted for publication to Nature.

HUMAN SEMEN AS A BIOLOGICAL PRODUCT

Paul D. Parkman

The scientific data that are presented here will be especially helpful to us in the Bureau of Biologics in the months ahead. The way in which it will be helpful may not be immediately apparent, so I thought it would be useful to summarize within the context of how it fits into the regulatory process. And, of course, I am speaking of the possible regulation of sperm banks by the Bureau of Biologics.

The original legislation concerning the regulation of biologics passed by Congress early in this century was a result of an incident in which diphtheria antitoxin inadvertently contaminated with tetanus organisms was inoculated into several children. This produced several tetanus deaths and, indirectly, the Biologics Control Act of 1902.

The Bureau of Biologics, as a component of the Food and Drug Administration (FDA), is responsible for establishing and maintaining the quality and safety of all biological products for human use. These products include vaccines, antitoxins, therapeutic serums, allergenic products, and human blood for transfusion, as well as the many components and derivatives of human blood and plasma. Human semen for use in artificial insemination would also be classed as a biological. Biologics safety is assured by:

1. *Regulation and research programs.* Research studies conducted by Bureau scientists are related to the development, manufacture, testing, and use of biological products and serve as a base for the promulgation of standards for new products. Such studies on human sperm have now been in progress for about 1 yr at the Bureau and were reported on earlier this morning.
2. *Monitoring of investigational new drugs (IND).*

Under FDA regulations, the Bureau maintains surveillance of experimental biological materials under clinical study. To date, we have not taken the position that human semen is an experimental material that requires an IND submission. However, if we were to embark on a regulatory program this could represent part of it.

3. *The development of written as well as physical standards.* Standards for the production and testing of biologic products are set forth in the *Federal Register* and in the *Code of Federal Regulations.* In addition, the Bureau provides more than 120 reference preparations annually to biologic laboratories throughout the world. Written standards relating to human semen are another possibility that could be pursued in the regulation of this biological.

4. *Control testing of samples of products submitted by manufacturers for release.* The testing of samples and close surveillance of production is a standard procedure for many of the products we regulate, since they are derived from living organisms, such as bacteria and viruses, and are by their nature potentially dangerous or ineffective if improperly prepared and tested. Most biologics are released by the Bureau on a lot by lot basis. The application of this procedure to sperm banking would obviously have to be modified because of the small volume of each semen sample. An approach similar to that which we take in blood banking would probably make sense. There we test random samples from each blood bank.

5. *Licensing of manufacturing establishments as well as the products they manufacture.* The physical facility where processing is performed is inspected prior to license; when certain general standards are met, it is licensed by the Bureau. A separate license for each product is issued following determination by the Bureau that the prescribed standards for safety, purity, and efficacy have been met for the product. The product license indicates in detail the methods used in processing and testing and forms another basis for assurance of the use of standard, consistent procedures.

6. *Inspection of manufacturer's facilities for compliance with standards.* Annual inspection of every establishment licensed to manufacture biologics, as well as those in the process of applying for license, is carried out to insure continued compliance with FDA regulations.

The remainder of my remarks today will focus on the safety of human semen used in artificial insemination,

since, from what has been said earlier today, there are still substantial scientific questions that need to be resolved on tests attempting to measure what for other biologicals we would term potency and efficacy. The two aspects of human semen for which there are now enough data to begin to ask some questions are the purity and safety of the preparations. These two issues--purity and safety--are often closely related, and, in the case of human semen, this may especially be true.

Generally speaking, in considering the safety of all biological products one is concerned with four kinds of problems: (1) inherent hazard of the biologic material comprising the product, (2) problems relating to known added materials such as preservatives and stabilizers, (3) problems relating to extraneous chemicals that may be present, and (4) problems concerning the presence of contaminating microbial agents or their metabolic by-products. It is recognized, of course, that while one is working toward complete purity of products, we are, in fact, talking in practical terms about the relative absence of toxic materials, and not the absolute. Let me say in approaching this subject that biologics are a peculiar and heterogeneous group of substances--live attenuated polio virus and allergenic extract for the treatment of hay fever and human semen have little in common. Also, there is no standard set of laboratory toxicity tests for biologics. The development of appropriate tests to assure safety is largely dependent upon what is known of man's natural experience with the substance. One should recognize that since the earliest time man has lived in a sea of naturally occurring biologic substances. For example, the relationship between man and poliovirus predates the development of polio vaccines by millions of years. Thus, when there was a need to develop tests for the safety of polio vaccines, one logically first thought of the known adverse effects of polioviruses and tailored tests to exclude them. Similar reasoning could well be applied to tests on semen. Like the blood bank products, human semen presents a particular problem of safety, since prerelease of multiple samples of a lot is not possible. Each donation is a separate lot. Safety of such products then is predicated in part on the concept that the principles followed in obtaining the donor sample will ensure product safety.

One major safety consideration in the regulation of blood and blood products relates to the possible presence

of pathogenic agents originating from the human donor. On the basis of the presentations at this conference, this would seem to be quite pertinent to semen. Prominent among those agents that potentially can be transmitted in semen are the gonococcus, cytomegalovirus, and the hepatitis group of viruses. Each of those agents presents special problems in detection, but I think it is clear that one should certainly attempt to determine their importance and explore means for excluding them from semen used in artificial insemination. Evidence for potential harm from other agents mentioned today is perhaps less firm.

Creative regulation is a perceptive blend of medical need, legal authority, public expectation, and scientific opportunity. There should be a positive, as well as a restrictive, component to regulation. The availability of products is often of vital public importance. Thus, the public is protected by the avoidance of unnecessary delays in certification, as well as avoidance of ill-conceived shortcuts in the collection of safety and effectiveness data. The creative regulatory agency operates as a catalyst in the process involving science, manufacture, and use. Good regulation requires a working familiarity with the pertinent scientific data and an ability to communicate the logic derived from these data to a broader world composed of administrators, lawyers, legislators, special interest groups of various sorts, the press, and, through these groups, to the general public.

In closing, I would leave you with four main points. First, we recognize that human semen used in artificial insemination could be classified as a biologic product subject to regulation by the Bureau of Biologics. Second, there appear to be scientifically valid reasons to take steps to ensure that donor semen is free of major potential pathogenic agents. Third, the testing of semen samples presents substantial problems because of the small volume per sample. Fourth, any regulatory action would necessarily take this fact into account. I would reemphasize our interest in your continued participation in decisions concerning the regulatory process by your willingness to attend meetings such as this, serve on committees, and in general to make your views known as we move towards a regulatory position on sperm banks. It is with your help that we hope to take that reasonable middle ground in which the safety of the public is assured, while at the same time the availability of the product is not unnecessarily compromised.

SUMMARY AND CONCLUSIONS: THE CAPABILITY FOR PROLONGED PRESERVATION OF THE SPERM OF MAMMALIAN SPECIES, INCLUDING MAN, AND SOME CONSEQUENT IMPLICATIONS

A. P. Rinfret

EVOLVING TECHNOLOGY IN THE CRYOPRESERVATION OF SPERMATOZOA AND RELATED ACTIVITIES

To those interested in upgrading the genetic potential of a species, the report of Polge, Smith, and Parkes[1] must have provided an enormous stimulus. The relatively simple expedient of combining glycerol with a suitably extended suspension of spermatozoa provided for the first time a means of preserving for prolonged periods germ plasm of known and desirable characteristics. Little time was lost in applying the concept to sperm of species other than fowl, with extraordinary success in cattle, as will be evident from perusal of contributions to these proceedings by Graham and several others. True, methods had to be developed with care, and the parameters by which recovery of viable (and presumably fertile) cells from the frozen state was determined, clearly indicated that not all passed through the freeze-thaw procedure in functional form. It became apparent that factors other than the presence of glycerol are necessary to maximize yields of normal cells. Among these are the rates at which heat is removed from or returned to the suspension. Further, the recoverability of such cells was influenced by heat-transfer conditions in the liquid state (cooling) and, independently, in the solid state even more precisely, over that range of temperature at which the transition from liquid to solid is effected. Again, having established desirable cooling and warming procedures, storage temperature would have an obvious role in assuring the integrity of the cells over protracted holding periods.

The readily apparent benefits in establishing banks of frozen spermatozoa for breeding purposes prompted both academic and commercial organizations to set up facilities

and obtain accessories suited to the special needs of this type of operation. The demand so created led to the production of equipment needed to limit variability in processing and to assure that efficient inventory control (an absolute necessity where hundreds or thousands of specimens from multiple donors might be placed in a single storage unit) was available in refrigerators providing suitably low temperatures. This latter equipment had to be designed to meet various conditions of use, including field transport of frozen material for weeks or, later, even months, where power was unavailable. Ruggedness and sophisticated insulation, the essential elements required in accessories of this kind, and economics, coupled with the requirement for cellular integrity over the full period of field use, dictated that liquid nitrogen serve as refrigerant. It is of interest to note that technology developed to meet the performance specifications of such equipment for use in cattle breeding operations also found application in various cryogenic aspects of the space program.

Within a few years after the original publication of Polge, Smith, and Parkes, a bovine artificial insemination industry based on sperm preserved and distributed in the frozen state had been established. An earlier observation by Rostand[2] on the protection afforded frog spermatozoa cooled to about -6°C, with partial freezing, seems not to have attracted widespread attention. By 1954, while not on a scale that in any sense approached that committed to cattle, Bunge, Keetel, and Sherman[3] reported on the use of frozen sperm in human insemination, describing four clinical cases. Twenty-eight years after the brief paper by Polge, Smith, and Parkes, the species yielding sperm shown to be amenable to cryopreservation include horse, sheep, swine, cattle, fowl, and man. During this same period, a multitude of other cells from a variety of sources were subjected to cryopreservation procedures, some with outstanding success. Among these latter must be included the human erythrocyte, certain bacterial preparations of commercial importance and the viral systems associated with Marek's disease in fowl. Currently, rapidly expanding interest in the implications for many biomedical research programs that lie in the capability for indefinitely prolonged preservation of cellular materials and their components, in a potentially viable or functional state, is evident in the frequent appearance in the literature of new methods to accomplish such ends.

Thus, at the time of this conference, there was available to the participants a significant body of basic cryobiological literature, to which several had themselves contributed; a much larger body of publications dealing with the arts of cryopreservation, to a considerable extent empirical and noncritical in nature; and a variety of apparatus and accessories for processing and storage over a broad range of temperature.

The economic stakes associated with artificial insemination (AI) in dairy and, later, beef cattle demanded intense study of every factor that could affect the yield and functionality of the sperm used in such operations. An enormous effort was made, and the multitude of variables at every phase, starting with the donor, through collection, dilution, packaging, low-temperature processing, storage, distribution, return to the liquid state, insemination procedure, gestation, subsequent observation of offspring for normal or abnormal characteristics, and, finally of their performance, demanded a very large investment in time, money, and facilities. Thanks to the incentives, much of the basic science underlying the present AI industry, and the procedures for preservation of materials of biological origin in the frozen state, has come and is continuing to come from workers in the field. It is probably not unreasonable to say that all the basic or applied problems confronting those who would use, or regulate the use of, AI in man, have already been dealt with, on an immensely larger scale and, as the technical evidence indicates, with a very large measure of success. A reservoir of knowledge, acquired at a cost in funds alone unlikely ever to be available for study in the human context, is at hand for researchers, practitioners, and regulators to draw upon in the pursuit of their respective objectives.

THE AREAS OF AGREEMENT

In undertakings as complex as those associated with AI, it is to be anticipated that substantial differences in purpose, viewpoint, data interpretation, methodology, business practice, research planning and implementation, and regulatory policy can arise and for good, or less than good, reason persist. There are, however, fundamentals that seem to be accepted by most, if not all, participants, in that they were not seriously challenged at the conference. That the source of the cellular component, the donor, is an intrinsically variable producer, yielding a product of

variable quality in terms of sperm number, fertility and stability to collection, preservation, and distribution procedures, even with animals of the most desirable genetic attributes, seems accepted as fact. As a point of departure, this requires building into any commercial operation, and even into any large-scale research program, flexibility in methods (not to be confused with lack of definition therein). That the donor, in addition to his ability to transmit highly desirable genetic information via his spermatozoa, can also transmit viral and other disease of microbiological origin via those same cells is a similarly indisputable fact. The potential for loss, in purely biological terms, within the bovine AI industry is very much greater than that likely to arise within the strictly medical operations of human AI.

The extraordinary technical achievement obvious in the separate presentations of Graham, Pickett, Foote, and Elliott, as these authors deal with preservation, will be better appreciated after reviewing the commentary offered by Bartlett. The potential for trouble is great through misuse of an effective technology, and his impatience with regulatory policy within the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture is easily understood. A qualitatively similar potential for trouble is also acknowledged to exist in human AI; and, indeed, what was perceived by some entrepreneurs as a financially attractive venture, a human sperm bank, might, in an almost totally unregulated commercial environment, offer some element of risk to an uninformed public. Fortunately, the original perceptions of financial attractiveness were later perceived to be in error, and the human sperm banks, soon diminished in numbers, seem to have passed to the medical profession. Although the motivation for operating such banks will probably differ, a significant risk exists, even in the hands of medical professionals. Thus, the need for regulatory action in economically or medically important AI was accepted without challenge by those in attendance. The manner in which such regulation is to be achieved over human AI could well involve heated debate, however, and Sell's contribution, with the commentary of Friedlander, suggested an approach common to that which might be applicable to other medically important cells and tissues.

The, at times, awe-inspiring presentations of Noguchi, Barile, and Lang further emphasized the ever-increasing complexity of what, in a more innocent time, seemed fairly

easy to comprehend, namely the concept of venereal disease. The risk incurred was largely to the conscience and, possibly, to the ego. Antibiotics eliminated any sustained hazard from infection. The list of viruses and other organisms known to contaminate human sperm, and which are unaffected by antibiotics, is of such dimensions that it becomes self-evident that the recipient of medical AI must be afforded a greater measure of protection than is currently the case. Parenthetically, one wonders how the sexual mores of these enlightened times can accommodate to the implications of this greatly enlarged body of knowledge. While a responsible and responsive Food and Drug Administration can do nothing about the latter, it can certainly play a critically important role in focussing attention of the pertinent sections of the medical community on the nature of the problems involved. As Parkman made clear in the concluding statement of the conference, the basic structure and procedure is already present within the Bureau of Biologics to render effective and efficient supervision of this area of medical practice through regulations rationally developed and scrupulously observed. Here too, there appeared no dissent on the need to protect the recipient through regulatory action.

Finally, no challenge was offered to the evidence repeatedly presented that spermatozoa of a number of species, including man, can be preserved in potentially functional form for prolonged periods of time in the frozen state. The material discussed indicated something less than complete agreement on optimal methods, but it is not unreasonable to conclude that, if banking of sperm could serve a medically useful purpose in human AI, the required technology is available. If the period necessary to determine that an ejaculate is free of potential disease-producing microorganisms is such as to require freeze-preservation for assurance of subsequent fertility at insemination, protocols that have been successfully developed and employed by others have been described in the literature. Sherman's presentations and that of Feldschuh, insofar as technology is involved, were based on these premises. However, Sherman's plea for support for research into the integrity of human spermatozoa preserved at cryogenic temperatures for very long periods of time, in excess of 5 or more years, lent emphasis to a point on which less than unanimous agreement was evident: The period of time for which spermatozoa can be stored in a liquid-nitrogen-cooled vessel without detectable evidence of change.

AN AREA OF DISPUTE

In many kinds of biological study, it is common practice to associate declining temperature with reduced activity of biochemical significance. Although processes involving diffusion are linearly related to temperature, those involving reaction rate are exponentially so, as the Arrhenius equation has indicated to every student in a first course in physical chemistry. Consequentially, when seeking to limit change with time in a biological system, it is conventional wisdom to maintain it at some temperature below ambient. Indeed, some very large and successful operations throughout the world are based on such procedures, the storage and transport of perishable food stuffs, for example. When the requirement is imposed that the system must function normally, as in its original state, on return from the reduced temperature to ambient, the problem may assume greater complexity, but the fundamental proposition retains its validity. If a reaction of degradative or other nature takes place in a living cell in the liquid state, that reaction will proceed more slowly at a lower temperature, by a factor of 2 to 3 for each decline of 10°C. If that same reaction can proceed at -200°C, it will do so at a rate about 2^{20} to 3^{20} slower than it does at, say, 20°C. The decline in rate is of such magnitude that measurable change in the system due to progress of the reaction is not likely to be detectable for periods measured in decades, if not greater units of time. It is, then, with considerable interest that one examines the data assembled in support of the view that detectable change, characteristic of biochemically significant reactions taking place at temperatures above the freezing point of water, occurs at or near the boiling point of liquid nitrogen over periods as short as 1 to 2 yr. The investigations of Salisbury and his colleagues on this subject over the past 10 yr were summarized by Salisbury. Their conclusions were based upon data and interpretation believed by the University of Illinois group applicable to the position they have taken.

For brevity, these comments will be limited to consideration of the evidence claimed to indicate the existence of reactions taking place at about -196°C that are qualitatively comparable to those occurring in sperm and other systems at temperatures above the freezing point of water. That change of a degradative nature will take place in sperm and other cellular entities while in the liquid state in an *in vitro* environment might reasonably be expected. The nature of such changes, particularly in the context of AI,

would be of very great theoretical and practical importance, and, if analyzed in sufficient detail, could probably be shown to be effected by a number of chemical reactions, interrelated or independent, with characteristic kinetics. That such a series of definable events is to be anticipated under the conditions specified (liquid state, *in vitro*), would hardly justify an assumption that they are also to be anticipated in the frozen state, *in vitro*, over a range of temperature extending to -196°C, albeit at a slower rate; and, as Salisbury emphasizes, with an altered kinetics, slower, to be sure, than would be the case in the liquid state, but orders of magnitude faster than an Arrhenius calculation would predict. Nevertheless, on the basis of statistical data obtained from field operations for the artificial insemination of cattle, conducted on such a scale as to make almost impossible direct supervision or control by their group while in the field and on the basis of laboratory experiments planned by the group, and involving spermatozoa studied for evidence of change, brought about for the most part in the liquid state, with time under a number of holding conditions, Salisbury and his colleagues argue that change of this kind is very likely to occur: "Our data with cattle clearly infer that such changes in effectiveness of the male gamete occurring with the passage of time occur at any temperature within the range of body temperature down to -196°C. Temperature influences the rate of change but does not postpone indefinitely the ultimate occurrence."

Surely the changes in effectiveness inferred are, or will one day be, defined in terms of chemical reactions. The inference must thus follow that despite total structural reorganization of the internal and external environments of the male gamete, due to the various changes of phase effected in the system as its temperature declines to -196°C, those same reactions taking place at body temperature, or *in vitro* in the liquid state, will also proceed at temperatures 200°C lower, although at a slower rate.

Quite apart from the special field of interest dealt with in this conference, it will be apparent that the theoretical implications of the concepts advanced by Salisbury are profound indeed. As Mulhaupt makes clear in his commentary, reactions at cryogenic temperatures are well known, none, however, being associated with systems of biological origin. Further, Mullhaupt proposes conditions under which changes of biological significance may occur over that range of temperature. These are not conventional biochemical reactions commonly associated with living cells *in vivo* or in the liquid state *in vitro*. Nor do those conditions call for

an inverted kinetics, in which reactions proceed tens of thousands of times more rapidly at extremely low temperatures than would be anticipated from their reaction constants under conventional laboratory temperatures. The construction placed by Salisbury on his observations over a period of many years must be regarded as a new hypothesis based on chemistry integral to living cells. Inasmuch as the changes induced by that chemistry are associated primarily with the storage function under conditions of temperature equilibrium, and not with inadvertent and undesired change effected during the nonequilibrium procedures of freezing and thawing, the importance of his concept to other fields of science can hardly be overstated.

Among the data cited in the contribution of Salisbury and Lodge are those in Table 7 from "Metabolic Activity of Spermatozoa at -196°C," a report by one of Salisbury's associates, Graves, that appeared in 1968. That it appeared in the *Journal of Animal Science* may account for the fact that a report with so arresting a title has not received the attention it calls for from the appropriate divisions within the chemical community. It is inevitable that when the implications of the concepts developed by Salisbury and his colleagues, buttressed by data presented in Graves, Table 7, are understood, they must attract major interest and intense scrutiny by many scientific groups. Clearly, current notions concerning extraterrestrial life, particularly experimental planning and data interpretation in connection with the life-detection program for the Mars Lander, may be subject to revision. A metabolism proceeding at -196°C may provide a new theoretical basis for speculation as to the origins of life on this planet, within the solar system, this galaxy, and beyond. Quite possibly, too, such a metabolism will find practical applications, conceivably in space exploration. Certainly, Salisbury's conclusions should be pertinent to investigations on the microbiology of the polar continent and ice caps.

Other speakers at the conference, holding strictly to the subject thereof, and to their own observations on the problems associated with the cryopreservation of sperm, its distribution for large-scale AI operations, and its withdrawal from storage and insemination under variable field conditions, were in obvious disagreement with Salisbury. The central point of issue did not rest on whether or not changes occur in spermatozoa stored and transported in, withdrawn from, and returned to vessels containing liquid nitrogen, since all were in agreement that

degradative changes could occur under such conditions, but on the physical basis for those changes. Foote and Pickett pointed out that, under completely controlled laboratory conditions, changes of the type described by Salisbury are not observed. Rather, changes with time under the conditions prevailing in field AI operations are due to variables in handling, which subjects the sperm suspensions to altered environments, at times because of inexperience on the part of the technician-inseminator. This was unassessed in the statistical studies made by Salisbury.

This area of disagreement is of evident concern to those considering the operational and regulatory aspects of the cryopreservation and banking of human spermatozoa. Commenting on the enhanced stability of sperm in field AI programs brought about by improvements in processing and handling, Salisbury admonished, "However, from such data we should not generate the view that now spermatozoa can be stored forever without detrimental effects to their ability to fertilize eggs and produce viable offspring." This statement seemed wholly in accord with the philosophy of all conference participants, none of whom gave evidence of generating a view toward storing spermatozoa forever. For the more limited objective of research on human spermatozoa preserved in the frozen state for periods in excess of 5 to 10 yr, as Sherman among others has advocated, the constructions placed on data obtained from field AI operations by Salisbury may serve as guideposts for doubtful storage technology. Under no circumstances can the data yet be considered of such quality as to justify, exclusively on their own merits, rejection of proposals for long-term research of this type.

TECHNICAL FACTORS AND OTHER CONSIDERATIONS IN THE CONTEXT OF REGULATION

Those biomedical scientists conducting investigations on the transfusion or implantation of cells and tissues in human subjects are highly conscious of the limitations of our knowledge in many aspects of these complex problems. None are more aware of the need for continued research in such sensitive areas as optimization of collection and isolation procedures, the maintenance of functional integrity from the time of collection of medically useful material until its transfer to a recipient, and the subsequent immunology inevitably associated with such therapy. This research will call for the gathering of basic and

applied data, much of it necessarily through experimental use of human patients. Clearly, this type of investigation falls within the purview of the Food and Drug Administration, and few of those engaged in it will challenge the necessity for that agency to assume a role in the protection of the public, as its legislative charter provides. There is, nevertheless, a certain apprehensiveness within this segment of the biomedical research community based on the notion that in the formulation of admittedly essential regulations its guidance may not be sought as fully as necessary to assure maximum contribution of the knowledge, understanding, and clinical experience at hand. Those who have occasion to peruse the *Federal Register* from time to time will appreciate the concern of the various regulatory agencies, including the FDA, to achieve contributions of the highest quality to the various panel and committee deliberations from which specific regulations must in part ultimately derive; regulations applying not only to the conduct of research, but also to those aspects of medical practice calling for the use of regulated products. There can be little question that Sherman's guidelines and the logic inherent in the proposal for peer group contribution to standards and regulations by Sell and Friedlander will be welcomed by the Bureau of Biologics as inputs of the highest professional quality, as Parkman made clear in his presentation. By the same token, Parkman's citing of the concept of creative regulation suggested the subtle and complex array of factors that enter into the process and that may well involve considerations of viewpoints in addition to those of the biomedical research professionals.

Muller's concept of the community blood bank as an existing institution administratively, technologically, and philosophically oriented to a broader banking role to meet the needs for medically useful cells and tissues among the population it serves was of particular interest. Already adapted to operations in a regulated environment, accustomed to the relationship of responsibility to physician and patient and to the standards of institutional and personal performance required by commitment to service, such banks in many communities unequipped with major medical research and teaching centers may serve a function not otherwise attainable. As the scientific and technical aspects of tissue banking are resolved in the years ahead, it is unlikely that separate facilities for each type of tissue will be operationally feasible or economically acceptable in hundreds of smaller communities in this country or in others. The role of the community and hospital blood banks may broaden.

As a topic on which to conclude these comments, it may be of interest to consider how the preservation function will be conducted in the cell, tissue, and blood banks of the future using a well-developed, even mature, technology to carry out specified procedures approved by the FDA. With general recognition that the steps constituting procurement, testing, prestorage processing, storage, retrieval, and poststorage processing of a defined unit of a cellular or other material are in fact a manufacturing operation and as such are required to conform to the criteria for good manufacturing practices (GMP), the necessity for control and for proof that such control has been achieved over each stage will become evident. With the need for data assuring standards of efficaccy and safety, the record-generating and retention procedures will require careful planning, quite possibly on a national basis, to secure in addition the advantages of uniformity for inventory management, interbank exchange, and the like. Implementation of these procedures at the level of the individual bank will be essential, not only for such assurance, but also to provide a basis for institutional and personal protection against possible litigation subsequently brought on behalf of the recipients of such products.

In determining the acceptability of protocols and the nature of the records to be integrated into the preservation function of biologic banks of this type, it will be important that the advisory panels and subcommittees of the FDA examine critically the conditions required to assure procedural reproducibility in the preservation of a given product. With high and uniform product quality to be achieved in all such banks, looseness of definition and failure to specify method in detail will not be tolerated. The notion that a technically undemanding procedure can be equated with variable and discretionary implementation can only lead to doubtful product quality. In those product types, such as suspensions of human sperm, where small volumes preclude direct and extensive quality-assurance testing, such manufacturing practices will have no place.

During the conference, the extraordinary breadth of experience of those participants who have studied the requirements for prolonged preservation of bovine sperm showed quite clearly that much can go awry. Sherman, with the confidence born of many years of experience and thousands of laboratory experiments under his demanding personal supervision, assures us that the preservation of human

spermatozoa in the frozen state is a rather simple affair. No doubt it is, in his laboratory, or in those of others where scientific exactitude is so obvious it is not deemed a matter requiring discussion. It would be unwise to assume that such a happy state will prevail universally among the biologic banks of the future, any more than it does today. For the least complex of protocols, as for the most complex, the GMP's must be specific and reproducibility readily attainable without taxing the ingenuity of the technical staffs of those banks.

REFERENCES

1. Polge, D., Smith, A. V., and Parkes, A. S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature *164:*666.
2. Rostand, J. C. 1946. Glycerine et resistance du sperme aux basses temperature. C. R. Acac. Sci. Paris *222:*1524-1525.
3. Bunge, R. C., Keetel, W. C., and Sherman, J. K. 1954. Clinical use of frozen semen. Report of four cases. Fertil. Steril. *5:*520-529.

Copyright © National Academy of Sciences. All rights reserved.

Copyright © National Academy of Sciences. All rights reserved.

Copyright © National Academy of Sciences. All rights reserved.

Copyright © National Academy of Sciences. All rights reserved.

Copyright © National Academy of Sciences. All rights reserved.

Copyright © National Academy of Sciences. All rights reserved.