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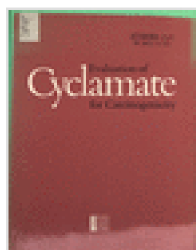

Evaluation of Cyclamate for Carcinogenicity (1985)

Pages
209

Size
8.5 x 11

ISBN
0309035465

Committee on the Evaluation of Cyclamate for Carcinogenicity; Commission on Life Sciences; National Research Council

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Evaluation of Cyclamate for Carcinogenicity

Committee on the Evaluation of Cyclamate for Carcinogenicity
Commission on Life Sciences
National Research Council

Order from
National Technical
Information Service,
Springfield, Va.
22161
Order No. PB86159159

NATIONAL ACADEMY PRESS
Washington, D.C. 1985

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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.

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The work on which this publication is based was performed pursuant to Contract No. 223-84-2063 with the Food and Drug Administration.

One volume only.

Library of Congress Catalog Card Number 85-61230
International Standard Book Number 0-309-03546-5

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Printed in the United States of America

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Preface

In November 1983, the Food and Drug Administration (FDA) asked the National Research Council to conduct a comprehensive review of the evidence on the carcinogenicity of cyclamate. In response, the Committee on the Evaluation of Cyclamate for Carcinogenicity was established in June 1984 under the auspices of the Food and Nutrition Board (FNB) in conjunction with the Board on Toxicology and Environmental Health Hazards (BOTEHH), both of the Research Council's Commission on Life Sciences. The committee was asked to evaluate all the data on this subject, including data from animal bioassays, epidemiological studies, and short-term tests, to determine whether or not cyclamate and its derivatives are carcinogenic. In the event that the data were found to be inconclusive, the committee was asked to recommend test protocols that would be sufficient for determining conclusively whether or not these substances are carcinogenic. In addition to the general charge to the committee, the FDA asked for answers to 11 specific questions relating to the interpretation of animal bioassays, including the advisability of combining data from different generations, tests, histogenic sites, and animal strains; the impact of bladder parasites and bladder stones on the interpretation of test results; the selection of significance levels; and weighting of negative and positive results in studies of differing quality.

To ensure that the committee included members with all the requisite expertise for the study, a multidisciplinary group was formed. Among the members are specialists in toxicology, animal bioassays, pathology, metabolism, genetic toxicology, biostatistics, and epidemiology. The committee reviewed all data on cyclamate relevant to its charge. Sources of these data included reports and reviews as well as published reports of original research relating to metabolic studies, animal bioassays, short-term tests, and epidemiological studies. The committee also contacted researchers directly to obtain information too new to have been incorporated in the open literature. In July 1984, a widely publicized public meeting was held at the National Academy of Sciences in Washington, D.C., to obtain the views of all interested individuals and organizations.

The committee is aware that the subject of this study is of interest not only to scientists but also to the public, to the food industry, and to the agencies responsible for regulating food additives. In particular, it recognizes the importance of the

possible carcinogenicity of cyclamate as a food safety policy issue and that public health can be protected adequately through consideration not only of carcinogenicity but also of all other possible adverse health effects such as reproductive abnormalities, teratogenicity, testicular atrophy, and gastrointestinal, neurobehavioral, and dermatological effects. Although the committee has considered in detail only those effects that may bear on its charge, i.e., the evaluation of data on carcinogenesis, its members are aware that important questions about other possible adverse health effects, particularly testicular atrophy in animals fed cyclohexylamine, may need to be considered in detail before the safety of cyclamate can be fully assessed.

Several subgroups of the committee were formed to study and evaluate data on cyclamate metabolism, short-term tests, animal bioassays for carcinogenicity, and epidemiological studies. Although each subgroup was given primary responsibility for one aspect of the study, all members of the committee contributed to each area of the study through discussions, comments, and shared report writing. The entire report was discussed, revised many times, and approved by all the committee members.

The Executive Summary (Chapter 1) describes the major findings, conclusions, and recommendations of the committee. Background information on the use of cyclamate and its regulatory status are given in the second chapter. The metabolism of cyclamate and the evidence from short-term tests are evaluated in the third and fourth chapters, respectively. Chapter 5 is an examination of the results of animal bioassays in mice, rats, hamsters, dogs, and monkeys. Epidemiological studies are reviewed in Chapter 6. Appendix A contains tables summarizing carcinogenicity bioassays of cyclamate in animals. The answers to the 11 specific technical questions posed to the committee by the FDA are included as Appendix B. Appendix C contains brief descriptions of the affiliations and major research interests of the committee and the Research Council staff.

The committee is grateful to all who contributed to this report. Special acknowledgments are due to John S. Wassom and Elizabeth S. von Halle of the Environmental Mutagen, Carcinogen, and Teratogen Information Program at Oak Ridge National Laboratory, Oak Ridge, Tenn., who conducted information searches on short-term tests and provided reference material; to Steven Hecht of the American Health Foundation; to Michael Waters and Ann Brady of the U.S. Environmental Protection Agency at Research Triangle Park, N.C., who generated spectra of the genetic activities of cyclamate and its derivatives from the tabulations made by the committee; and to R. Marian Hicks of the Middlesex Hospital Medical School in London, Andrew G. Renwick of the University of Southampton Faculty of Medicine in Southampton, and the staff of the Calorie Control Council, who provided requested background material.

The people listed below presented useful information and participated in the public meeting held on July 31, 1984: James Emerson of the International Life Sciences Institute, Washington, D.C.; William Havender, William Havender Associates, Berkeley, Calif.; Robert H. Kellen, Calorie Control Council, Atlanta, Ga.; Albert Kolbye, Jr., The Nutrition Foundation, Washington, D.C.; Rodney E. Leonard, Community Nutrition Institute, Washington, D.C.; and Robert W. Morgan, Environmental Health Associates, Oakland, Calif.

Thanks are also due to members of BOTEHH and FNB, and to Devra Davis, James A. Frazier, Stephen L. Brown, and Alvin G. Lazen, CLS staff who provided advice and assistance in planning the study.

The committee also acknowledges the assistance of Sanford A. Miller, Robert J. Sheuplein, Robert J. Lorentzen, Patricia M. McLaughlin, and Gary W. Flamm at the FDA and their colleagues in providing access to information throughout the study.

In addition, the committee commends the assistance and the support of the FNB staff, including Sushma Palmer, Farid E. Ahmed, Virginia Hight Laukaran, Marianne E. La Veille, and Frances M. Peter. The dedicated secretarial and administrative assistance provided by Drusilla E. Alston, Janet J. Crooks, Susan G. Barron, and Shirley Ash are especially acknowledged.

Richard Havel
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Cyclamate for Carcinogenicity

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Chapter 1

Executive Summary

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Executive Summary

The National Research Council Committee on the Evaluation of Cyclamate for Carcinogenicity concludes that the weight of experimental and epidemiological evidence does not indicate that cyclamate by itself is carcinogenic.[1] However, there is suggestive evidence from *in vivo* and *in vitro* studies in animals that cyclamate has cancer-promoting or cocarcinogenic[2] activity, and from epidemiological studies in humans that the use of cyclamate-saccharin mixtures may be associated with a small increase in risk of bladder cancer.

The committee was asked by the Food and Drug Administration to focus exclusively on carcinogenicity. During the course of its evaluation, however, the committee noted that other adverse effects such as testicular atrophy had been observed in laboratory animals exposed to cyclohexylamine. Some of these observations are mentioned in the report. Although they were not evaluated in this study, the committee recognizes that they would need to be considered in detail in the overall evaluation of cyclamate for widespread use.

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[1] By *carcinogenesis* the committee means the capacity of cyclamate to increase the risk of cancer. The measures of increased risk for carcinogenicity in animal experiments include the induction of neoplasms that are not usually observed, the earlier induction of neoplasms that are usually observed, and the induction of more neoplasms than are usually found.

[2] By *cocarcinogenesis* is meant augmentation of the neoplastic response brought about by a noncarcinogenic factor operating in conjunction with a carcinogen. Operationally, cocarcinogenesis is defined here as enhancement of carcinogenicity resulting from administration of the noncarcinogenic modifying factor (cyclamate) either just before or together with the carcinogenic agent. *Tumor promotion*, on the other hand, is used operationally herein to refer to enhancement of the tumorigenic response to an agent when the second agent (cyclamate) is administered later.

Following is a brief description of the committee's major findings and recommendations. The remainder of this chapter contains a historical perspective on cyclamate use, a more complete description of the major areas of inquiry, and the overall recommendations of the committee.

- The epidemiological evidence indicates that cyclamate-saccharin mixtures, as they have been consumed in the past, have not resulted in a clear increase in risk of bladder cancer in most categories of users. A small increase in risk among nonsmoking women and among users of unusually large amounts has been observed. These observations could be due to chance, but they are compatible with weak carcinogenicity for the human bladder. Data are insufficient to separate the effects of cyclamate from those of saccharin. There is also insufficient information to assess the carcinogenic effects of these substances at sites other than the bladder. The committee recommends that in those countries where cyclamate is being used or has been used, epidemiological monitoring should be continued to determine whether the risk of human cancer is increased in heavy or long-term users or after a long period since first exposure. In such monitoring, consideration should be given to other cancer sites in addition to the bladder.

- Despite a few earlier studies in rodents suggesting tumorigenicity, the totality of the evidence from studies in animals does not indicate that cyclamate or its major metabolite cyclohexylamine is carcinogenic by itself. In one bioassay, rats dosed with a 10 to 1 cyclamate-saccharin mixture were reported to develop urinary bladder cancer. Two replicate studies by others, however, did not confirm this result, and there is no clear evidence for a synergistic effect of cyclamate on saccharin-induced bladder cancer. The committee concluded that no further animal bioassays of this type are necessary.

- Two studies in rodents suggest that cyclamate possesses tumor-promoting and cocarcinogenic activity in the urinary bladder. The committee recommends that one and, possibly, both of these studies be repeated. Whether or not the results are confirmed, a wider analysis will be required to learn the significance of studies of this type to human health.

- Overall, the short-term test data indicate that the likelihood of cyclamate or cyclohexylamine being DNA-reactive carcinogens is small. Nevertheless, positive results for cytogenetic and certain other effects were considered to be consistent with the possibility that cyclamate could be a promoter of neoplasia. Many tests have been performed, but there have been no assays for mammalian cell DNA damage or gene mutation for cyclamate and no DNA damage tests for cyclohexylamine. These should be done. More definitive cytogenetic studies should also be conducted.

● The evidence from metabolic studies does not indicate that potentially hazardous metabolites are formed in humans. The committee recommends, therefore, that no further studies on the metabolism of cyclamate are necessary for evaluating carcinogenicity.

HISTORICAL PERSPECTIVE

The committee was formed in June 1984 at the request of the Food and Drug Administration (FDA) to determine whether or not there is sufficient evidence to establish the carcinogenicity of cyclamate. This substance has been the subject of controversy for several decades because of reports that it may have some carcinogenic potential.

Cyclamate was initially marketed as a nonnutritive sweetener in 1949. In 1953, a mixture of 10 parts cyclamate to 1 part saccharin replaced the cyclamate-only product. During the 1960s, cyclamate-sweetened soft drinks were first marketed, and there was a rapid increase in cyclamate use. Results of one test conducted in the 1960s suggested possible increases in tumor incidence in rats fed the cyclamate-saccharin mixture. Pending confirmation of these results, however, cyclamate continued to be used. Because of concern about gastrointestinal effects, it was suggested that its use in the United States be limited to 70 mg/kg body weight per day for adults.

In 1968, a Research Council committee concluded that there was no evidence for the carcinogenicity of cyclamate in rats. One year later, however, that committee reconvened to review new data and concluded that the cyclamate-saccharin mixture used in the study was carcinogenic in rats, while noting the possibility that saccharin may have been responsible for the carcinogenicity. Later in 1969 the FDA banned the use of cyclamate in foods. The data on carcinogenicity were reexamined in 1976 by a National Cancer Institute committee, which concluded that the evidence had not demonstrated that cyclamate is carcinogenic. In response to a petition from the manufacturers to allow cyclamate to be used as a food additive, the FDA Commissioner in 1980 concluded that "cyclamate has not been shown not to cause cancer; and ... cyclamate has not been shown not to cause heritable genetic damage."[3]

Considering new data generated after the 1980 decision, an FDA Cancer Assessment Committee in 1984 concluded that "no newly discovered toxic effects of cyclamate were likely to be revealed if additional standardized studies were performed."[4] Later that year,

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[3] Federal Register, p. 61475, September 16, 1980.

[4] Scientific Review of the Long-Term Carcinogen Bioassays Performed on the Artificial Sweetener, Cyclamate. Food and Drug Administration, April 1984.

the FDA approached the Research Council with a request for the present study, which was conducted under the auspices of the Food and Nutrition Board in conjunction with the Board on Toxicology and Environmental Health Hazards.

METABOLISM OF CYCLAMATE

There is a substantial body of information on the absorption and excretion of cyclamate and its major metabolite cyclohexylamine in humans and several other mammals. No major differences have been observed among species. Cyclamate is absorbed only partially from the intestine, and a variable but usually small amount is converted to cyclohexylamine by microorganisms in the large bowel. In humans, the conversion of cyclamate to cyclohexylamine is usually very limited (less than 1%), but varies markedly from person to person and in the same person from time to time. Absorbed cyclamate and cyclohexylamine are both rapidly excreted as such by the kidneys, and there is little accumulation in body fluids, even during long-term administration. Both cyclamate and cyclohexylamine can cross the placental barrier, thereby exposing the fetus.

In some mammals, trace amounts of another cyclamate metabolite, *N*-hydroxycyclohexylamine (which is structurally similar to certain activated carcinogens), may be found in the urine. However, there is no credible evidence that this metabolite is formed in humans.

SHORT-TERM TESTS

Cyclamate and its major metabolite cyclohexylamine have been extensively evaluated in a variety of short-term tests for DNA damage, gene mutations, and chromosome aberrations. Positive results from these tests would provide some indication for carcinogenicity as a result of DNA damage or reactivity, but negative results do not exclude carcinogenicity by other mechanisms.

Tests for gene mutations in bacteria have been uniformly negative for cyclamate and cyclohexylamine. A significant deficiency in the data, however, is the absence of assays for mammalian cell DNA damage and gene mutation for cyclamate and a gene mutation assay for cyclohexylamine. Positive results have been obtained in mammalian cytogenetic tests and in some tests for recessive lethals and chromosome abnormalities in fruit flies (*Drosophila melanogaster*). Many of these cytogenetics tests had limitations that indicate a need for more refined studies. The combined evidence from short-term tests, as evaluated by two different techniques, indicates that there is little likelihood that cyclamate or cyclohexylamine could be DNA-reactive carcinogens. The positive cytogenetic effects taken by themselves are of uncertain significance with regard to carcinogenicity, but they are consistent with the possibility of a neoplasm-promot-

ing action. Likewise, the committee also noted that results of five *in vitro* studies conducted to explore effects associated with tumor-promoting properties were positive for cyclamate. The observations made in these studies are described on pages 37-38 and 61-62.

ANIMAL BIOASSAYS FOR CARCINOGENESIS

The committee evaluated animal bioassays by assessing the design, conduct, and reporting of results in light of contemporary standards for the performance of such assays. Included in the evaluation were 22 long-term studies in which cyclamate alone was fed to rats and mice. A few positive results were reported in some of the earlier studies, but taken together with more recent studies, these long-term studies do not indicate that cyclamate or cyclohexylamine by themselves are carcinogenic in rats and mice. Multigeneration studies designed specifically to evaluate the production of tumors of the urinary bladder provided no evidence for carcinogenicity.

In studies of cyclohexylamine, no evidence for carcinogenicity has been obtained in rats or mice. This compound has not been as extensively studied in animals as has cyclamate.

Studies of cyclamate-saccharin mixtures were also examined. In one older study, there was an increased incidence of bladder tumors in rats, but in two more detailed recent studies, an increase in bladder tumors was not obtained. Overall, the studies conducted with mixtures of cyclamate and saccharin do not provide convincing evidence that such mixtures are carcinogenic in rats.

Two studies, one in rats and one in mice, suggest that cyclamate may enhance the carcinogenic effect of other substances in the urinary bladder. In one study, cyclamate was incorporated into a cholesterol pellet and implanted into the bladders of mice; in the other, it was fed to rats after a carcinogen, *N*-methyl-*N*-nitrosourea, had been instilled into the bladder. In both experiments, more bladder cancers formed in animals receiving cyclamate in combination with other agents than in those receiving the other agent alone, suggesting that cyclamate has cocarcinogenic and tumor-promoting activities, respectively. The committee recommends repetition of the experiment that suggested tumor promotion and, perhaps, the study that suggested a cocarcinogenic effect. If the findings are confirmed, uncertainty would still exist about the assessment of risk of cyclamate use for humans, because there are no data bases for extrapolating results obtained in these model systems of promotion or cocarcinogenesis in the urinary bladder to natural human exposures. The committee also recommends that more research be carried out to gain an understanding of the predictive value for human health of results from such test systems and that generic research should be performed to develop well-validated, relevant systems for the assessment of cancer-promoting agents.

EPIDEMIOLOGICAL STUDIES

Most epidemiological studies to examine the association between cyclamate use and human neoplasms have involved mixtures of saccharin and cyclamate and have dealt almost exclusively with cancer of the urinary bladder. Overall, studies in which the effects of cyclamate and saccharin could not be distinguished were judged to be inconclusive. Most showed no significant increase in the risk of bladder cancer for persons who had ever used artificial sweeteners, and most failed to show a relationship between the risk of bladder cancer and the amount consumed per day or duration of use. However, in otherwise low risk (nonsmoking) women in four studies, an increase in risk was found among users of nonnutritive sweeteners. In the largest of these studies, the risk of bladder cancer in female nonsmokers increased with the total amount of artificial sweetener consumed. In the same study, the risk of bladder cancer was increased in those persons who consumed unusually high doses of artificial sweeteners.

Since there have been practically no investigations of human cancer sites other than the urinary bladder, it is impossible to estimate what effect cyclamate may have on other parts of the body. There is little reason to assume that cyclamate, if it were carcinogenic in humans, would affect the bladder specifically or exclusively.

In three studies of mixtures, attempts were made to examine the effects of cyclamate alone on risk of bladder cancer. The data in these studies were judged to be too limited to be used for this purpose.

Since cyclamate was used by large numbers of people only during the 1960s and was banned in the United States in 1969, data are insufficient to rule out an elevated risk associated with long-term use. In addition, the data are inadequate to rule out excess risk long after initial exposure. Because saccharin and cyclamate were often used together, it is impossible to separate the effects of cyclamate from saccharin in the epidemiological studies conducted to date.

RECOMMENDATIONS

- In countries where cyclamate is or has been used, epidemiological monitoring should be continued to determine whether the risk of human cancer is increased in heavy or long-term users or after a long period since first exposure. In such monitoring, consideration should be given to other cancer sites in addition to the bladder.

- The committee does not recommend further animal bioassays to test for the carcinogenicity of cyclamate by itself.

- The committee recommends repetition of one and, possibly, both studies in rodents that suggested a promotional or cocarcinogenic effect of cyclamate. Because the significance to humans of a positive outcome of such studies is uncertain, the committee also recommends that additional research be carried out to gain an understanding of the predictive value for human health of such results, and also that generic research be performed to develop properly validated, relevant systems for the assessment of cancer-promoting agents.

- Although many short-term tests have been conducted, there have been no assays for mammalian cell DNA damage and gene mutation for cyclamate and no DNA damage test for cyclohexylamine. The committee recommends that those tests be done. In addition, more definitive cytogenetic studies should be carried out.

- Evidence from metabolic studies does not indicate that potentially hazardous metabolites of cyclamate are formed in humans. The committee recommends, therefore, that no further studies on metabolism be conducted for the purpose of evaluating the carcinogenicity of cyclamate.

Chapter 2
Historical Perspective

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Historical Perspective

Cyclamate was accidentally discovered in 1937 by Michael Sveda, a University of Illinois graduate student at the time. This substance, which proved to be approximately 30 times sweeter than sugar, was first marketed in 1949 as sodium cyclamate in tablet form for use by diabetics. In 1953, a mixture of 10 parts cyclamate to 1 part saccharin largely replaced the cyclamate-only product, because taste panels had judged that combination to be more palatable. Apparently, the 10:1 cyclamate-saccharin mixture masked the bitter aftertaste that had been experienced by some people with the use of either product alone. In November 1959, cyclamate was placed on the Food and Drug Administration's (FDA) list of GRAS[1] (Generally Recognized as Safe) substances.

In the early 1960s, the FDA began to require testing of new substances for effects on reproduction, fertility, and teratogenicity. In accordance, a multigeneration study was initiated at a private laboratory, the Food and Drug Research Laboratory (FDRL), to investigate effects of cyclamate in rats. When it was discovered in 1966 that some humans metabolize a portion of ingested cyclamate into cyclohexylamine, the protocol for the study was changed to add cyclohexylamine to the diet of half the rats during the last 6 months of observation.

Before any results of the FDRL study were available, an Ad Hoc Committee on Nonnutritive Sweeteners was established in 1968 within the Food and Nutrition Board of the National Research Council to review the status of research on cyclamate. This group concluded that there was no evidence for carcinogenicity, but judged that only the studies in rats had been adequate (NRC, 1968). In 1969, the FDRL reported that an increased incidence of bladder tumors had been observed in both male and female rats at the high-dose level. The ad hoc committee reconvened and, on the basis of the FDRL study, concluded that the mixture of saccharin and cyclamate was carcinogenic

[1] GRAS refers to all food additives that because of years of widespread use in food are generally recognized by qualified experts as safe for their intended use (Wodicka, 1980).

(NRC, 1969). The committee reported, "Although ... the evidence strongly suggests that cyclamate or cyclohexylamine is the active substance, the most persuasive experiment was done using the cyclamate-saccharin mixture. There is, therefore, a slight possibility that saccharin is involved" (NRC, 1969).

Cyclamate was removed from the GRAS list in October 1969 and was limited to use as a therapeutic drug. In 1970 cyclamate was taken off the market completely after the Medical Advisory Group of the Department of Health, Education, and Welfare concluded that there was no valid evidence that cyclamate had therapeutic value in the treatment of obesity or diabetes (Egeberg _et al._, 1970).

In 1975 the National Cancer Institute (NCI) convened a committee to review the data on the carcinogenicity of cyclamate. This group examined evidence from human studies, animal studies, short-term tests, and _in vitro_ assays. To assess the animal studies, committee members visited key laboratories to validate the experimental methods. In its report of this study, the NCI committee concluded that "the present evidence does not establish the carcinogenicity of cyclamate or its principal metabolite, cyclohexylamine, in experimental animals" (NCI, 1976). It also expressed concern about the increased incidence of urinary tract cancers in some animal studies, but could not judge the meaning of these findings because the increases were not statistically significant. The NCI group noted several studies in which chromosome damage was found in human and rodent cells treated with cyclamate or cyclohexylamine.

The manufacturer, Abbott Laboratories, requested a formal hearing by the FDA, which was held in 1977. After written and oral testimony, an initial decision was issued in 1978 by the Administrative Law Judge, who ruled, "It has not been shown to a reasonable certainty that cyclamate does not cause cancer in man or animals" (FDA, 1980). This order was remanded to the Administrative Law Judge by FDA Commissioner Donald Kennedy in 1979, and the hearing was reopened. After further written and oral testimony, another decision was issued in February 1980. Again the judge ruled that cyclamate had not been demonstrated to be noncarcinogenic.

The final FDA decision on this series of hearings was published in the _Federal Register_ in September 1980 by Kennedy's successor, Jere Goyan. The commissioner also concluded, "The data ... do not establish to a reasonable certainty that cyclamate does not cause cancer" (FDA, 1980).

At the time cyclamate was banned in the United States, many other countries followed suit. Some countries continued the unrestricted use of cyclamate, however, and others enacted special restrictions on its sale or distribution, e.g., by requiring a medical prescription or

limiting sales to pharmacies (IARC, 1980). Since that time, several countries have lifted their ban on cyclamate and others are currently reviewing their position on its use (R. Haigh, Commission of the European Communities, personal communication, 1984).

In 1982, the FDA was once more petitioned for approval of cyclamate as a food additive. An internal Cancer Assessment Committee (CAC) appointed by the FDA again reviewed the data on cyclamate and drew a conclusion different from that reached by the commissioner (FDA, 1984). The CAC committee stated, "The collective weight of many experiments intending to discriminate between the carcinogenicity and non-carcinogenicity of cyclamate indicates that cyclamate is not carcinogenic." It is in this context of examination and reexamination that the Research Council was asked to evaluate the data on cyclamate.

USE OF ARTIFICIAL SWEETENERS IN THE UNITED STATES

Information on the consumption of cyclamate would be helpful in estimating artificial sweetener use during the period before cyclamate was banned and in estimating the levels of use that would be anticipated if cyclamate was again approved. Although it is known that artificial sweetener use has increased considerably in the United States since diet soft drinks were introduced in the 1950s, data on artificial sweetener consumption during the 1960s are limited. Unpublished data from the USDA Nationwide Food Consumption Survey indicate that 3% of the U.S. population had used artificially sweetened soft drinks during a 24-hour recall period in 1965 (NRC, 1978). No other data were available to the committee to permit comparison of sweetener consumption in the 1960s with later patterns for which considerable information is available.

The percentage of persons using artificial sweeteners in the form of saccharin during the 1970s was determined in two surveys conducted by the Market Research Corporation of America (MRCA, 1978a,b). Although these data are derived from saccharin use, they can be regarded as an indication of the extent to which artificial sweeteners in general are used, i.e., by about one-third of adults. Table 2-1 provides data on artificial sweeteners used by different age groups as determined by surveys conducted in 1972-1973 and 1977-1978 (MRCA, 1978a,b). In each age group except the elderly, the percentage of use increased between the two surveys. There also seemed to be an increase over time in the average amount consumed by each person. There was little variation in percentage of use by race, income, education, and region (Table 2-2), and the increase in use over time was relatively consistent within each of these categories. Since more than one artificial sweetener is currently being marketed, it would be difficult to predict the level of exposure to cyclamate if it were to be approved for use.

TABLE 2-1. Percentage of Adults Currently Using Saccharin by Age, 1972-1973 and 1977-1978[a]

Age Group, years	1972-1973 N	%	Mean Consumption, mg/day[b]	1977-1778[a] N	%	Mean Consumption, mg/day[b]
18-24	993	23	20.1	685	28	35.7
25-34	1,713	28	32.6	1,272	35	35.5
35-44	1,284	28	29.0	875	31	39.7
45-54	1,453	28	32.2	909	33	36.0
55-64	1,402	35	33.6	969	33	33.7
>65	1,342	33	31.1	1,239	33	30.9

[a] Based on two surveys conducted by Market Research Corporation of America (MRCA, 1978a,b). Includes both food and beverage sources.
[b] One artificially sweetened beverage contained approximately 125 mg of saccharin in 1975.

A different picture is given in Table 2-3, which is based on the foods reported in a 3-day recall period in the USDA's Nationwide Food Consumption Survey. Only information on diet beverages is given because tabletop artificial sweeteners were not included in the USDA data. The percentages here are much lower than in Tables 2-1 and 2-2, reflecting actual use over a 3-day period during the 1977-1978 survey rather than the percentage of the population that uses artificial sweeteners. These figures thus reflect frequency of use and reveal, when compared with the previous table, that there is a substantially higher frequency of use among women than among men and, thus, a much greater lifetime exposure. This difference between sexes becomes evident at about age 10. The data also indicate a substantial amount of use by children beginning in infancy (Pao _et al._, 1982).

In a study conducted by the American Cancer Society in 1982 on 1.2 million adult men and women, similar estimates were obtained, i.e., approximately one-third of adults used artificial sweeteners at that time (S. Stellman, American Cancer Society, personal communication, 1985). This study, although it was not based on a random sampling scheme, confirms that the levels of use for adults in 1977-1978 did not change substantially until 1982.

TABLE 2-2. Percentage Currently Using Saccharin, According to Selected Demographic Characteristics[a]

Demographic Characteristic	1972-1973 N	1972-1973 %	1977-1978 N	1977-1978 %
Race:				
White	3,271	29	2,647	35
Nonwhite	221	23	274	36
Income/year:				
<$4,000- $6,999	476	26	250	33
$ 7,000- $9,999	658	27	355	33
$10,000-$14,999	1,089	28	615	32
$15,000-$24,999	830	33	1,113	37
≥$25,000	149	29	446	37
Education:				
<9th grade	530	26	343	32
9th-12th grade	1,640	27	1,356	34
Some college	1,322	31	1,222	37
Region:				
Northeast	819	28	719	36
North central	1,007	26	813	33
South Atlantic	497	29	494	39
Mountain	161	28	111	30
Pacific	481	33	376	35

[a] Data from MRCA, 1978a,b. Includes both food and beverage sources.

It can thus be assumed that if cyclamate were again approved for use, exposure would occur primarily through its incorporation into existing or similar products. Therefore, recent patterns of artificial sweetener use provide a reasonable guide to expected future patterns of cyclamate consumption. Like other artificial sweeteners, they would probably again be used in diet drinks, in foods, and as tabletop sweeteners.

Diabetics are by far the heaviest users of artificial sweeteners. Market Facts, Inc. (1978) estimated that 91% of diabetics use tabletop artificial sweeteners and foods containing nonnutritive sweeteners.

These products are not restricted to use by diabetics, however, but are widely used by all major segments of society.

The marked increase in artificial sweetener consumption during the 1960s was due largely to the increased use of diet drinks, and until 1970 many of them contained cyclamate. Women generally consume more artificial sweeteners than do men (MRCA, 1978a,b). Past and current consumption patterns suggest that, if reintroduced, cyclamate would probably be used widely by large numbers of people. Thus, even if cyclamate were to exert only a weak carcinogenic effect, its use would alter risk in millions of people.

TABLE 2-3. Consumption of Artificially Sweetened Soft Drinks in a 30-Day Period, by Age and Sex [a]

Age Group, years	Sample Size	Sex	Percent Using
1	498	Both	0.4
1-2	1,045	Both	2.5
3-5	1,719	Both	3.8
6-8	1,841	Both	3.1
9-14	2,089	Male	3.0
	2,158	Female	4.7
15-18	1,394	Male	3.4
	1,473	Female	11.4
19-34	3,928	Male	5.2
	5,346	Female	15.7
35-64	4,929	Male	7.0
	7,069	Female	14.2
65-74	1,118	Male	2.2
	1,738	Female	5.1
≥75	536	Male	2.2
	993	Female	4.1

[a] Data from the USDA Nationwide Food Consumption Survey 1977-1978 (Pao *et al.*, 1982).

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Chapter 3

Metabolism

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Metabolism

Whether administered to animals or to humans as cyclamic acid or as a sodium or calcium salt, cyclamate (CHS) is largely excreted unchanged. A generally much smaller but highly variable amount is converted to cyclohexylamine (CHA) and thence to _cis_ and _trans_ 3- and 4-aminocyclohexanol, _trans_-cyclohexane-1,2-diol, cyclohexanone, and cyclohexanol. Some evidence indicates that it is also converted to _N_-hydroxycyclohexylamine (_N_-hydroxyCHA) and dicyclohexylamine (diCHA). However, not all metabolites have been observed in all species. In humans only cyclohexanol, cyclohexanone, and cyclohexane-1,2-diol have been found and diCHA and _N_-hydroxyCHA have been reported but not confirmed. No other products have yet been identified, and numerous experiments with ^{14}C-labeling have shown that it is not appreciably converted to carbon dioxide or other volatile products. The patterns of metabolism in humans are similar to those in the animals that have been studied.

CHEMICAL AND PHYSICAL PROPERTIES

The chemical and physical properties of cyclamate compounds and derivatives (cyclamic acid, sodium cyclamate, calcium cyclamate, cyclohexylamine, and dicyclohexylamine) are described in detail in a number of publications (e.g., IARC, 1980; National Formulary Board, 1970; NRC, 1966; Wade, 1977; and Windholz, 1983). Their chemical structure is illustrated in Figure 3-1.

In addition, IARC (1980) has provided information on impurities of cyclamate manufactured in the United States and abroad, and on their production, use, occurrence, and analysis. The metabolic products of cyclamate and the presumed order of their formation are shown in Figure 3-2.

ABSORPTION AND EXCRETION

Many studies with varied dosages and periods of administration have been conducted in human subjects. Results have shown that orally

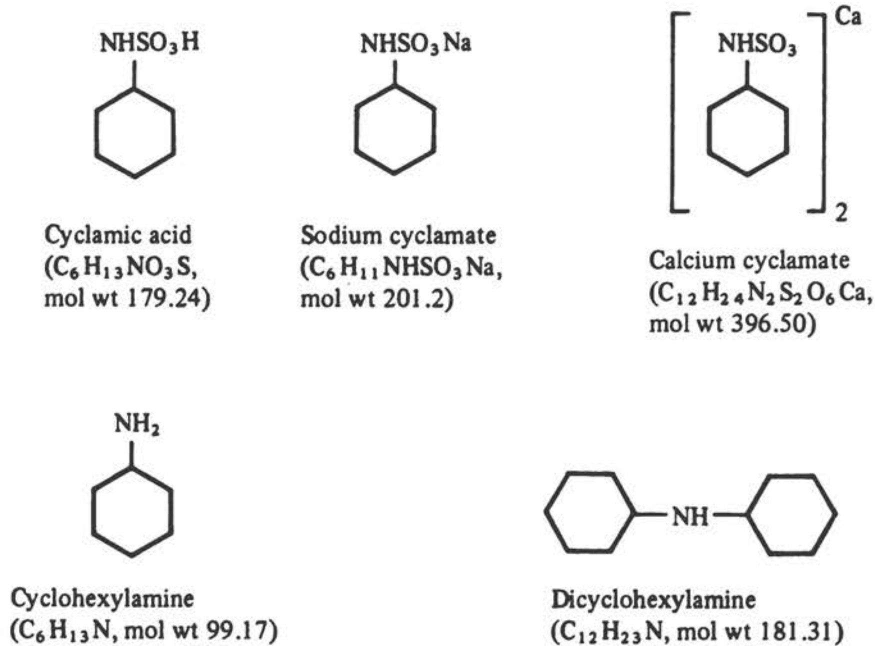

FIGURE 3-1. Chemical structure of cyclamate and related substances.

administered CHS is readily and rapidly excreted in both urine and feces, but the relative amounts can vary widely (Renwick and Williams, 1972a,b; Richards *et al.*, 1951; Smith, 1971; Williams, 1971; Wills *et al.*, 1981). After intravenous injection, clearance is very rapid; from 70% to 90% appears in the urine within 3 hours (Schoenberger *et al.*, 1953).

Absorption from the gut is incomplete, but total excretion in urine and feces is virtually complete in 1 to 2 days. Ninety-six hours after a 5-g oral dose of ^{14}C-labeled CHS was administered to two human subjects, total recovery was 98% and 102%, respectively, of which 52% and 74% was excreted as such in the urine (Sonders and Wiegand, 1967, 1968; Sonders *et al.*, 1967). Plasma CHS reached a peak of 20 mg/ml between 6 and 8 hours and declined with a half-life ($t_{1/2}$) of 8 hours. The $t_{1/2}$ of oral CHS in rats and dogs was 6.8 and 8 hours, respectively (Miller *et al.*, 1966).

In rats and dogs, more than 95% of an oral dose of ^{35}S-labeled CHS was excreted unchanged in the urine and feces (Taylor *et al.*, 1951). The balance had been distributed in very small amounts to all tissues except the brain; less than 0.5% was retained after 24 hours. Only 0.15% was converted to urinary ^{35}S sulfate. Miller *et al.* (1966) also found nearly complete excretion of oral CHS in the urine and feces of rats and dogs. Accumulation in tissues was negligible. Renwick and Williams (1972b) reported that 80% to 100% is excreted

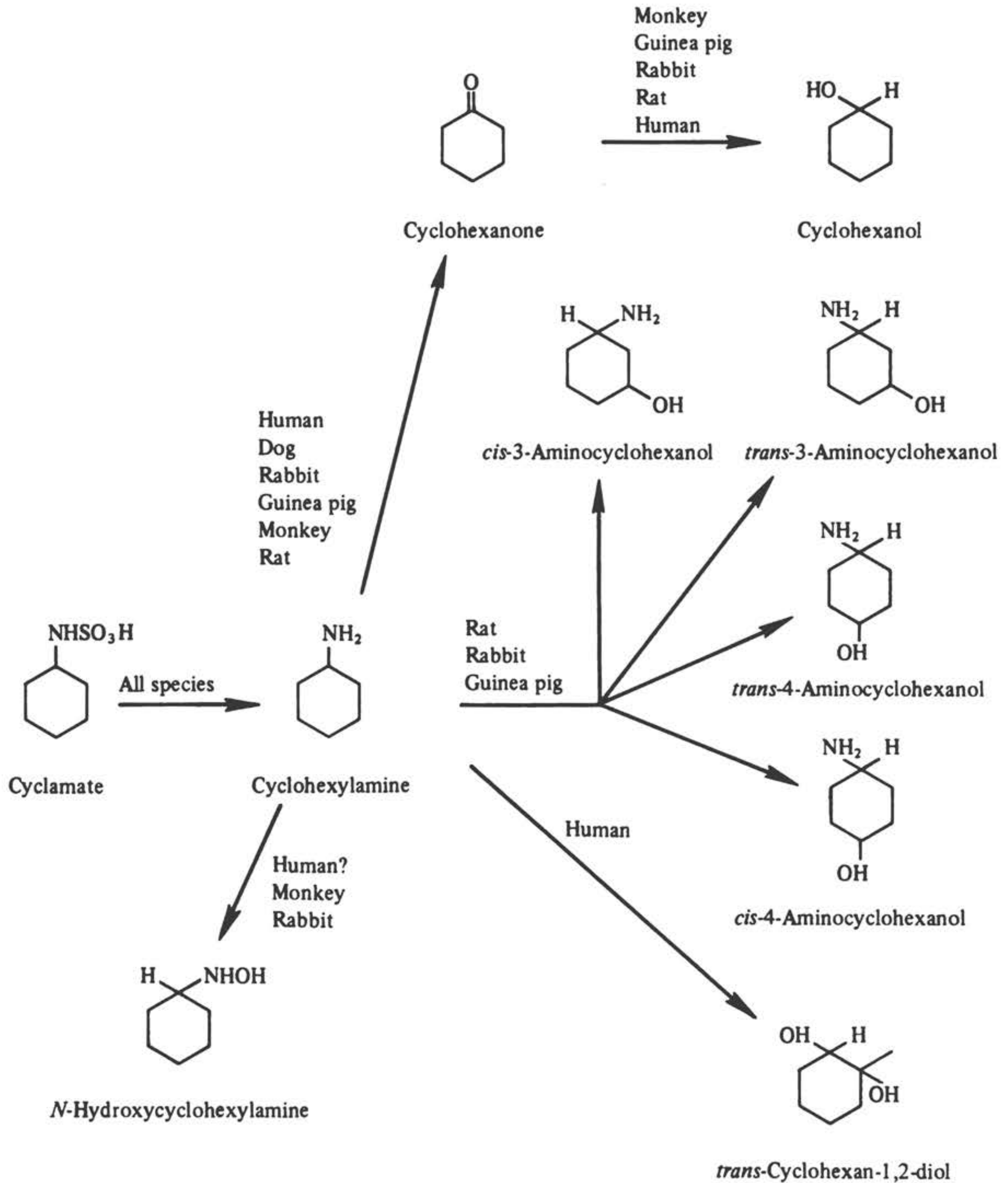

FIGURE 3-2. Products of cyclamate metabolism. Adapted from Renwick, 1983a.

from various species 2 to 3 days after administration. The percentages found in the feces and urine were 30% and 65%, respectively, in the guinea pig, 50% and 40% in the rat, 5% and 90% in the rabbit, 50% and 30% in one human, and 60% and 40% in another human. In rats, 46% of the CHS in the plasma was bound to plasma protein (Postorino and Estep, 1967); binding to bovine serum albumin has been observed *in vitro* (Kojima and Ichibagase, 1968a). By ligation of renal arteries, the volume of CHS distribution in rats was determined to be 0.57 liter/kg, corrected for protein binding. This value is approximately the same as total body water (Posterino and Estep, 1967).

Intravenously injected CHS equilibrates rapidly in plasma and milk of lactating rats (Ward and Zeman, 1971), dogs (Sonders and Wiegand, 1967, 1968), and pigs (Derse, 1971). CHS also traverses the placental barrier to a somewhat limited extent in early human pregnancy (Pitkin *et al*., 1969), in the pregnant rat (Schechter and Roth, 1971; Taylor *et al*., 1951), and in the pregnant rhesus monkey (Pitkin *et al*., 1969). Because of the rapid excretion, accumulation in organs, milk, blood, and fetus is generally extremely low. In humans, urinary excretion occurs both by glomerular filtration and tubular excretion (Schoenberger *et al*., 1953).

In six rabbits given an intravenous injection of sodium cyclamate (NaCHS) in a dose of 100 mg/kg body weight (bw), the urinary output over 24 hours ranged from 81.5% to 98.0% (mean, 91.8%). The half-life of 3.9 hours was not affected appreciably by simultaneous administration of caffeine, theophylline, theobromine, albumin, casein, or citric acid in doses ranging from 150 to 2,000 mg (Kojima *et al*., 1966a,b). Absorption from the isolated small intestine of the rat was rapid at pH 5 and 7 and was linear with time over 2 hours. It was not affected by the other substances administered. Absorption from the isolated rat stomach was rapid at pH 1.0, was nil at pH 3.0, and was enhanced by all the other substances administered.

CONVERSION TO CYCLOHEXYLAMINE

Hydrolysis of the sulfamide bond of CHS,

$$R\text{-}NHSO_3H + H_2O \longrightarrow RNH_2 + H_2SO_4,$$

yields CHA, which was first detected by Kojima and Ichibagase (1966) in the urine of a human subject given oral CHS. Although the amount was only 0.7% of the administered dose, this observation was the first evidence of CHS metabolism and stimulated a host of further confirmatory studies in humans and animals (Asahina *et al*., 1971; Davis *et al*., 1969; Glogner, 1970; Golberg *et al*., 1969; Hengstmann *et al*., 1971; Kojima and Ichibagase, 1966, 1968b, 1969; Kojima *et al*., 1966a,b; Litchfield and Swan, 1971; Pawan, 1970; Renwick and Williams, 1972a,b; Sonders, 1968; Sonders and Wiegand, 1967; Sonders *et al*.,

1969; Wills et al., 1981). CHA was absent from the urine of control subjects receiving no CHS (Renwick and Williams, 1972a).

A property common to all species studied was the wide range in the degree to which CHA was formed. Only a small percentage of humans converted CHS to CHA in appreciable quantities. Of those that were converters, i.e., those that excreted at least 0.2% of the administered dose, the degree of conversion ranged widely and tended to increase markedly with chronic ingestion, then fell off gradually after cessation of feeding. Although nonconverters or low converters generally maintained their status, there were frequent exceptions. For example, Sonders et al. (1969) fed 1.5 g of CHS to one subject each day for 14 days. During that time, urinary CHA rose from 1.4% to 41% of the daily dose and fecal CHA rose from 1.7% to 6.0% of the dose. In another subject, 30% of the dose was converted after 5 days on CHS, but dropped to 1.5% after 5 days of abstinence. Renwick and Williams (1972b) found that one human subject excreted as CHA 5.2% of a 1-g dose of CHS administered on 10 consecutive days, whereas two others excreted only 0.02% after 30 days of treatment. As the dose of CHS increases, the amount converted increases, but the percentage conversion decreases (Collings, 1971; Davis et al., 1969; Litchfield and Swan, 1971).

The accumulated data indicate that humans convert appreciable amounts of CHS to CHA only when CHS is given orally. The degree of conversion depends upon the individual, but not necessarily on the dosage and the duration of feeding.

Wills et al. (1981) have reported extensive data on CHS ingestion in 32 male inmates of a correctional institution given daily doses of up to 16 g for as long as 213 days. No untoward effects were observed, except for softening of the stool, diarrhea, frequent bowel movements, and some perianal irritation in subjects ingesting more than 5 g/day. These symptoms disappeared quickly when the dose was reduced. Diarrhea also occurred in rats fed diets containing 10% CHS (Wallace et al., 1970).

All subjects excreted some CHA at some time after administration, and the excretion was not affected either by basification with sodium bicarbonate or acidification with ammonium chloride (Wills et al., 1981). No changes were observed in hematocrit, in hemoglobin, in erythrocyte or white cell number, or in number and type of white blood cells. There were also no differences in routine blood chemistry, prothrombin time, glutamate-pyruvate aminotransferase, or protein-bound iodine. No significant changes were observed in sperm count or motility, in ratio of urinary amino nitrogen to creatinine (indicating proper kidney tubular functions), or in other parameters of kidney function. No changes were observed in total chromosomes or in the proportion of abnormal chromosomes in leukocytes cultured from the circulating blood. However, no data were provided by these authors (see Chapter 4).

Among 300 urine samples from 32 humans assayed for both CHS and CHA, 215 contained CHA. Of 148 samples from excreters of 0.25% of the administered dose or more, CHA concentration was as high as 75.4% of the total urinary CHA and CHS output. The average was 17.2% (Wills _et al._, 1981). In 55 urine samples, CHS was detected, but no traces of CHA were found. The concentration of CHA in the urine ranged from 0.03 to 3,490 mg/100 ml.

Five consistent excreters of CHA were given 3.3 g of CHS per day. In a single collection of urine during the second hour after the second dose of the day, samples from four of these subjects contained CHA in concentrations ranging from 0.8 to 5.8 mg/100 ml, but one sample contained 103 mg/100 ml (Wills _et al._, 1981).

The extreme range of conversion can be seen in Table 3-1. Over a 26-week period, one volunteer consuming 3 g of CHS per day excreted urine containing CHA in concentrations varying from 2 mg/100 ml to

TABLE 3-1. Urinary Excretion of CHS and CHA by a Man Receiving Three 1-g Doses Daily for 26 Weeks[a]

Weeks of CHS Administration	Urinary Excretion, mg/100 ml morning urine		
	CHA	CHS	Proportion of Urinary CHA to CHS, %
8	3	1,443	0.4
14	2	350	1.1
16	6	1,318	0.9
17	103	1,480	12.2
20	7	1,690	0.8
22	139	580	38.9
23	23	1,699	2.7
24	24	740	6.1
25	140	1,760	13.7
26	27	1,840	0.3

[a]Adapted from Wills _et al._, 1981.

140 mg/100 ml. The proportion of CHA to CHS in the urine ranged from 0.3% to 38.9% (Wills et al., 1981). Despite the large chronic daily dosages, conversion to CHA was low and variable in this long-term study.

Among 141 subjects given 0.5 g of NaCHS per day for 4 days, Collings (1971) found that CHA excretion was less than 0.15% of the administered NaCHS dose in 105 subjects (including the only three children in the study), 0.15% to 1.0% in 14 subjects, 1% to 20% in 15 subjects, 20% to 60% in 5 subjects, and more than 60% in 2 subjects. Thus conversion was 1% or less in 84% of the subjects tested.

Davis et al. (1969) administered 1 to 3 g of CHS daily to 11 subjects and found that maximal CHA excretion ranged from 0.04 mg to 154 mg. Leahy et al. (1967) measured CHA excretion in 35 subjects given 1 g of NaCHS daily for 3 days. The urine samples collected during the subsequent 3 days indicated that only four people excreted from 0.2% to 3.1% of the administered dose as NaCHA; the others excreted no detectable CHA. Litchfield and Swan (1971) screened 69 subjects given 2 g of NaCHS for 7 or 8 days. The only eight converters found in this group excreted from 0.26% to 1.5% CHA. The others excreted less than 0.1%, the limit of detection. Only five of the eight were converters in a repeated experiment (Leahy et al., 1967).

In several studies of acute exposures, Pawan (1970) reported CHA excretions ranging from 0.4 to 2.6 mg in the 8-hour urines of only 9 of 104 volunteers receiving 45 mg of NaCHS per kilogram of body weight. None was present in urine of the 95 others. In another group of 52 subjects receiving 50 mg/kg bw, CHA was present in eight samples in amounts ranging from 0.5 to 4.6 mg, but was absent from the other 44. Ten volunteers, including three excreters, were given 1 g of NaCHS per day for 38 days. Peak urinary excretion of 7 to 8 mg over a 24-hour period occurred by 9 days, but only in the excreters; it plateaued at approximately 6 mg. Of the seven nonexcreters, three sporadically excreted 0.6 to 1.7 mg, whereas the other four subjects had no detectable excretion over the 28-day period.

Renwick and Williams (1972b) administered calcium cyclamate (CaCHS) to three volunteers at a dosage of 3 g/day for 17 to 30 days. Each subject was then given a single dose of 1 g of ^{14}C-CaCHS. In each case 96% to 99% of the radioactivity was recovered in urine and feces. One of the subjects excreted 8% of the ^{14}C-labeled dose as CHA over the next 4 days after the 17 or 30 days, whereas the two others excreted only 0.02%.

In four separate studies, Sonders and Wiegand (1967) found that urinary CHA excretion after CHS ingestion was generally low in the subjects, except for a small number of excreters. In the first study, 13 subjects were given a 5-g single dose of NaCHS. In the urine samples collected over the next 2 days, CHA was found for only one of these

subjects as 2.1% of the administered dose. In a similar experiment extending over 9 days, there were no CHA excreters. In a third study, the one excreter ingesting 3 g/day for 15 days excreted increasing amounts of CHA, reaching 41.4% on the fourth day, while 23% was excreted in the stool. In the fourth study, 84 subjects were given 3 g/day for 4 days. Urinary assay for CHA on the fourth day revealed that 71 (or 85% of the subjects) excreted less than 0.02%, 9 (or 11%) excreted between 0.02% and 0.38%, and 4 excreted 1.15%, 14.8%, 38.0%, and 42.6%.

Renwick (1983b) summarized data on metabolism of CHS to CHA in humans. Of 480 subjects who received a single oral dose of CHS, 76 (or 16%) excreted measurable amounts of CHA in the urine during the subsequent 25 hours. A more reliable figure for CHS metabolism was obtained by assaying the urine of subjects who received CHS regularly for at least 3 days. The 219 subjects studied in this way exhibited a wide range of CHA excretion. As shown in Figure 3-3, approximately 76% excreted 0.1% or less of the CHS ingested, and less than 10% excreted 1% or more. Only about 4% excreted 20% or more. Wide

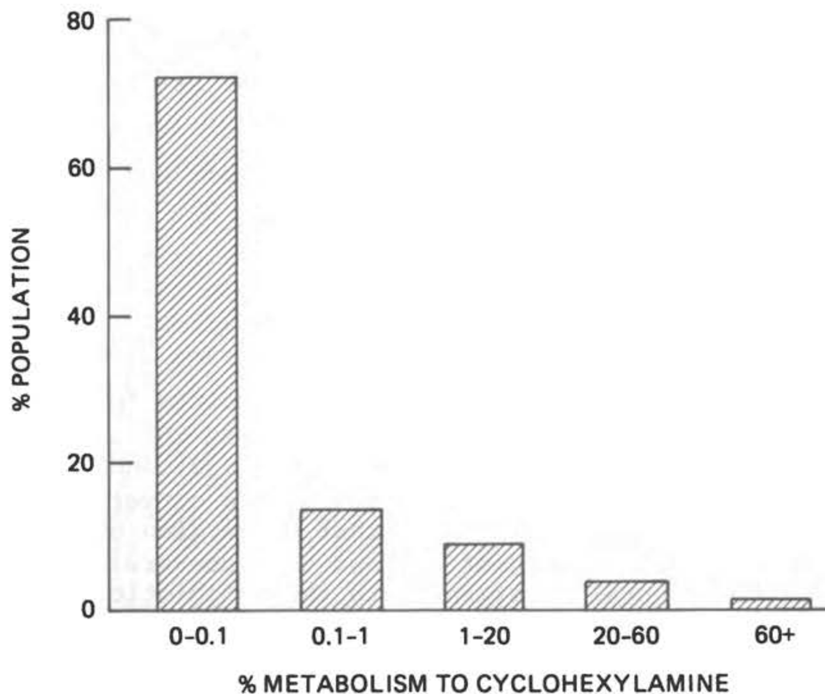

FIGURE 3-3. The distribution of cyclamate-metabolizing ability in 219 humans receiving cyclamate regularly for at least 3 days. From Renwick, 1983a,b.

variability has also been observed among 50 Japanese subjects regularly using CHS as a food additive (Asahina et al., 1971).

From these data it is clear that conversion to CHA is generally low in most individuals, but highly variable in those that do convert. Although convertibility generally requires chronic feeding of CHS, the duration of administration does not appear to affect the conversion capability.

In a study of CHS metabolism in 255 patients suffering from diabetes or obesity, Hengstmann (1971) found that during 2 days after oral administration of 1 g of NaCHS, the urinary excretion of CHA was less than 100 μg in 65% of the subjects and between 1 and 100 mg in 17% of them. Eleven percent excreted between 1 and 10 mg, and 8% excreted more than 10 mg. Thus only 8% of these diabetic subjects excreted more than 1% of the dose as CHA.

In a study of children belonging to employees of Abbott Laboratories, 24 boys and 25 girls aged 3 to 18 years were given 507 mg of CaCHS in a soft drink daily for 4 days, after which 24-hour urine samples were collected and assayed (Sonders, 1968). Four (8%) of the children converted trace amounts, 1.4%, 8.8%, and 21.6% of CHS to CHA; the others exhibited no urinary CHA. Although one girl converter was the daughter of a converter, three of her siblings were nonconverters. A 10-year-old boy was a converter, but his 5-year-old sister was not. The presence of 8% converters among the children and the wide range of activity are comparable with the results of studies in adult populations.

In summary, these studies indicate that in most healthy humans ingesting 5.0 g of CHS or less daily, the conversion of CHS to CHA is very low or negligible.

THE INTESTINE AS THE CONVERSION SITE

The overwhelming preponderance of evidence points to the microflora of the colon and cecum as the site where CHS is hydrolized to CHA (Renwick, 1977). Although Davis et al. (1969) and Leahy et al. (1967) were unable to establish the gut flora as a conversion site, Drasar et al. (1972) later demonstrated that conversion occurs in feces of men who chronically ingest CHS and that conversion in vivo is markedly suppressed by oral treatment with the antibiotic ampicillin (Collings, 1971). Drasar et al. (1972) found that three samples of feces from three subjects unable to convert CHS to CHA failed to yield CHA when incubated anaerobically with CHS. When one of the subjects became an active converter after chronic CHS ingestion, however, his feces were active in forming CHA from CHS.

Much of our detailed knowledge of CHS metabolism has been derived from experiments in rats. A number of investigators have shown that

under various conditions nearly 100% of administered oral CHS dosages is excreted unchanged in the urine and feces in an approximate ratio of 1 to 3. Only a fraction of a percent of labeled CHS is retained after 5 days (Klaverkamp and Dixon, 1969; Miller et al., 1966; Renwick, 1977; Renwick and Williams, 1972a,b; Sonders et al., 1968b; Taylor et al., 1951; Wallace et al., 1970; Ward and Zeman, 1971). The half-life for excretion after oral ingestion is approximately 6.6 hours (Miller et al., 1966). Only about 0.04% is excreted in the bile (Wallace et al., 1970).

As with humans, some rats are converters and others produce little or no CHA from oral CHS (Collings, 1971; Dalderup et al., 1970; Kojima and Ichibagase, 1966; Oser et al., 1968; Renwick and Williams, 1972a,b; Sonders et al., 1968a,b, 1969; Wallace et al., 1970). This trait persists but with much variability through repeated tests. Again, as in humans, however, the conversion can be greatly increased (up to 62% of the dose) by chronic dietary inclusion of CHS and lowered after its removal (Bickel et al., 1974; Renwick and Williams, 1972a,b; Sonders et al., 1969). Several detailed studies described below are representative of the many investigations on CHS metabolism in the rat.

Wallace et al. (1970) studied the excretion and conversion of ^{14}C-labeled CHS in rats that had been fed CHS in the diet for a year or more. The labeled compound was administered in three levels: 0.4%, 2.0%, and 10.0% of the diet. Both the sodium and calcium salts were given to both male and female rats. Urine and feces were collected each day for 3 days. For all three levels of both salts and for both sexes, more than 95% was excreted--approximately one-third in the urine and two-thirds in the feces.

Of 63 rats on CHS for at least 1 year and given the labeled material, 12 excreted from 1% to 38% as CHA in the urine, 18 excreted 0.1% to 1.0%, and 22 excreted less than 0.1%. As a striking example of conversion variability, one rat converted 38% the first week, but only 0.33% 9 weeks later. Another rat converted 8% the first week and 29% 9 weeks later.

The bile is not an important route of excretion for CHS, since less than 0.05% of the ^{14}C-labeled CHS dose and no CHA appeared in the bile of bile-duct cannulated rats. Taylor et al. (1951) found less than 0.5% of ^{35}S-CHS in the bile of dogs given oral doses.

A detailed and informative study was conducted by Bickel et al. (1974), who followed CHA formation in four groups of six rats each and one group of two rats for 6 to 15 months. Each rat received 100 mg of CHS daily in drinking water, and urine was assayed periodically for CHA. Of the 26 rats studied, none metabolized more than 0.2% of a single oral dose of CHS. More than half was excreted as CHS in the feces. However, 24 of the animals became converters within 1 to 7 months, excreting from 1% to 70% of the dose as CHA. Ability to

convert was lost within 2 weeks after withdrawal of CHS, but was regained within 1 week after resumption of CHS feeding. The addition of the antibiotics neomycin or gentamycin to the drinking water inhibited CHA formation, and the ability to convert required 10 to 58 days after stopping antibiotic treatment, depending on the dose of antibiotic. Incubation of feces with CHS yielded CHA. This also was prevented by low doses of neomycin, gentamycin, or polymixin, consistent with the activity of gram-negative species.

Intragastric administration of feces from converters to normal rats resulted in conversion in 1 to 2 weeks, but not if feces from normal or preconverter rats were used, or if feces from converter rats were heated to destroy the microflora. The fecal microflora did not change with respect to _Clostridium_, _Enterococcus_, _Lactobacillus_, _Bacteriaceae_, _Escherichia coli_, and _Proteus_ content during the long-term course from nonconverter to converter. In converter rats given intraperitoneal injections of ^{14}C-labeled CHS, negligible conversion occurred.

In nonconverter rats given a single 2.5-g dose of CHS per kilogram of body weight, accumulation of CHS in tissues 24 hours later ranged from 8 µg/g in the lung to 91 µg/g in the small intestine, with 10 µg/g in the liver, 28 µg/g in the kidney, 35 µg/g in the subcutaneous and abdominal fat, and 55 µg/g in the adrenal glands. In two converter rats after long-term chronic treatment, approximately 8% of a daily dose of CHS was present in the tissues, 41.4% in the urine, 8.2% in the feces, and 13.0% in the gut contents. CHA was present at 10% in the tissues, 63.1% in the urine, 0.4% in the feces, and 0.5% in the gut contents. The amount of CHA in the individual tissues ranged from 28 µg/g in the liver to 178 µg/g in the kidney. Evidently there is little chronic buildup of either CHS or CHA in the tissues during a long period of daily CHS administration.

Additional experiments in rats confirm the conversion site as the gastrointestinal tract. After 10 months on a 5% CHS diet, two rats excreted 39% and 62% of the compound in the urine as CHA (Sonders _et al_., 1969). When given intraperitoneally, however, from none to only 2% was converted to urinary CHA. Intravenous CHS also was excreted quantitatively unchanged in the urine of high converters of oral CHS (Sonders _et al_., 1969). When high converter rats were given neomycin for 3 or 4 days, the yield of CHA was markedly reduced (Bickel _et al_., 1974; Sonders _et al_., 1969).

Tesoriero and Roxon (1975) found that bacteria isolated from feces of converter rats but not of nonconverters metabolized CHS to CHA _in vitro_. Using ^{35}S-labeled CHS they found that approximately 2% of the sulfate moiety is reduced and incorporated into the bacterial protein within 50 hours. Incorporation of ^{35}S is inhibited by cysteine in the medium, presumably through the inhibition of its assimilation by sulfate-metabolizing anaerobes. The ^{35}S was present in cysteine and methionine. A significant portion, up to 11%, was

converted to volatile material absorbed by sodium hydroxide, but this substance was not identified.

When nonconverters are caged together with converters, they become converters (Collings, 1971; Dalderup et al., 1970), presumably by exchanging intestinal microflora through coprophagia. Intestinal contents of converters form CHA from CHS under anaerobic conditions (Collings, 1971; Drasar et al., 1971, 1972). Clostridia, the apparently responsible organisms, multiply rapidly upon chronic oral CHS feeding (Drasar et al., 1971, 1972).

In dogs, the pattern of CHS metabolism is similar with regard to rapidity and routes of its excretion and conversion to CHA (Golberg et al., 1969; Kojima and Ichibagase, 1966; Miller et al., 1966; Taylor et al., 1951). Conversion in canine intestinal contents incubated anaerobically in vitro has been attributed to Clostridium perfringens (Golberg et al., 1969).

In general, guinea pigs are low converters, but they respond somewhat to chronic oral administration of CHS (Asahina et al., 1972a,b; Drasar et al., 1972; Renwick and Williams, 1972b). Excretion decreases upon the feeding of antibiotics or by intraperitoneal injection of CHS.

Fifteen strains of Pseudomonas and Corynebacterium isolated from the feces of converter guinea pigs had assimilated CHS, even when they had been grown with CHS as their sole source of carbon and nitrogen (Asahina, 1972; Asahina et al., 1972a,b). When these microorganisms were fed to guinea pigs in drinking water, conversion was promoted. CHA injected directly into the cecum of guinea pigs was absorbed readily and excreted in the urine. One of the Pseudomonas strains served as the source of a purified sulfamatase--an enzyme that converts CHS to CHA and also hydrolyzes other sulfamates with 3 to 8 carbons (Niimura et al., 1974).

Rabbits also follow similar patterns of CHS metabolism, namely, rapid excretion of oral CHS (Audrieth and Sveda, 1944; Kojima et al., 1966a), rapid clearance of intravenously injected CHS with a half-life of 3.9 hours (Kojima et al., 1966a), and variable conversion to CHA accompanied by trace amounts of cyclohexanol and cyclohexanone (Ichibagase et al., 1972; Kojima and Ichibagase, 1968a,b; Kojima et al., 1966a,b; Renwick and Williams, 1972b). In accord with the weight of other evidence, the wide variation in CHA conversion occurs in the cecum and colon of the rabbit and is associated with Clostridium and Enterobacterium (Drasar et al., 1972; Matsui et al., 1980). According to Kojima and colleagues, however, the low response to chronic CHS administration in rabbits was not affected by antibiotics (Ichibagase et al., 1972; Kojima and Ichibagase, 1968b; Suenaga et al., 1972). They provided evidence to suggest that conversion occurs in the liver.

Kojima and Ichibagase (1968b) and Ichibagase et al. (1972) reported that liver homogenates from converter rabbits and, to a lesser extent, converter rats are able to convert CHS to CHA and to produce trace amounts of cyclohexanone and cyclohexanol. However, these data were reported in extremely sketchy fashion and without appropriate control data. Wallace et al. (1970) gave ^{14}C-labeled CHS to 11 good converter rats with ligated urethras and cannulated bile ducts. The collected bile contained only 0.01% to 0.11% of the administered dose (mean, 0.04%). The authors also stated, without experimental details, that various rat tissues incubated for 0.2 and 4 hours with ^{14}C-labeled CHS exhibited no evidence of CHA formation either by radioactivity or paper chromatography; only a single band characteristic of CHS was observed. The authors also reported that there was no volatile radioactivity and that no $^{14}CO_2$ was released by rats given ^{14}C-labeled CHS. Other investigators were unable to produce in vitro conversion in the tissues of rats (Drasar et al., 1972; Tokieda et al., 1979), guinea pigs (Asahina, 1972; Asahina et al., 1972a,b; Drasar et al., 1972), rabbits (Drasar et al., 1972), or in perfused rat liver (Prosky and O'Dell, 1971).

The rhesus monkey is also reported to be a converter, but after long-term CHS feeding, only 0.1% to 0.2% of the oral dose was converted to CHA, and only trace amounts to cyclohexanol, cyclohexanone, and N-hydroxyCHA (Parekh et al., 1970). Monkey feces contain microbial strains that assimilate CHS and convert it to CHA (Hayashi et al., 1973; Matsui et al., 1980).

In a recent study of CHS conversion in 12 male monkeys (rhesus, cynomolgus, and African green) given single CHS doses ranging from 100 to 500 mg/kg, Bopp and Patterson (1985) observed a pattern of urinary excretion similar to that of rats and humans. One monkey excreted none, five excreted less than 0.1%, and another five excreted between 0.1% and 1%. Only one monkey excreted 3%. After six consecutive daily doses, three monkeys still converted less than 0.1% and three converted less than 1%. Two converted 2.5%, and two others excreted 13.5% and 36.6%.

The pig also converts oral (but not subcutaneous) CHS to CHA, and the conversion is reduced by administering antibiotics. The contents of a pig's colon were also found to convert CHS in vitro (Collings, 1971).

OTHER METABOLITES OF CYCLAMATE

Using gas-liquid chromatography, Kojima and Ichibagase (1968b) identified low levels of cyclohexanone and cyclohexanol in the accumulated urines of rabbits and rats fed CHS for 7 days. In a

subsequent paper, Kojima and Ichibagase (1969) identified these two deaminated products in 24-hour urines of three men and two women, who voluntarily ingested 2 g of NaCHS. CHA excretion ranged from 40 mg to 10.8 mg, whereas cyclohexanol and cyclohexanone were all less than 216 μg, equivalent to approximately 0.02% of the dose. Most prevalent was conjugated cyclohexanol, which ranged from 1,710 to 5,140 μg or from 0.3% to 0.9% of the dose. Trace amounts of cyclohexanol in addition to CHA have also been detected in the urine of two human converters who ingested single oral 5-g doses of ^{14}C-labeled CHS (Sonders and Wiegand, 1968; Sonders *et al.*, 1968a).

Trace quantities of these deaminated products were observed to accompany CHA formation in the urines of guinea pigs (Asahina *et al.*, 1972b), monkeys (Parekh *et al.*, 1970), and men (Golberg *et al.*, 1969). After oral ingestion of CHS, *N*-hydroxyCHA has also been found in very small amounts in the urine of men (Golberg *et al.*, 1969) and rabbits (Elliott *et al.*, 1968), but not in the rat (Prosky and O'Dell, 1971).

DiCHA has been reported as a trace CHS metabolite in the rat (Prosky and O'Dell, 1971; Suenaga *et al.*, 1972) and in the rabbit (Suenaga *et al.*, 1983), but it was not found by Oser *et al.* (1968) in the rat or by Leahy *et al.* (1967) or Sonders *et al.* (1967) in the urine of humans. Its absence from urine may not signify absence of formation, however, since Suenaga *et al.* (1983) found that diCHA administered orally to rats and rabbits or injected directly into the small intestine is rapidly absorbed but that only 0.04% is excreted by the rat and only 0.14% by the rabbit. The same low yield of fecal or urinary diCHA occurred after intravenous injection. These results suggest that this metabolite is transformed *in vivo*. This was confirmed by experiments in which diCHA was incubated *in vitro* with the microsomal fraction (10,000 x g supernatant) of rabbit and rat liver. Aerobically, diCHA disappeared rapidly in the rabbit preparations, but much more slowly in those of rat liver. No metabolism occurred anaerobically. These findings leave open the possibility that diCHA may be formed from CHS and further metabolized in higher amounts than might be anticipated from its extremely low excretion after CHS ingestion.

The toxicity and carcinogenicity of diCHA have been reported (Pliss, 1958), and *N*-hydroxyCHA is structurally similar to certain activated carcinogens (Miller and Miller, 1977). The carcinogenicity of diCHA is questioned by the committee in Chapter 5. Renwick failed to find a trace of either compound in the urine of humans who had consumed CHS during an extensive search using various techniques of chromatography, radioactivity labeling and carrier addition, and gas-liquid chromatography (A. G. Renwick, University of Southampton, personal communication, 1984). Renwick (1983a) pointed out that the CHS used in the earlier experiments might have contained diCHA sulfamate as an impurity.

METABOLISM OF CYCLOHEXYLAMINE

CHA administered orally to humans is rapidly absorbed and excreted. Its plasma $t_{1/2}$ is 4 to 5 hours, and peak blood levels of 0.08 to 0.1 µg/ml are reached 1 to 2 hours after feeding (Sonders *et al.*, 1968a). Approximately 88% to 97% appears in the urine by 48 hours; 98% of this amount is unchanged CHA. Only about 2% is excreted in the feces. At a plasma concentration of 1.7 µg/ml, only 8% was bound to protein.

According to Renwick and Williams (1972a), ^{14}C-labeled CHA fed to humans at doses of either 25 mg or 200 mg was rapidly excreted nearly entirely unchanged in the urine within 24 hours and only 1% appeared in the feces. In humans, therefore, urinary excretion must account for nearly all CHA formed from CHS. Only 1% to 2% is converted to two products--0.2% cyclohexanol and 1.4% *trans*-cyclohexane-1,2-diol. These are excreted in both free and conjugated forms. Metabolites have been sought and identified by radioactivity after carrier addition and purification, paper chromatography and mass spectrometry, gas-liquid chromatography and mass spectrometry, and color reactions. No *N*-hydroxyCHA was detected. Rats, rabbits, and guinea pigs fed proportionately much larger doses (50 to 500 mg/kg bw) also excreted nearly all the compound in the urine; only 1% to 7% was present in the feces. Approximately 4% to 5% is metabolized in the rat and guinea pig, but 30% is converted to other products in the rabbit. Five rat metabolites were identified in the 24-hour urine: cyclohexanol, 0.05%; *trans*-3-aminocyclohexanol, 2.2%; *cis*-4-aminocyclohexanol, 1.7%; *trans*-4-aminocyclohexanol, 0.5%; and *cis*-3-aminocyclohexanol, 0.1%. Eight metabolites were identified in the rabbit: cyclohexanol, 9.3%; *trans*-cyclohexane-1,2-diol, 4.7%; cyclohexanone, 0.2%; *trans*-3-aminocyclohexanol, 11.3%; *cis*-3-aminocyclohexanol, 0.6%; *trans*-4-aminocyclohexanol, 0.4%; and *cis*-4-aminocyclohexanol, 0.2%. *N*-hydroxyCHA was also found to account for 0.2% of the dose by carrier addition and crystallization to constant specific activity, by infrared spectroscopy, and by gas-liquid chromatography. In the guinea pig, six urinary metabolites were identified: cyclohexanol, 0.5%; *trans*-cyclohexane-1,2-diol, 2.5%; *trans*-3-aminocyclohexanol, 1.2%; *cis*-3-aminocyclohexanol, 0.2%; *trans*-4-aminocyclohexanol, 0.2%; and *cis*-4-aminocyclohexanol, 0.2%. The hydroxy metabolites were excreted in part as glucuronides.

The only reaction observed in humans is oxidative deamination, but all other species form amino alcohols. The authors (Renwick and Williams, 1972a) suggest that this may be due simply to our inability to detect the small amounts that would be formed by the much lower dose of CHS ingested by humans, i.e., 3 mg/kg bw. If amino alcohols are formed by humans, the amount produced must be too small to detect.

According to Collings (1971), 23 mg of CHA was rapidly and nearly completely excreted by the rat within 16 hours after oral administration. The blood levels of CHA reached a peak of approximately 12

μg/ml in about 20 minutes and decreased to 2 μg/ml in 2 hours. The author pointed out, however, that CHA formed by the metabolism of CHS in the gut would enter the blood at a relatively slow, steady rate without the marked peak in concentration observed after the oral administration of CHA itself.

Litchfield and Swan (1971) fed 5 g of NaCHS to human converters who produced CHA in amounts ranging from 0.1% to 0.9% of the daily intake for 7 or 8 days. However, they were unable to detect CHA in the blood at a detection limit of 0.1 μg/ml. It appears unlikely therefore that a significant buildup of CHA could occur in human users ingesting anticipated dietary levels of CHS.

A number of reports have indicated that ^{14}C-labeled CHA orally administered to dogs at 5 mg/kg bw was excreted nearly completely as such in the urine. Its $t_{1/2}$ was 3 hours, and a peak plasma concentration of 1.7 μg/ml was reached 1 hour after dosing (Estep, 1967; Estep and Wiegand, 1967; Sonders, 1969; Sonders and Wiegand, 1968; Sonders et al., 1968a). Eight hundred micrograms of cyclohexanol and 200 μg of cyclohexanone were produced in the urine. Pitkin et al. (1970) observed the same pattern in rats fed ^{14}C-labeled CHA in doses of 54 mg/kg bw. Radioactivity in volatile matter and respired carbon dioxide were negligible. Plasma concentration peaked in 1 hour at 0.5 to 0.7 μg/ml, and 17% of the administered dose was bound to plasma proteins. In both dogs and rats, unknown urinary products were detected by gas-liquid chromatography. These products accounted for approximately 17% of the administered dose of CHA in dogs and 1% in rats.

These results indicate that CHA is absorbed and excreted extremely rapidly, presumably from its site of formation in the colon and cecum. This has been confirmed by direct insertion of CHA in the intestine of rats (Elliott et al., 1968) and guinea pigs (Asahina, 1972). Rapid excretion was also demonstrated by intravenous injection of ^{14}C-labeled CHA. In rats, the ^{14}C was rapidly distributed throughout the tissues, the highest concentrations collecting in the liver, kidney, spleen, and lung (Drasar et al., 1972; Sonders et al., 1968a). The possibility of CHA retention has been raised by Leighty and Fentiman (1983), who found that in vitro rat liver microsomes fortified with coenzyme A formed an amide linkage with the nitrogen of CHA and also conjugated with both the nitrogen and oxygen of N-hydroxyCHA. The conjugates were identified by mass spectrometric and gas chromatographic comparison with the synthetic substances. In view of the rapid and nearly quantitative excretion of injected CHA, however, such conjugates are not likely to be formed in appreciable quantity or are hydrolyzed rapidly.

After feeding ^{14}C-labeled CHA to rabbits, Elliott et al. (1968) identified 0.2% of the dose as N-hydroxyCHA in the urine and 2.5% as cyclohexanone oxime, which they regarded as an artifact arising from the glucuronide of N-hydroxyCHA during the hydrolysis. However, the

oxime was also detected during oxidative deamination of CHA by rabbit liver microsomes (Kurebayashi et al., 1979). Approximately 0.5% of the initial radioactivity was respired: 0.3% as carbon dioxide and 0.2% as alcohol-soluble volatile material.

Hengstmann et al. (1971) administered CHA orally in doses of 2.5, 5.0, and 10.0 mg/kg bw to 11 normal humans and observed rapid absorption, reaching peak blood levels at 90 minutes. This was followed by a rapid decline at a rate of 0.026 to 0.037 mg/minute, corresponding to a $t_{1/2}$ of 206 to 288 minutes. More than 95% was excreted as CHA, and neither cyclohexanol, cyclohexanone, nor N-hydroxyCHA was detected.

Pitkin et al. (1969) studied the transfer of CHS and CHA across the hemochorial placenta of four rhesus monkeys during the last trimester of pregnancy (140th to the 160th day of gestation) to assess a possible risk during pregnancy. ^{14}C-labeled CHS (4 mg) and CHA (10 mg) were infused into the internal antecubital veins of two animals over 110 minutes and two animals over 180 minutes. Radioactivity of ^{14}C-labeled CHS peaked in maternal blood at about 60 minutes and dropped by 75% in 140 minutes, whereas fetal blood peaked at about 110 minutes at only 25% of the maternal blood peak, then dropped to approximately 75% by 140 minutes. Radioactivity in the amniotic fluid rose slowly and at 110 minutes was only about 5% of that in the maternal blood. In contrast, CHA radioactivity in maternal and fetal bloods rose identically, reaching a peak at 180 to 190 minutes and dropping by approximately one-third in 230 minutes. Activity in amniotic fluid was low, plateauing within 10 minutes at approximately 15% of the peak activity in the maternal and fetal blood. Thus, although both substances could traverse the placental barrier, CHS transmission was limited, whereas CHA diffused freely. The pathways of CHA metabolism are shown in Figure 3-2.

SITE OF CYCLOHEXYLAMINE METABOLISM

The site of CHA metabolism is not entirely certain. Ichibagase et al. (1972) found that rabbit and rat liver homogenates convert CHA to cyclohexanol and cyclohexanone. In the rabbit liver preparation, the conversion rate is high, increasing two- to threefold upon prolonged chronic feeding of CHA. In rat liver, however, the conversion rate is extremely low and is not affected by chronic CHA administration. Kurebayashi et al. (1979) reported that in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen, rabbit liver microsomes oxidatively deaminated CHA to cyclohexanone, presumably by a cytochrome P450-dependent monooxygenase. This system also reduces cyclohexanone to cyclohexanol. The cyclohexanone oxime detected by these investigators was assumed to be the primary oxidation product.

On the other hand, Tokieda *et al.* (1979) reported that urinary excretion of cyclohexanol by rabbits fed CHA is suppressed by oral administration of neomycin or metronidazole. When the cecal contents of rabbits chronically exposed to CHA in drinking water were incubated with CHA, both cyclohexanone and cyclohexanol accumulated. Furthermore, cyclohexanone was converted to cyclohexanol and other products not identified, and various isolated and cultured cecal microbial species, anaerobes, and facultative anaerobes possessed the ability to deaminate CHA or to desulfate CHS.

EFFECTS OF CYCLAMATE ON BIOCHEMICAL SYSTEMS

In view of the strong current interest in growth factors as putative products of oncogenes (Hunter, 1984), it is noteworthy that CHS as well as saccharin are reported to inhibit both the binding of the nerve growth factor to embryonic chick sensory ganglia cells and the outgrowth of dendrites in a dose-dependent manner (Ishii, 1982a,b). These effects, according to the author, are shared by tumor promoters and are presumed to support a suspected promoter action of these sweeteners.

Lee (1981a,b) found that saccharin and CHS inhibited binding of the epidermal growth factor (EGF) to 18 cell lines of various species--an effect shared by tetradecanoylphorbol-13-acetate (TPA). However, he cautioned against the assumption that TPA and the sweeteners act by a common mechanism and the corollary assumption that the latter are tumor promoters. He pointed out that the receptor for TPA is distinct from that for the sweeteners, which inhibit EGF binding only at extremely high concentrations. Although TPA did not inhibit the binding of multiplication stimulating activity (MSA), insulin, concanavalin A, or human growth hormone to various cell lines, saccharin and cyclamate did inhibit the binding of insulin and MSA at very high concentrations (Lee, 1981b). At saccharin and CHS concentrations between 7 and 10 mg/ml, inhibition of insulin and MSA binding to Hela cells was 50%.

Boyland and Mohiuddin (1981) pointed to the tumor-promoting action of surface-active compounds, such as Tweens and bile salts, and noted that saccharin and cyclamate, which they regard as tumor promoters, and CHA have a surfactant effect in reducing the interfacial tension between water and *n*-octanol. The same effect was shown also for ethanol and for sodium salts of various bile acids and fatty acids. The authors speculated that all surface-active substances, including saccharin and cyclamate, may therefore have tumor-promoting activity.

Knowles *et al.* (in press) reported that a 0.25% CHS solution induced marked epithelial cell hyperplasia in rat bladder organ culture. This was characterized by small granular cells with large, often multinucleated polymorphic cells at the advancing edge of the

outgrowth, and by 60 to 70 days, large areas of the dishes were covered by pure epithelium. Explant cultures of rat urothelium treated on day 4 with N-methyl-N-nitrosourea (MNU) at 250 μg/ml and maintained for at least 120 days developed proliferating epithelial foci of two morphological types (squamous and nonsquamous) that were identified from day 45 and were 2 cm in diameter by day 60. MNU-treated cultures, in contrast to untreated controls, had foci that appeared earlier, in higher incidence, and were primarily of the squamous type. In cultures treated with MNU and CHS, nonsquamous foci were more frequent in 70-day cultures treated with a combination of MNU and CHS than in those treated with CHS alone and were two- to threefold higher in the CHS-treated group than in controls. The authors indicated that the ability to induce hyperplasia _in vivo_ in the target organ, even without prior initiation, is a property of promoting agents, including saccharin, in the various systems. Evidence for promotion of carcinogenesis by CHS is discussed in Chapter 5.

CHS and saccharin have been reported to inhibit glucose-6-phosphate phosphohydrolase in beef liver microsomes, affecting both its hydrolase and pyrophosphate-glucose phosphotransferase activities (Lygre, 1974). However, the concentrations required for inhibition (K_i of 26 mM and 68 mM, respectively) were so high for both substances and for both activities, the committee believes that they are unlikely to be physiologically significant.

Parvu and Sendrea (1981) fed NaCHS via stomach catheter to 10 guinea pigs at a daily dosage of 1 g/kg bw for 6 weeks and measured indices of toxicity in liver and serum. In the serum, lactate dehydrogenase increased by 56%, alkaline phosphatase decreased by 52%, aldolase increased by 74%, and choline esterase decreased by 11%. In the liver, aldolase decreased by 38% and alkaline phosphatase decreased by 33%, but there were no changes in lactate dehydrogenase or choline esterase. In the serum, total lipids, triglycerides, and cholesterol increased by approximately 22%, whereas phospholipids fell by 16%. In the liver, total lipids decreased by 15%, cholesterol remained constant, and phospholipids increased by 58%. Histologic examination revealed no structural changes in liver, and none of the internal organs (liver, kidney, heart, and spleen) differed in weight from those of control animals. Considering the massive doses fed, the impact is minimal--perhaps a minor hepatotoxicity.

Carr _et al._ (1970) studied effects of CHS and CHA on the metabolism of cultured embryonic lung cells from humans. Five hours after exposure to CHS at 100 μg/ml or higher and to CHA at 10 μg/ml or higher, levels of cytidine deaminase, thymidine kinase, DNA polymerase, and uridine kinase increased, but by 24 hours they fell to control levels. At CHS concentrations of 750 μg/ml or at CHA concentrations of 500 μg/ml, no changes were observed in total DNA, in total protein, or in _de novo_ DNA and RNA after periods as long as 48 hours. No quantitative data were given.

Kitchin and Ebron (1983) adapted the system of whole rat embryo culture _in vitro_ coupled with a microsomal preparation of rat liver to study embryotoxicity and teratogenicity. Concentrations of saccharin up to 1 mM and CHA up to 0.3 mM had no significant effects on growth or morphology or on DNA content of yolk sac or embryo after a 48-hour exposure. When CHA was 1.0 mM, however, the DNA content of the yolk sac was decreased by 51% and the DNA content of the embryo by 69%. In the presence of the activating system, the same CHA concentration caused greater decreases in DNA content--60% in the yolk sac and 84% in the embryo. At 1 mM, CHA caused profound deleterious effects on growth and morphogenesis. This exposure level is extremely high, approaching the LD_{50} level for CHA, and is not likely to be encountered at the usual cyclamate (or saccharin) dosages, which the authors conclude are without significant embryotoxicity or teratogenicity.

SUMMARY

In humans and a variety of animal species, CHS has been shown to be incompletely absorbed from the intestinal tract. The absorbed CHS is rapidly excreted in the urine without appreciable buildup in the blood or tissues. Most of the unabsorbed CHS is usually excreted in the feces, but a variable amount is converted to CHA by a variety of microbial organisms dwelling in the colon and cecum. CHA thus formed is rapidly absorbed and excreted by the kidneys, with little if any fecal excretion. The urinary excretion rates of both CHS and CHA indicate that little if any of either compound remains in the tissues or body fluids even after long periods of chronic administration of high doses. This is borne out by the rapid and virtually complete urinary excretion of parenterally administered CHS and CHA. Both CHS and CHA are transported across the placental barrier. Humans exhibit heterogeneity in their ability to convert CHS to CHA, a property dependent on the intestinal flora. About three-fourths of regular users convert less than 0.1% of an ingested dose of CHS, approximately 8% to 10% of them convert 1% or more, and 4% of them convert 20% or more.

In humans, several products of CHA metabolism have been identified, namely, cyclohexanol, cyclohexanone, and _trans_-cyclohexane-1,2-diol. The presence of trace amounts of diCHA and _N_-hydroxyCHA in human urine have also been reported, but has not been substantiated by more thorough investigations. A series of amino alcohols have been identified as CHA metabolites in rats, rabbits, and guinea pigs; their formation in humans is possible, but has not yet been demonstrated.

The preponderance of evidence points to the colon and cecum as the only site of CHS metabolism. The site of further oxidative metabolism of CHA is uncertain, but both tissues and intestinal contents have been reported to be active.

Results of some short-term tests described in this chapter (Boyland and Mohiuddin, 1981; Ishii, 1982a,b; Knowles et al., in press; Lee, 1981a,b) were considered by the committee to support a role of cyclamate as a promoter of carcinogenesis.

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Chapter 4

Evidence from Short-Term Tests

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Evidence from Short-Term Tests

Effects of cyclamate and its derivatives in short-term tests were evaluated by the committee. Many chemical carcinogens have the ability to act as electrophilic reactants either in their parent form or following biotransformation and, therefore, produce DNA damage, gene mutations, and chromosome mutations in such tests (de Serres and Ashby, 1981; Upton et al., 1984). Carcinogens of this type have been designated as DNA-reactive or genotoxic (Weisburger and Williams, 1981) or as primary (Kolbye, 1980). For this reason, the activity of chemicals in short-term tests for genotoxic effects has been used to measure their potential carcinogenicity. Although positive results do not prove carcinogenicity, they can indicate the existence of an important biochemical property that may be relevant to the carcinogenicity of the compound being tested.

For a number of carcinogens there is no evidence of DNA reactivity nor have there been consistent positive findings in short-term tests for genotoxic effects (IARC, 1983; Upton et al., 1984). Among these are agents that modify endocrine systems such as hormones and amitrole, neoplasm promoters such as phenobarbital and saccharin, and diverse synthetic materials such as nitrilotriacetic acid. These substances may enhance the formation of neoplasms by mechanisms not involving their reaction with DNA. Agents of this type have been called epigenetic carcinogens (Weisburger and Williams, 1981) or secondary carcinogens (Kolbye, 1980). Thus, the lack of activity in short-term tests does not constitute proof that a chemical is without carcinogenic potential.

One proposed epigenetic mechanism of particular relevance to assessment of the cyclamate data is the enhancement of carcinogenesis through a promoting action. For example, Ashby et al. (1978) have suggested that saccharin produces neoplasms through such a process. Assays of in vitro effects, especially the inhibition of metabolic cooperation (Murray and Fitzgerald, 1979; Williams, 1980; Yotti et al., 1979), are being developed to identify promoters (Sivak, 1982).

There is extensive literature on the effects of cyclamate, cyclohexylamine, and N-hydroxycyclohexylamine in short-term tests.

(See review by Cattanach, 1976.) Cyclamate and cyclohexylamine were also among the chemicals included in the U.S. Environmental Protection Agency Gene-Tox Program. In its review, the committee drew upon the Gene-Tox data base as well as on all the literature available. The results of all the studies that were reviewed were interpreted by several approaches to determine whether cyclamate and its derivatives might have carcinogenic potential, either through production of genetic effects or by other mechanisms.

DATA COLLECTION

Gene-Tox Data Base

The Gene-Tox reports (Waters, 1979; Waters and Auletta, 1981) are summaries of published data up to about 1981 that had to satisfy rigorous criteria with respect to experimental design and peer review. Table 4-1 lists the information on cyclamate and cyclohexylamine contained in these reports.

Total Data Base

The committee reviewed papers on short-term tests containing usable primary information, including abstracts with minimum data. Whenever possible, the end points were enumerated and designated in a manner analogous to the one used by the Gene-Tox working groups. The completeness of the search was checked by comparing the references collected with reviews on cyclamate and its metabolites by Bungard (1976), Cattanach (1976), and IARC (1980).

Papers containing redundant information, i.e., either previously published information or reconfirmation of previous results by the same investigators, were not entered separately in the data base. Rather, they were either listed as co-entries with the earlier report (see Appendix 4A) or were removed from further consideration.

Committee's Approach to Data Evaluation

For its analysis, the committee first dealt with the results contained in the Gene-Tox data base, in view of its rigorous nature. The analysis was then extended to all the data found acceptable by the committee. Finally, although not described in this report, the committee examined the information contained in the excluded sources--even though they contained no quantitative data--and concluded that consideration of these reports did not affect the interpretation of the results or the final evaluation.

There are three major differences between the Gene-Tox data base and the total data base. (1) The Gene-Tox data compilation did not

TABLE 4-1. Summary of Data in Gene-Tox Reports of the Environmental Protection Agency.

Assay System	Reference Describing Criteria in Gene-Tox Data Base	Specific References Included in Gene-Tox Reports: Cyclohexylamine Result	Cyclohexylamine Reference	Sodium Cyclamate Result	Sodium Cyclamate Reference	Calcium Cyclamate Result	Calcium Cyclamate Reference
Escherichia coli plA	Leifer *et al.*, 1981	i[a]	Fluck *et al.*, 1976				
Drosophila chromosome mutations	Valencia *et al.*, 1984	-	Felix and de la Rosa, 1971b				
Drosophila nondisjunctions	Valencia *et al.*, 1984	i	Knaap *et al.*, 1973	i	Felix and de la Rosa, 1971a,b		
Drosophila recessive lethal	Lee *et al.*, 1983	-	Vogel and Chandler, 1974	i	Vogel and Chandler, 1974	i	Majumdar and Freedman, 1971
In vitro mammalian cytogenetics	Preston *et al.*, 1981	+	Green *et al.*, 1970				
In vivo cytogenetics--spermatogonia	Preston *et al.*, 1981	+	Legator *et al.*, 1969				
In vivo cytogenetics--bone marrow	Preston *et al.*, 1981	-	Brewen *et al.*, 1971				
In vivo cytogenetics--spermatocytes	Preston *et al.*, 1981			-	Leonard and Linden, 1972		
In vivo cytogenetics--leukocytes	Preston *et al.*, 1981	-	Brewen *et al.*, 1971			+	Majumdar and Solomon, 1971a
Host-mediated	Legator *et al.*, 1982	-	Buselmaier *et al.*, 1972	-	Buselmaier *et al.*, 1972		
Mouse micronucleus	Heddle *et al.*, 1983					i	Bruce and Heddle, 1979
Transformation, virus-enhanced	Heidelberger *et al.*, 1983	+	Casto, 1981				

[a] i = inconclusive. Each Gene-Tox working group set its own criteria for listing a chemical as "+," "-," or "i."

continue after 1981. (2) The committee interpreted results from the total data base in report or abstract forms, whereas only approximately 25% to 30% of the published data met the rigid standards for inclusion used in the Gene-Tox Program. Thus, the Gene-Tox data base contains information on only approximately 2,700 chemicals of the 11,000 published in the open literature. (3) Not all Gene-Tox reports have been published. One example is a report on *Salmonella* mutagenesis.

The experimental results from all studies reviewed by the committee are summarized in the next section of this chapter. Greater descriptive emphasis has been given to the reports of cytogenetic studies because they comprise the majority of positive results.

CYCLAMATE

The committee evaluated genetic bioassay data on the calcium and sodium salts of cyclamate.

Bacteria

Salmonella. Negative results were obtained when cyclamate was tested in *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 with and without metabolic activation (Bruce and Heddle, 1979; Herbold, 1981; J. McCann, University of California, Berkeley, personal communication, 1976).

Host-Mediated Assay

Sodium cyclamate did not cause mutation in *S. typhimurium* or *Serratia marcescens* in the host-mediated assay (Buselmaier *et al.*, 1972).

Dominant Lethal Test

Dominant lethal mutations were not produced in mice by either calcium cyclamate (Epstein *et al.*, 1972) or sodium cyclamate (Lorke, 1973; Lorke and Machemer, 1975; Machemer and Lorke, 1975b).

Drosophila melanogaster

Most workers reported that cyclamate did not induce sex-linked recessive lethals in *Drosophila melanogaster* (Rotter and Mittler, 1973; Sram and Ondrej, 1968; Vogel and Chandler, 1974). Majumdar and Freedman (1971) cited positive results in the same test.

Heritable translocations were not observed in *D. melanogaster* treated with calcium cyclamate (Rotter and Mittler, 1973). When treated with sodium cyclamate in another study, crossing-over occurred

(Chinnici, 1975). After *Drosophila* were fed high doses of sodium cyclamate, nondisjunction did not occur (Felix and de la Rosa, 1971a).

Cell Transformation

Cyclamate without metabolic activation (Styles, 1979) and sodium cyclamate with metabolic activation (Styles, 1977) did not induce transformation in BHK21/C13 (baby hamster kidney) cells.

Sperm Morphology

Abnormal morphology was not observed in studies of sperm from mice treated with calcium cyclamate (Bruce and Heddle, 1979; Wyrobek and Bruce, 1975) or sodium cyclamate (Topham, 1980).

In Vitro Cytogenetics

Human leukocytes. Cytogenetic effects have been studied in cultures of human leukocytes from whole peripheral blood in which cell division is stimulated by phytohemagglutinin to induce mitotic figures. In most studies the cells were cultured for 3 to 4 days with cyclamate present for part or all of the time.

In 1969, Stone *et al*. reported the effect of sodium and calcium cyclamates (source and purity not given) on cultures from more than 20 persons. The cultures were exposed to doses of 50, 100, 250, and 500 µg/ml for 72 to 84 hours. The authors scored as a break both single chromatid or isochromatid (chromosome) breaks. Breaks in control cultures ranged from 0 to 12.3% of the cells (average, 5.2%). In cultures exposed to 50 or 100 µg/ml, the average incidence of breaks was 106% and 112% of controls, respectively. Combining the exposures at 250 and 500 µg/ml, 11% of cells had breaks, representing 212% of the average incidence of breaks in controls. Very few quadriradial figures or double fragments were noted. Toxicity was not studied. A negative control compound was not included. The authors concluded that cyclamate induced breaks at a minimum concentration of 200 µg/ml.

Stoltz *et al*. (1970) studied human leukocytes from a 25-year-old male exposed to sodium cyclamate (source and purity not given) at 10^{-3}, 10^{-4}, and 10^{-5} M for either the final 5 or 25 hours of a 72-hour culture period. The data were reported as total chromosome abnormalities, including gaps and breaks. The authors mentioned, but did not quantify, exchange figures and unusual chromosomes, which were observed infrequently and only in exposed cultures. However, the study contained data on effects of cyclamate, cyclohexylamine, and *N*-hydroxycyclohexylamine without specifying which of the chemicals produced the exchanges or unusual chromosomes. In the control

cultures, there was an approximately 6% incidence of cells with chromosome abnormalities. The results after a 5- or 25-hour exposure to cyclamate were comparable. The incidence of chromosome abnormalities was approximately as follows: at 10^{-5} M, 9%; at 10^{-4} M, 10%; and at 10^{-3} M, 15%. Toxicity was not studied. A negative control compound was not included. The authors concluded that high concentrations of cyclamate can induce chromosome aberrations, but noted that either cytotoxicity or mutagenesis could be involved.

The effect of sodium cyclamate (source and purity not given) on leukocytes from 15 people was studied by Ebenezer and Sadasivan (1970). After 2-day exposures, gaps and breaks were counted as one aberration and acentric fragments as two aberrations. At four doses between 0.01 mg/ml and 0.08 mg/ml, cyclamate produced a dose-dependent inhibition of mitosis. At 0.08 mg/ml of culture, the mitotic index was too low for assessment of aberrations. The range of aberrations was as follows: 6 to 10 per 100 cells in control cultures, 7 to 10 at a cyclamate concentration of 0.1 mg/ml, 12 to 15 at 0.2 mg/ml, and 18 to 27 at 0.4 mg/ml. A negative control compound was not included. The authors concluded that cyclamate concentrations of 0.2 mg/ml or higher increased aberrations. They interpreted these findings as evidence that humans are susceptible to cyclamate.

Tokumitsu (1971) studied the effect of 0.01 M sodium cyclamate (source and purity not given) in 3- or 6-day cultures of leukocytes from a male donor. This concentration was chosen because it did not inhibit growth of an established cell line. At 3 days, two chromatid breaks were found in 176 cells (1.1%) but none in controls. At 6 days, 27 chromatid breaks were present in 149 cells (18.1%) and none in controls. At 3 days, 3.4% of the nonexposed cells were aneuploid, and at 6 days, 4.4% were aneuploid. This was increased by cyclamate to 22.7% at 3 days and 38.3% at 6 days. A negative control compound was not included. The author concluded that breaks were increased by cyclamate at 6 days and that aneuploidy was produced by cyclamate. He considered it likely that cyclamate has some mutagenic and carcinogenic action.

In a study by Collin (1971a), who exposed human leukocyte cultures (number not specified) to doses of the compound ranging from 1 mg to 500 mg per culture of a 22-ml medium, sodium cyclamate (source and purity not given) doses of 200 and 500 mg were found to suppress mitoses. The incidence of breakage per 20 cells was as follows: 1 mg, none; 5 mg, 1; 10 mg, 4; and 100 mg, 4. The incidence of achromatic regions per 20 cells was as follows: 1 mg, 2; 5 mg, 4; 10 mg, 6; and 100 mg, 11. In 100 control cells, two breaks and nine achromatic regions were observed. Saccharin at 0.2% produced no cytogenetic effect. The author concluded that the number of chromosome aberrations was correlated with the concentration of cyclamate in the medium.

Perez-Requejo (1972) studied leukocyte cultures from both males and females between the ages of 25 and 45 years. All types of chromosome aberrations were scored. In control cultures, the incidence of anomalies was 1.25% of mitoses. In cultures exposed to sodium cyclamate (source and purity not given), the incidence was 1.15% at 0.9 mg/ml, 5.3% at 4.5 mg/ml, and 9.96% at 9 mg/ml. Glucose at the same concentration as cyclamate did not produce anomalies. The author concluded that only excessively high doses induce chromosome alterations.

In a study not specifically concerned with cyclamate, Shamberger _et al_. (1973) scored all types of chromosome abberations in leukocytes from one male subject exposed to sodium cyclamate. Most of the aberrations were gaps and breaks, which were apparently scored together. Breaks were found in 23 (10.9%) of 211 leukocytes from this person. After exposure to 100 mM sodium cyclamate (Sigma Chemical Co., purity not given) for 15 hours, 26 breaks were present in 222 metaphases (11.6%). In this and a later publication of the same data (Shamberger, 1973), the investigators stated that cells exposed to cyclamate had only a slightly higher percentage of breaks than unexposed ones, but no statistical evaluation was performed.

Jemison _et al_. (1984) studied exposure of human leukocytes to 10^{-4}, 10^{-3}, and 10^{-2} M calcium cyclamate (source and purity not given). After exposure during the culture intervals of 24-48 hours, 48-72 hours, and 72-96 hours, 10^{-3} and 10^{-2} M cyclamate substantially inhibited DNA synthesis, as measured by cell number and tritiated thymidine incorporation. At an unspecified duration of exposure, the percentages of cells with chromosome changes were 1.82 at 10^{-4} M, 1.54 at 10^{-3} M, and 3.00 at 10^{-2} M. In an experimental group for which the concentration and duration of exposure to cyclamate were not given, the types of aberrations in cells were more numerous and different than in a control group. A negative control compound was not included. The authors concluded that cyclamate increased chromosome aberrations.

Human fibroblasts. Stone _et al_. (1969) reported the effect of 200 μg/ml concentrations of sodium cyclamate (source and purity not given) for an unspecified duration on monolayer cultures of skin from a human subject. Scoring both chromatid and chromosome breaks as breaks, these investigators reported an approximate doubling in the presence of cyclamate. A negative control compound was not included. The authors concluded that cyclamate in a minimum concentration of 200 μg/ml can stimulate chromosome breakage in human cells _in vitro_.

In a study by Meisner and Inhorn (1972), sodium cyclamate was added to cultured fibroblasts of unspecified origin from two persons. After 3 days, the compound was removed and metaphase spreads were made at a later interval. Chromatid and isochromatid breaks were scored together; rearrangements were scored separately. Breakage in control cultures ranged from 0.5% to 4.0% (mean, 2.2%). Exposure to 500

μg/ml concentrations of cyclamate (source and purity not given) for 3 days followed by 5 days of culture resulted in 3.2% of cells with breaks, compared to 2.0% in controls. When cultures were maintained for 12 days after exposure, 0.8% of the cells had breaks. In cultures exposed to 500 μg/ml and then 1 week later to 250 μg/ml for 3 days, 5.0% of the cells had breaks after 5 days, compared to 4% in controls. Similar exposure to 500 μg/ml followed by a second exposure to 500 μg/ml produced breaks in 6.0% of the cells, compared to 4% in controls. No increase in aberrations was found with any of these treatments. In cultures exposed only to 500 μg/ml for 3 days followed by 5 days of maintenance, chromatid breakage was similar to that for controls. Similar effects were observed with alcohol and benadryl, and aspirin produced chromosome rearrangements. The authors suggested that the concentration of cyclamate at the time of harvest is critical to obtaining a high incidence of breakage. Since the breaks did not result in aberrations, the genetic significance of the breaks was considered to be uncertain. The authors suggested that nonspecific cytotoxicity may be involved.

Chinese hamster fibroblasts. Chinese hamster (Cricetulus griseus) fibroblasts were exposed to sodium cyclamate or calcium cyclamate for up to 124 days (Dixon, 1973). The growth rate of cells was not significantly affected by the presence of cyclamate (source and purity not given) for 72 hours at concentrations up to 1,000 μg/ml. Metaphase indices showed that concentrations as low as 1 μg/ml for periods longer than 90 days caused a greater than 50% decrease in growth rate. Exposures for 72 hours to sodium cyclamate at 500 μg/ml or to calcium cyclamate at 100 μg/ml caused a statistically significant increase in breaks. Extended periods of exposure to sodium cyclamate at 10 μg/ml also produced breakage. A negative control compound was not included.

Chinese hamster cells. Monolayer cultures of embryonic lung cells from a Chinese hamster were exposed to sodium cyclamate (technical product, source not given) concentrations between 100 and 1,000 μg/ml for 1 to 3 days (Kristoffersson, 1972). The main goal of the study was to identify chromosome breaks and gaps and to localize them to individual chromosome segments. Control cells displayed a 9.2% incidence of breaks and a 6.3% incidence of gaps. In the presence of cyclamate (concentration not specified), the incidences were: breaks, 12.8% and gaps, 8.4%. Toxicity was not recorded. The percentages of cells with at least one break or gap for the different exposures were as follows: no cyclamate, 17.9%; 100 μg/ml, 23.0%; 250 μg/ml, 25.3%; 500 μg/ml, 32.8%; 1,000 μg/ml, 37.8%. Saccharin produced similar effects. The authors concluded that there was a correlation between the breaks and the concentration of cyclamate but not with the duration of treatment.

Rat kangaroo cells. The effect of cyclamate on a cell line derived from the rat kangaroo (Potorous tridactylis) kidney was

reported by Green et al. (1970). On the first day of the culture, calcium cyclamate (City Chemical Corp., purity not given) was added for 24 hours at concentrations of 100 or 200 μg/ml. The chromosomes of the cells were examined 48 hours after removal of the cyclamate. Cyclamate did not inhibit mitosis or cause chromosome breaks.

Human larynx carcinoma cells. The effect of cyclamate on chromosomes of human larynx carcinoma cells was reported by Stone et al. (1969). In two separate experiments, exposure to 200 μg/ml concentrations of sodium cyclamate (source and purity not given) for an unspecified period produced a two- to threefold increase in combined breaks of the chromatid and chromosome type. A negative control compound was not included. The authors concluded that a mininum concentration of 200 μg/ml caused chromosome breaks.

In Vivo Cytogenetics

Rat bone marrow. Collin (1971a) fed 5% sodium cyclamate (source and purity not given) to four adult female rats for 2 to 6 months. An examination of the femoral bone marrow from these animals indicated an absence of the satellite of chromosome 3 in three rats; fragmentation and a double centromere of chromosome 1 in one animal; small fragmentation of chromosome 3 in one animal; and one fragmentation of the large chromosomes of one animal. In the same report, Collin (1971a) also noted that leukocytes from 13-day fetuses whose mothers were fed 5% sodium cyclamate had breakage in their large chromosomes. A negative control compound was not included. The authors concluded that cyclamate exerts a deleterious effect on chromosomes.

As part of a study on the toxicity of cyclamate in rats, Friedman et al. (1972) fed calcium cyclamate (City Chemical Corp., purity not given) at 0.5 g/kg bw per day (1% of diet) to male Holtzman rats in a semisynthetic diet for 75 weeks. At the end of the study, bone marrow cells from 6 controls and 10 treated rats were examined by the method of Legator et al. (1969). Only chromatid breaks were observed. The range of these breaks was 0 to 3% for the control rats and 0 to 5% for the treated rats. Thus, no difference in chromosome aberrations were found.

Mouse bone marrow micronucleus. As part of the evaluation of 61 chemicals in different assays, Bruce and Heddle (1979) studied the production of micronuclei by cyclamate. At the age of 11 to 14 weeks, female B6C3F$_1$ [(C57BL/6 x C3H/He)F$_1$)] hybrid mice were exposed to the following doses of calcium cyclamate: 300, 600, 1,250, and 2,500 mg/kg. The highest dose was an approximate LD_{50} for these animals. The doses were injected intraperitoneally for 5 days to eight mice per group. Controls (three animals per group) were injected with water and dimethyl sulfoxide (DMSO). On the last day, femoral bone marrow cells were harvested. Cyclamate did not increase the percentage of micronuclei above that of controls.

Chinese hamster bone marrow sister chromatid exchange. In a study on the cytogenetic effects of saccharin in hamsters, Renner (1979) mentioned results with cyclamate. Five Chinese hamsters 10 to 11 weeks of age (30 g bw) were given 10 g/kg doses of sodium cyclamate (source not given; stated to be "pure quality") by stomach tube. The frequency of sister chromatid exchanges in femoral bone marrow cells from controls was 3.82 $\pm$ 0.16 per cell; in treated animals, it was 3.99 $\pm$ 0.08 per cell. The authors concluded that there was no evidence of a mutagenic effect.

Gerbil bone marrow. In a study by Majumdar and Solomon (1971a), calcium cyclamate (Ruger Chemical Co., purity not given) doses of 10, 30, 50, 70, and 100 mg/kg were injected intraperitoneally into 75- to 80-g adult Mongolian gerbils (*Meriones unguiculatus*) of both sexes daily for 5 days. There were 10 animals in each of the treatment groups and in the control group. At the end of the 5-day period, chromosome preparations were made from bone marrow cells following the procedure of Legator *et al*. (1969). Breaks and hyperaneuploidy were scored. Approximately 0.6% of the control cells had breaks. At the 50 mg/kg dose, 1.1% of the cells had breaks; at 150 mg/kg, 5.8%; at 250 mg/kg, 6.3%; at 350 mg/kg, 6.8%; and at 500 mg/kg, 7.4%. The percentage of hyperaneuploid cells in controls was 0.3%; at 50 mg/kg, 0.9%; at 150 mg/kg, 4.8%; at 250 mg/kg, 5.7%; at 350 mg/kg, 6.2%; and at 500 mg/kg, 6.8%. A negative control compound was not included. The authors concluded that cyclamate produced chromosome aberrations in bone marrow cells.

Human leukocytes. Dick *et al*. (1970, 1974) studied the cytogenetic effects of cyclamate in three groups of two men and two women: controls (Group 1), subjects who did not convert cyclamate to cyclohexylamine (Group 2), and subjects who did convert cyclamate to cyclohexylamine (Group 3). Groups 2 and 3 were given sodium cyclamate (Abbott Laboratories, 99% pure with 9.4 ppm cyclohexylamine) in capsules 3 times a day for 4 days for a total dose of 5 g for men and 4 g for women--approximately 70 mg/kg bw. Blood for chromosome analyses was collected before and immediately following the 4 days of cyclamate ingestion. The abnormalities most commonly seen were gaps; however, they were within normal limits. In all 12 subjects the frequency of chromosome abnormalities before exposure to cyclamate was 0 to 4% (mean, 1.6%). After 4 days of treatment, the frequency of chromosome abnormalities was 0 to 4% (mean, 1.8%). The authors concluded that these doses of cyclamate did not induce chromosome damage within the limited period of this treatment.

Wills *et al*. (1981) conducted a detailed metabolism study in men given daily doses of up to 16 g of sodium cyclamate (Abbott Laboratories, purity not given) for as long as 213 days. They observed no changes in the production of abnormal chromosomes or the total chromosome count in peripheral blood leukocytes, but no data were presented.

Rat spermatogonia. In the study of cyclamate toxicity described above, Friedman et al. (1972) also examined spermatogonial cells from 6 controls and 10 treated rats using the method of Legator et al. (1969). Again only chromatid breaks were observed. The range of the breaks was 0 to 3% for control rats and 0 to 5% for rats receiving cyclamate for 75 weeks. Thus, there was no effect of cyclamate on chromosome aberrations.

Hamster spermatogonia. Machemer and Lorke (1975a) studied the cytogenetic effects of cyclamate on spermatogonia. At 3 to 4 months of age, male Chinese hamsters (C. griseus) were given five oral 2,000 mg/kg bw per day doses of sodium cyclamate (source not given; purity conformed to German pharmacopoeia). On the sixth day, the spermatogonia from these animals were prepared by the method of Hoo and Bowles (1971). The investigators found that 4.33% of the metaphases had aberrations (2.5%, gaps; 1.8%, other). No translocations were seen. Among the untreated controls, aberrant metaphases occurred in 2.5% of the spermatogonia. One translocation, a tricentric chromosome, was found. Excluding gaps, the percentage of cells with aberrations was 1.7%. Thus, the results obtained in the cyclamate group did not differ significantly from the control data. The authors concluded that cyclamate does not have a mutagenic effect on spermatogenic cells of Chinese hamsters.

Mouse spermatocytes. Leonard and Linden (1972) administered sodium cyclamate (stated to be a commerical product, but source and purity not given) to adult male BALB/c mice in their drinking water at concentrations of 2.7, 5.3, or 10.7 g/liter for 30, 60, or 150 days. Two mice were used for each test period and for each concentration. The testes were treated according to the technique of Evans et al. (1964). No data were presented, but the investigators reported that they detected no chromosome anomalies among 600 spermatocytes studied from control mice and 1,800 from treated mice. The rate of univalents was ± 5% in the different groups. The authors concluded that there was no evidence of chromosome abnormalities and suggested that substances capable of inducing chromosome anomalies in somatic cells may not necessarily act on reproductive cells.

Metabolic Cooperation

Studies by Murray and Fitzgerald (1979) and Yotti et al. (1979) first documented that phorbol ester tumor promoters inhibited the transfer of nucleotides between cultured cells. Among substances that produce such an effect is saccharin (Trosko et al., 1980). Malcolm and Mills (1982, 1983) studied the effect of sodium cyclamate on the transfer of the toxic 6-thioguanine metabolite between competent Chinese hamster V79 cells containing hypoxanthine-guanine phosphoribosyl transferase and cocultured deficient mutant cells. At doses of 1 to 4 mg/ml, the recovery of mutants was increased, indicating that the transfer of the toxic metabolite to the mutant cells was inhibited. Effects of this type have been described for a variety of

tumor promotors (Murray and Fitzgerald, 1979; Trosko *et al.*, 1981; Umeda *et al.*, 1980; Williams, 1981; Yotti *et al.*, 1979).

CYCLOHEXYLAMINE

Bacteria

Bacteriophage induction. Cyclohexylamine did not induce temperate bacteriophage (Mayer *et al.*, 1969).

Salmonella. Mutations were not produced in *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 by treatment with cyclohexylamine in the presence and absence of metabolic activation (Herbold, 1981; J. McCann, University of California, Berkeley, personal communication, 1976).

Host-Mediated Assay

Negative results were reported for cyclohexylamine in host-mediated assays using *Salmonella typhimurium* and *Serratia marcescens* (Buselmaier *et al.*, 1972) and human leukocytes (Brewen *et al.*, 1971).

Drosophila melanogaster

No increase in sex-linked recessive mutations were found in *Drosophila melanogaster* that had been either fed or injected with cyclohexylamine (Browning, 1972; Knaap *et al.*, 1973; Vogel and Chandler, 1974). Weakly positive results were obtained in a mouse spot test (Fahrig, 1982). Cyclohexylamine did not induce aneuploidy in *D. melanogaster* (Felix and de la Rosa, 1971b).

Gene Mutation

Negative results were obtained for cyclohexylamine in Chinese hamster ovary cells (Chu and Bailiff, 1970).

In Vitro Cytogenetics

Human leukocytes. Stoltz *et al.* (1970) studied exposure of human leukocytes to cyclohexylamine (source and purity not given) at 10^{-5}, 10^{-4}, or 10^{-3} M for either the final 5 or 25 hours of a 72-hour culture period. They reported the data as total chromosome abnormalities, including gaps and breaks. Exchange figures and unusual chromosomes were stated to occur only in exposed cultures. The paper included studies on cyclamate and *N*-hydroxycyclohexylamine, however,

and the authors did not specify which chemical produced these effects. In the control cultures, there was an approximately 6% incidence of cells with chromosome abnormalities. The results with 5- or 25-hour exposures were comparable. The incidence of chromosome abnormalities was approximately as follows: 10^{-5} M, 11%; 10^{-4} M, 12%; and 10^{-3} M, 14%. Toxicity was not studied. A negative control compound was not included, but the maximal effect with cyclohexylamine was the same or less than with cyclamate. The authors concluded that high concentrations of cyclohexylamine can induce chromosome aberrations, but noted that either cytotoxicity or mutagenesis could be involved.

Brewen et al. (1971) reported that high doses of cyclohexylamine did not influence the number of aberrations in leukocytes exposed in vitro.

Chinese hamster fibroblasts. Chinese hamster fibroblasts were exposed to cyclohexylamine for up to 124 days (Dixon, 1973). Cyclohexylamine (source and purity not given) concentrations of 100 µg/ml decreased growth rates significantly, and 1,000 µg/ml was lethal. Metaphase indices showed that concentrations as low as 1 µg/ml significantly decreased growth rate. Exposure for 72 hours to 100 µg/ml produced a significant increase in the number of cells with chromosome breaks (data not given). Sucrose did not cause a significant number of breaks. Exposure to low concentrations produced breaks only after long periods, according to this author.

Rat kangaroo cells. A cell line derived from the rat kangaroo (P. tridactylis) kidney was exposed to cyclohexylamine (City Chemical Corp., purity not given) on the first day of culture for 24 hours at concentrations between 1 and 500 µg/ml (Green et al., 1970). Chromosomes were examined at 1, 24, and 48 hours after the cyclohexylamine was removed. A break was defined as a deletion greater than 1 chromatid width. Mitotic inhibition (concentration not specified) was found at 1 and 24 hours after recovery, but not at 48 hours. At 48 hours after treatment, the percentages of cells with breaks were as follows: control, 7.64%; at 1 µg/ml, 8.24%; at 10 µg/ml, 11.64%; at 50 µg/ml, 14.00%; at 100 µg/ml, 16.97%; and at 500 µg/ml, 20.24%. Relative to the chromosome length, there was a disproportionate number of chromosome breaks in chromosome group 3. In comparison, triflupromazine produced breaks and chromosome rearrangements. The authors concluded that cyclohexylamine produced single chromatid breaks, but that this was not conclusive evidence that the agent would be mutagenic in humans.

In Vivo Cytogenetics

Rat bone marrow. Legator et al. (1969) administered various doses of cyclohexylamine (City Chemical Corp.; completely pure by gas chromatography) by intraperitoneal injection daily for 5 days to groups of 20 to 30 Holtzman strain male rats. Bone marrow cells were

collected from the femur by aspiration. In control rats, the incidence of chromosome breaks was 2.72%. After administration of cyclohexylamine, this was increased to 4.0% by a 5 mg/kg total dose, 5.12% by 50 mg/kg, 8% by 100 mg/kg, 12.16% by 200 mg/kg, and 16.28% by 250 mg/kg. Single chromatid breaks predominated with infrequent exchange figures. The authors concluded that cyclohexylamine has potential mutagenic, carcinogenic, or teratogenic effects.

The effect of cyclohexylamine on bone marrow cells was studied by Dick et al. (1974) in 170- to 200-g male Holtzman rats. The animals were dosed daily for 2 days. One day later femoral bone marrow cells were collected. In groups of 12 rats given cyclohexylamine hydrochloride (City Chemical Co., 99% pure) in 50 mg/kg doses, either intraperitoneally or per os, the frequency of gaps and breaks was 2.1% and 2.4%, respectively, compared to 3.2% in controls. In 17 rats given 68.4 mg/kg doses of cyclohexylamine hydrochloride intraperitoneally and 14 rats given 50 mg/kg doses of cyclohexylamine intraperitoneally, the incidence of gaps and breaks was 1.8% and 1.3%, respectively, compared to 2.8% in controls. In these studies, the positive controls, given triethylenemelamine and tris(2-methyl-1-aziridinyl) phosphine oxide, produced high percentages of aberrations. The authors concluded that cyclohexylamine is not mutagenic.

Ford et al. (1971) administered a 50 mg/kg dose of cyclohexylamine base (City Chemical Co., purity not given) intraperitoneally, or a 68.4 mg/kg dose of two samples of cyclohexylamine hydrochloride (City Chemical Co. or Baker Chemical Co., purity not given) intraperitoneally daily for 5 days to three groups, each consisting of three male Holtzman rats. On the final day of dosing, rats were injected with colchicine and killed 4 hours later. Both femurs were used to make chromosome preparations. A spectrum of chromosome abnormalities was scored. The average percentages of total abnormalities with the three cyclohexylamine groups were 2.0, 0, and 0.7 compared to 2.0 in controls. Several positive control compounds at lower doses greatly increased abnormalities.

No increase in chromosome aberrations was found in bone marrow following in vivo exposures of rats (Ford et al., 1971), fetal lambs (Turner and Hutchinson, 1974), and Chinese hamsters (Brewen et al., 1971).

Leukocytes. Turner and Hutchinson (1974) studied cytogenetic effects of cyclohexylamine in 10 fetal hybrid sheep compared to 2 controls. Between 116 to 134 days of gestation, five dose levels of cyclohexylamine (source and purity not given) were given to cannulized fetuses for 5 or 18 hours. After exposure, fetal blood was removed for lymphocyte culture. The percentage of cells with aberrations was increased in cultures from exposed fetuses and was greater after the 18-hour exposure than after the 5-hour exposure. For example, the percentage with aberrations after 250 mg/kg for 18 hours was 4.0 compared to 0 in the control. Positive or negative control compounds

were not studied. The authors concluded that cyclohexylamine is a strong clastogen.

Cyclohexylamine also produced chromosome aberrations in circulating lymphocytes when Chinese hamsters (van Went-de Vries *et al.*, 1975a,b) were exposed *in vivo*. In rats, the same assay did not produce an elevation in aberrations (Mostardi *et al.*, 1972).

Rat spermatogonia. Legator *et al.* (1969) administered various doses of cyclohexylamine (City Chemical Corp.; completely pure by gas chromatography) by intraperitoneal injection daily for 5 days to groups of 20 to 30 Holtzman strain male rats. Spermatogonia were prepared by squashing isolated seminiferous tubules. In control rats, the incidence of chromosome breaks was 1.8%. After administration of cyclohexylamine, this was increased to 4.4% by a 5 mg/kg total dose, 7.6% by 50 mg/kg, 11.2% by 100 mg/kg, 16.2% by 200 mg/kg, and 19.2% by 250 mg/kg. Single chromatid breaks predominated with infrequent exchange figures. The authors concluded that cyclohexylamine has potential mutagenic, carcinogenic, or teratogenic effects.

In spermatogonia, chromosome aberrations did not increase after *in vivo* exposures of Holtzman rats (Ford *et al.*, 1971; Mitzutani *et al.*, 1970) and Chinese hamsters (Machemer and Lorke, 1976) to cyclohexylamine.

Mouse spermatogonia. Cattanach and Pollard (1971) reported the effect of cyclohexylamine on mouse spermatogonia. F_1 hybrid males were derived from the cross of C3H females with strain 101 males. At the end of 3 to 4 months, they were given five equal daily intraperitoneal injections of cyclohexylamine (source and purity not given) at one of two dose levels: 250 mg/kg or 500 mg/kg. Translocations in spermatogonia were measured with the technique of Evans *et al.* (1964). The testes from treated and control mice were prepared 8 or more weeks after injection. This insured that the meiotic metaphase I cells were spermatogonia at the time of treatment. One translocation was scored among control mice; none was found in mice exposed to either dose of cyclohexylamine. The authors concluded that cyclohexylamine, even at the extremely high doses used in this study, did not induce any detectable amount of heritable chromosome breakage.

Rat spermatocytes. No effects on spermatocytes from SD rats exposed *in vivo* were reported (Mitzutani *et al.*, 1970).

Dominant Lethal Test

Dominant lethal mutations generally were not produced in mice given cyclohexylamine (Cattanach and Ford, 1971; Ford *et al.*, 1971; Lorke and Machemer, 1975; Machemer and Lorke, 1975b). Positive results were reported in two experiments (Petersen and Figge, 1970; Petersen *et al.*, 1972). Results from one dominant lethal test in

Holtzman albino rats showed no effects from cyclohexylamine exposure (Green et al., 1972).

In Drosophila melanogaster, exposure to cyclohexylamine did not produce heritable translocations (Browning, 1972; Knaap et al., 1973), mosaic lethals (Knaap et al., 1973), or nondisjunctions (Knaap et al., 1973).

Cell Transformation

Transformation to anchorage independence was not induced in BHK21/C13 (baby hamster kidney) or WI-38 human cells (Styles, 1977). Virus-enhanced transformation was induced by cyclohexylamine, whereas transformation to anchorage independence was not induced in BHK21/C13 cells (Casto, 1981).

N-HYDROXYCYCLOHEXYLAMINE

Bacteriophage Induction

N-Hydroxycyclohexylamine did not induce temperate bacteriophage (Mayer et al., 1969).

Drosophila melanogaster

Sex-linked recessive lethal mutations were not found in D. melanogaster following exposure to N-hydroxycyclohexylamine (Browning, 1972; Knaap et al., 1973).

In D. melanogaster, heritable translocations (Browning, 1972; Knaap et al., 1973) and lethal mosaics (Knaap et al., 1973) did not result from treatment with N-hydroxycyclohexylamine (Knaap et al., 1973).

Gene Mutation

A weak positive response was obtained for N-hydroxycyclohexylamine in Chinese hamster ovary cells (Chu and Bailiff, 1970).

In Vitro Cytogenetics

Stoltz et al. (1970) studied human leukocytes from a 25-year-old male exposed to N-hydroxycyclohexylamine (source and purity not given) at 10^{-5}, 10^{-4}, and 10^{-3} M for either the final 5 or 25 hours of a 72-hour culture period. The data were reported as total chromosome abnormalities, including gaps and breaks. The authors mentioned, but did not quantify, exchange figures and unusual chromosomes, which were observed infrequently and only in exposed cultures. However, the study contained data on effects of cyclamate, cyclohexylamine, and

N-hydroxycyclohexylamine without specifying which of the chemicals produced the exchanges or unusual chromosomes. In the control cultures, there was an approximately 6% incidence of cells with chromosome abnormalities. The results after a 5- or 25-hour exposure to N-hydroxycyclohexylamine were comparable. The incidence of chromosome abnormalities was approximately as follows: at 10^{-5} M, 8%; at 10^{-4} M, 11%; and at 10^{-3} M, 11%-15%. Positive or negative control compounds were not included, although cyclamate yielded comparable results. The authors concluded that at high concentrations N-hydroxycyclohexylamine had effects similar to cyclamate in inducing chromosome aberrations. They noted, however, that cytogenetic damage may be associated with either mutagenesis or cytotoxicity.

In leukocyte cultures from human donors treated *in vitro* with N-hydroxycyclohexylamine, there was no significant increase in aberrations (Brewen *et al.*, 1971).

Dominant Lethal Test

Injections of mice with N-hydroxycyclohexylamine did not produce dominant lethal mutations (Petersen *et al.*, 1970).

REPRESENTATION OF THE TEST RESULTS: GENETIC SPECTRA

To simplify the presentation of the test results as well as to provide an overview of the magnitude of the responses in the various assays, the committee used genetic spectra--a recently developed methodology (Garrett *et al.*, 1984)--to summarize the Gene-Tox results for cyclamate and cyclohexylamine and the total data base results for cyclamate, cyclohexylamine, and N-hydroxycyclohexylamine. The genetic spectra for cyclamate, cyclohexylamine, and N-hydroxycyclohexylamine are presented in Figures 4-1, 4-2, and 4-3, respectively. Concentrations for the *in vitro* assays are expressed as micrograms per milliliters. For microbial plate incorporation assays, a volume of 2 ml is assumed for the top agar, and 1 ml volumes are assumed for differential toxicity tests. Doses for *in vivo* bioassays are expressed in milligrams per kilogram of body weight.

Spectral line heights were derived as follows: for a negative test result, the highest dose tested is defined as the highest ineffective dose (HID). If there was evidence of extreme toxicity, the next highest dose was used. A single dose tested with a negative result was considered equivalent to the HID. Similarly, the lowest effective dose (LED) was recorded for positive results. The dose required to produce an effect was estimated as follows: when a dose-related positive response was observed after two or more doses, the lower of the doses was taken as the LED. A single dose resulting in a positive response was considered equivalent to the LED. When two or more studies were conducted with the same assay, an average of the

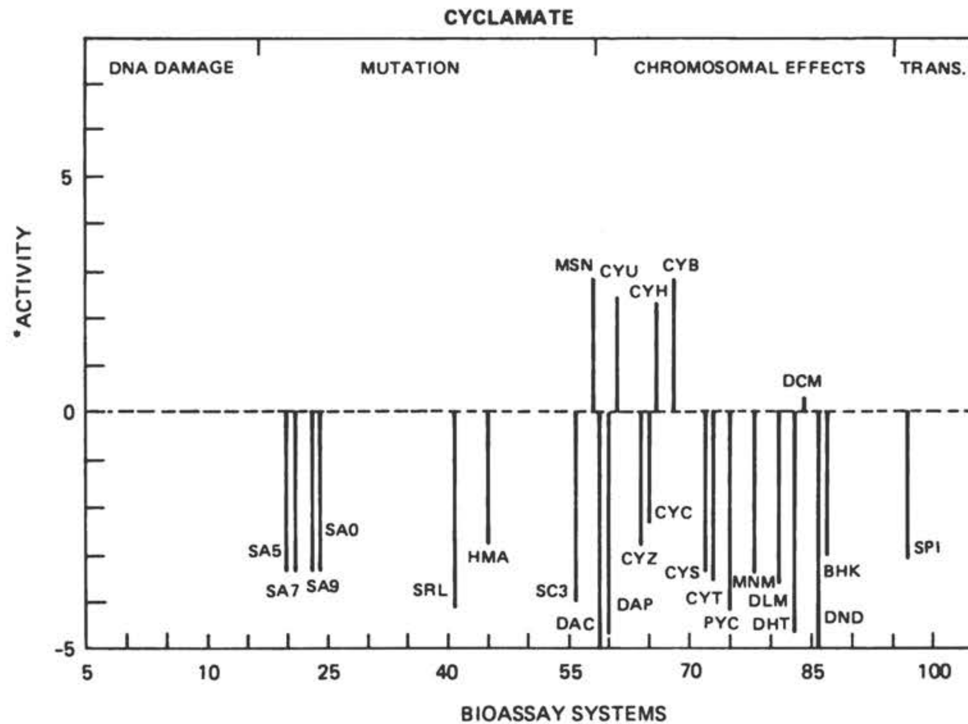

FIGURE 4-1 Genetic spectrum of cyclamate. (For abbreviations, see legend.)

*Because of the wide range of doses, a logarithmic scale is used. The lowest effective dose is plotted on an inverted scale (100,000 - 1 μg/ml) on the positive y-axis, and the highest ineffective dose (1-100,000 μg/ml)on the negative y-axis.

Legend for Figures 4-1, 4-2, and 4-3.

TRANS— Transformation:

BHK — BHK21/C13 cells

CTL — established cell lines

Chromosome aberrations in:

CYB — bone marrow, mammals *in vivo*

CYC — other cell types, mammals *in vitro*

CYH — lymphocytes or leukocytes, human *in vitro*

CYL — lymphocytes or leukocytes, mammals *in vivo*

CYS — spermatogonia treated and observed, mammals *in vivo*

CYT — spermatocytes treated and observed, mammals *in vivo*

CYU — Chinese hamster cells *in vitro*

CYZ — other human cells

Drosophila melanogaster:

DAC — aneuploidy, whole sex chromosome loss

DAP — aneuploidy, partial sex chromosome loss

DCM — crossing-over

DHT — heritable translocation

Dominant lethal mutation:

DLM — mouse

DLR — rat

Drosophila melanogaster:

DMM — mosaics

DND — nondisjunction

Host-mediated assay:

HMA — bacterial mutagenesis

HML — chromosome aberrations, human lymphocytes

MNM — micronucleus test, mouse *in vivo*

MSN — aneuploidy, mammals *in vivo*

MST — mouse spot test

PYC — plant chromosome studies

Salmonella typhimurium mutation:

SA0 — TA100

SA5 — TA1535

SA7 — TA1537

SA9 — TA98

SC3 — sister chromatid exchange, mammals *in vivo*, except humans

SPI — sperm morphology, mouse

SRL — sex-linked recessive lethals, *Drosophila melanogaster*

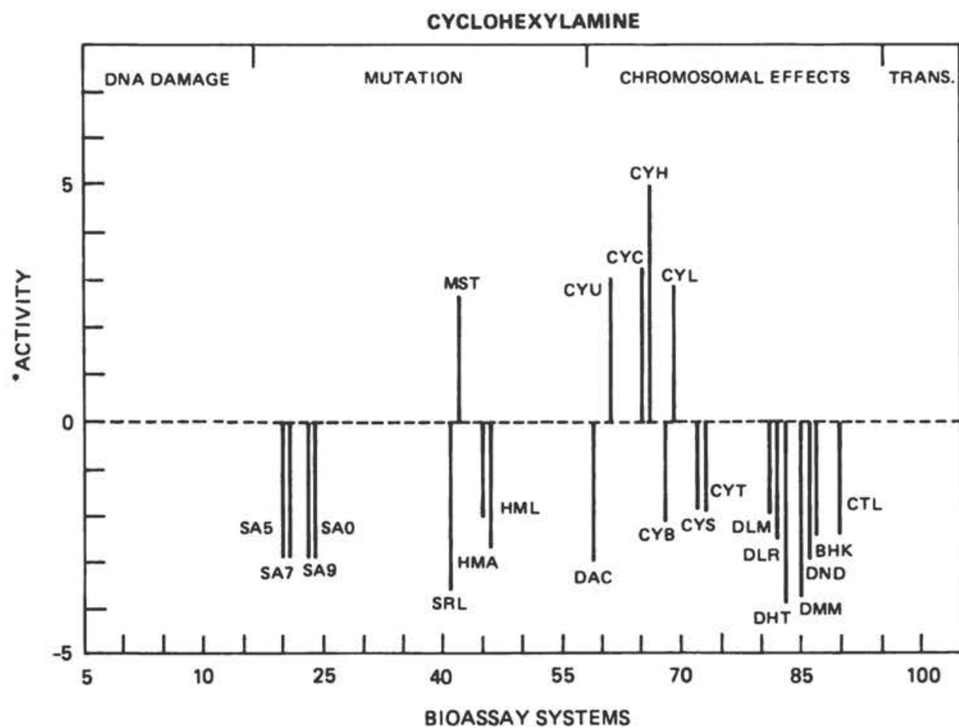

FIGURE 4-2 Genetic spectrum of cyclohexylamine. (For abbreviations, see legend.)

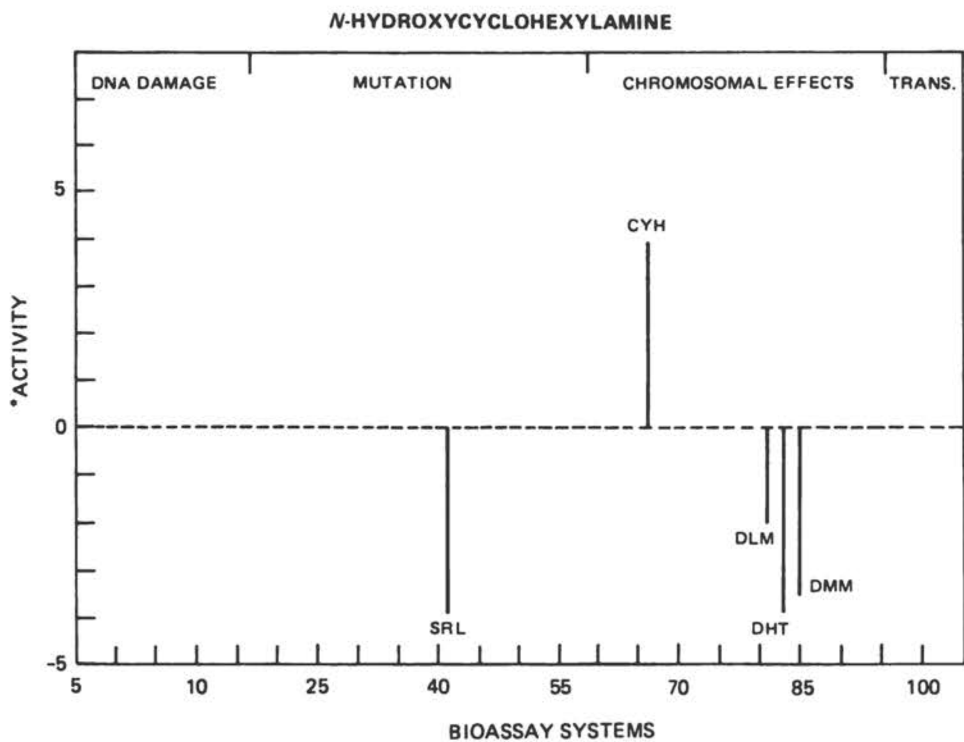

FIGURE 4-3 Genetic spectrum of *N*-hydroxycyclohexylamine. (For abbreviations, see legend.)

logarithmic values of the data subsets was calculated. When conflicting results were encountered for a given assay, the subset of data corresponding to the positive or negative result was used.

Because of the wide range of doses encountered, a logarithmic scale is used. The LED is plotted on an inverted scale (100,000-1 μg/ml) on the positive y-axis and the HID (1-100,000 μg/ml) on the negative y-axis. Positive or negative results obtained with less than 1 μg/ml are arbitrarily assigned to a dose of 1 μg/ml.

INTERPRETATION OF RESULTS

In the application of short-term assays to the prediction of carcinogenicity, most authorities recommend the use of multiple tests. A variety of approaches has been suggested. Some utilize a tier approach, whereas others require a fixed-set battery. In both approaches, the spectrum of tests should include a variety of genetic end points (e.g., DNA damage, gene mutation, chromosome effects) and should contain test systems of varied phylogenetic complexity (e.g., prokaryotes, eukaryotic cell systems, and whole animals) (Ashby, 1982, 1983; Bochkov *et al.*, 1976; Bora, 1976; Bridges, 1976; Brusick *et al.*, 1980; Dean, 1983; Flamm, 1974; Fox, 1979; Heinze and Poulsen, 1983; Ray, 1982; U.S. Congress, 1981; Weisburger and Williams, 1978, 1981).

For the fixed-set battery, which is *de facto* what published data represent, it is generally considered to be essential that all results be considered in arriving at a final judgment (Grice and Krewski, 1984). In order to err on the side of safety, however, some people would consider a single positive response to outweigh all negative ones (DHHS, 1984).

To evaluate cyclamate and its derivatives, the committee has used the decision point approach analysis (Weisburger and Williams, 1981), which is similar to the testing requirements of most national regulatory agencies (Grice and Krewski, 1984). This approach consists of a battery of assays for carcinogen testing: a DNA damage test in mammalian cells, gene mutation tests in bacteria and mammalian cells, and a mammalian cell chromosome test, with a mammalian cell transformation assay as a supplementary test. Different activation systems, including both subcellular preparations and intact cells, should be used.

In the decision point approach battery, various combinations of results have been assigned degrees of reliability in the prediction of carcinogenicity based on analysis of data for a spectrum of structural classes of carcinogens (Williams and Weisburger, 1981). For example, positive results for all four end points tested are considered a virtually certain indication of carcinogenicity. A positive result in only one assay is considered to be equivocal. Of course, the prediction is limited to the DNA-reactive (genotoxic) type of carcinogen.

The International Agency for Research on Cancer (IARC, 1984) has developed criteria for categorizing data from short-term tests as sufficient, limited, inadequate, or no evidence. These are defined as follows:

> "(i) Sufficient evidence is provided by at least three positive entries, one of which must involve mammalian cells *in vitro* or *in vivo* and which must include at least two of three end-points--DNA damage, mutation, and chromosomal effects.
>
> "(ii) Limited evidence is provided by at least two positive entries.
>
> "(iii) Inadequate evidence is available when there is only one positive entry or when there are too few data to permit an evaluation of an absence of genetic activity or when there are unexplained, inconsistent findings in different test systems.
>
> "(iv) No evidence applies when there are only negative entries; these must include entries for at least two end-points and two levels of biological complexity, one of which must involve mammalian cells *in vitro* or *in vivo*."

These criteria refer to the degree of evidence for three types of genetic activity (DNA damage, mutagenesis, and chromosomal effects) in systems at six different levels of biological complexity--prokaryotes, fungi and green plants, insects, mammalian cells (*in vitro*), mammals (*in vivo*), and humans (*in vivo*). IARC (1984) emphasized that "short-term tests should not be used by themselves to conclude whether or not an agent is carcinogenic."

Statistical approaches have also been used to interpret the experimental results obtained with batteries. These approaches are dependent upon the known performances of the assays with known carcinogens and noncarcinogens (Heinze and Poulson, 1983; Lave *et al.*, 1983a,b; Mendelsohn and Moore, 1984; Purchase, 1982; Rosenkranz *et al.*, 1984a). The committee selected the carcinogenicity prediction and battery selection (CPBS) statistical approach (Rosenkranz *et al.*, 1984a), which was devised to alleviate some, if not all, of the subjective decisions involved in the tier or hierarchical approaches. This approach is based upon Bayes' decision theory (Kendall and Stuart, 1967) and takes into consideration the performance characteristics of each assay with respect to various carcinogens and noncarcinogens that have been tested (Chankong *et al.*, 1985; Krewski *et al.*, 1982; Rosenkranz *et al.*, 1984a,b). Of all the approaches available for use by the committee, this approach appeared to be the most compatible with the available short-term test results on cyclamate and its metabolites.

In summary, the committee used three published methods for analyzing the results of short-term assays: the decision point approach, the IARC criteria, and the CPBS method.

Decision Point Approach Analysis

In analyzing the reported *in vitro* effects of cyclamate, the committee noted a serious deficiency in most of the publications: the source and purity of cyclamate was not given. In contemporary genetic toxicology, this omission would disqualify a study in the opinion of many.

The data base for cyclamate contains no reports on DNA or gene mutation in mammalian cells and a negative score for gene mutations in bacteria (Table 4-3 and Appendix 4A). Cyclamate has been reported to produce a positive effect in *in vitro* mammalian cytogenetics tests and in *in vitro* mammalian bone marrow cytogenetics tests.

In most of these cytogenetic studies, the authors did not report the types of chromosome aberrations, as is the norm in rigorous cytogenetic studies (NRC, 1983). In some of them, no distinction was made between chromosome and chromatid aberrations. Many studies (e.g., Stone *et al.*, 1969; Stoltz *et al.*, 1970) did not report chromatid gaps separately. This is critical, because gaps (achromatic regions) are considered to be very different from true breaks (deletions). Many studies did not give the frequency of aberrations per cell, but only the percentage of aberrent cells (Stone *et al.*, 1969; Stoltz *et al.*, 1970). The latter represents an all-or-none observation and does not express the actual number of aberrations. Moreover, in most studies the cyclamate was present up to the time the cells were harvested for chromosome preparations. Consequently, nonspecific toxic effects, rather than heritable alterations, could be the basis for the findings. Studies in which this could be a problem did not contain a negative compound to control for nonspecific effects. In light of all these limitations, great weight could not be assigned to the reports of chromosome effects, even though they were numerous. *Drosophila* recessive lethal tests and chromosome mutation tests were also positive. However, the *Drosophila* results were not confirmed for cyclohexylamine or *N*-hydroxycyclohexylamine. This suggests a false positive or some special metabolism in *Drosophila*.

Overall, the data base for cyclamate is not adequate for assessment according to the criteria of the decision point approach. The positive findings in cytogenetic tests and in *Drosophila* are not sufficient to indicate carcinogenicity and would require complementation by other tests. The total data bases for cyclohexylamine or *N*-hydroxycyclohexylamine are less extensive than that for cyclamate. In addition, *Drosophila* tests score as negative. Accordingly, assessment by decision point approach criteria also leads to the conclusion

that the data are inadequate, but not sufficient to indicate carcinogenicity.

International Agency for Research on Cancer Criteria

In 1982, IARC reviewed the data on cyclamate obtained in short-term tests (IARC, 1982). Of the studies available at that time, only tests for chromosome anomalies in mammalian cells *in vitro* were regarded as positive and the data were categorized as inadequate. The addition of positive results in *Drosophila* for recessive lethals and chromosome mutations could raise the categorization to limited. In any event, IARC does not use *in vitro* data to predict carcinogenicity at present. The more limited data on cyclohexylamine and *N*-hydroxycyclohexylamine would probably be judged inadequate for classification.

The Carcinogenicity Prediction and Battery Selection Method (CPBS)

The CPBS method was designed to evaluate the probability that a chemical is or is not a carcinogen, based on the results of short-term assays (Chankong *et al.*, 1985; Rosenkranz *et al.*, 1984a). It appears to be useful when the test results on a chemical are mixed, i.e., when some of the results are positive while others are negative. In this procedure, Bayes' decision theory is used to calculate the probabilities that a test chemical is or is not a carcinogen, taking advantage of the known performance characteristics of the various assays.

The CPBS method has been applied to 465 known carcinogens and 84 noncarcinogens for which short-term test results are listed in the Gene-Tox data base. For batteries consisting of three assays, between 80% and 98% of the same group of chemicals were identified correctly, depending on the actual composition of the battery (Pet-Edward *et al.*, 1985a). Sample calculations made by the committee indicate that common chemicals such as ascorbic acid and sucrose had 99.6% and 100% probabilities of noncarcinogenicity, respectively.

Application of the CPBS method to the chemicals of interest in this study leads to the following approximate predictions of noncarcinogenicity through DNA reactivity by using either the Gene-Tox or total data base: cyclamate, 97%; cyclohexylamine, 97%; and *N*-hydroxycyclohexylamine, 83%. Detailed calculations of these probabilities are also described in Appendix 4B.

CONCLUSIONS

Analysis of the results of short-term assays by the criteria of the decision point approach leads to the conclusion that the data are not sufficient for assessing the potential carcinogenicity of

cyclamate, cyclohexylamine, or *N*-hydroxycyclohexylamine. Not represented for cyclamate were mammalian cell DNA damage assays and gene mutation tests and for cyclohexylamine, a DNA damage test. These tests should be conducted to complete the data base.

The positive effects, mainly cytogenetics, observed with these compounds are not sufficient to indicate potential carcinogenicity. Because of the deficiencies in many of the studies on cytogenetic effects, it is recommended that more definitive studies be performed in which the types of chromosome aberration are assessed in more detail with regard to nonspecific cytotoxicity and the mechanisms of any effect are elucidated. Results from the additional testing recommended would contribute significantly to a judgment.

Application of the CPBS method to these chemicals leads to the prediction that cyclamate, cyclohexylamine, and *N*-hydroxycyclohexylamine have low probabilities of being carcinogens through a mechanism of DNA reactivity.

Most tests of cyclamate and its derivatives were tests for genetic effects stemming from DNA damage. Thus, the absence of sufficient positive findings relates mainly to the possibility that these chemicals are DNA-reactive carcinogens (Weisburger and Williams, 1981). Cyclamate has been found to inhibit metabolic cooperation and induce chromosome aberrations, which are postulated to be effects of promoters (Kinsella and Radman, 1978; Parry *et al.*, 1981; Trosko *et al.*, 1981). The predictive power of these determinations has not been established.

None of the positive genetic effects reported for cyclamate can serve as a basis for predicting its potential germ cell mutagenicity in mammals. Cyclamate was uniformly inactive as a gene mutagen in *Salmonella*. Although there is a good correlation between *Salmonella* mutagenicity and gene mutations in cultured mammalian cells, the committee found no data on mammalian gene mutation tests. The negative effects in rodent dominant lethal tests and cytogenetic tests in spermatogonia and spermatocytes argue against the possibility of germ-cell mutagenicity.

APPENDIX 4A

Summary of the Data on the Mutagenicity and Genotoxicity of Cyclamate and Its Metabolites

Assay System	Result	Reference
CYCLAMATE:		
Bacteria		
E. coli PolA	Inconclusive	Rosenknanz and Leifer, 1980
Salmonella	-	Bruce and Heddle, 1979; Heddle and Bruce, 1977
	-	Herbold, 1981; Herbold and Lorke, 1980
	-	J. McCann, University of California, Berkeley, personal communication, 1976
Host-mediated assay		
Salmonella/mouse	-	Buselmaier *et al.*, 1972
Drosophila		
Chromosome mutation	+	Chinnici, 1975
Chromosome mutation	-	Felix and de la Rosa, 1971a
Chromosome mutation	+	Wu and Smith, 1981
Aneuploidy	-	Felix and de la Rosa, 1971a
	-	Rotter and Mittler, 1973
	-	Wu and Smith, 1981
Recessive lethal	+	Majumdar and Freedman, 1971
Recessive lethal	-	Sram and Ondrej, 1968
In vitro cytogenetics		
Human leukocytes	+	Collin, 1971a
	+	Ebenezer and Sadasivan, 1970
	+	Jemison *et al.*, 1984
	+	Perez-Requejo, 1972
	-	Shamberger *et al.*, 1973
	+	Stoltz *et al.*, 1970
	+	Stone *et al.*, 1969
	+	Tokumitsu, 1971
Human fibroblasts	+	Meisner and Inhorn, 1972
	+	Stone *et al.*, 1969
Chinese hamster fibroblasts	+	Dixon, 1973
Chinese hamster cells	+	Kristoffersson, 1972
Rat kangaroo cells	-	Green *et al.*, 1970
Human larynx carcinoma cells	+	Stone *et al.*, 1969

Assay System	Result	Reference
In vivo cytogenetics		
Rat bone marrow	+	Collin, 1971a
	-	Friedman et al., 1972
Mouse micronucleus	-	Bruce and Heddle, 1979; Heddle and Bruce, 1977
Chinese hamster sister chromatid exchange	-	Renner, 1979
Gerbil bone marrow	+	Majumdar and Solomon, 1971a,b
Human leukocytes	-	Dick et al., 1970; Dick et al., 1974
Rat spermatogonia	-	Friedman et al., 1972
Chinese hamster spermatogonia	-	Machemer and Lorke, 1975a
Mouse spermatocytes	-	Leonard and Linden, 1972
Dominant lethal test		
Rat	-	Friedman et al., 1972
Mouse	-	Epstein et al., 1972
	-	Lorke, 1973; Lorke and Machemer, 1975
	-	Machemer and Lorke, 1975b
Sperm morphology		
Mouse	-	Bruce and Heddle, 1979; Heddle and Bruce, 1977
	-	Topham, 1980
	-	Wyrobek and Bruce, 1975
Cell transformation		
BHK21/C13	-	Styles, 1977; Styles, 1979
Metabolic cooperation	+	Malcolm and Mills, 1982, 1983; Malcolm et al., 1983
Plants		
Haworthia	-	Majumdar and Lane, 1970; Majumdar and Schlosser, 1972
CYCLOHEXYLAMINE:		
Bacteria		
Bacteriophage induction	-	Mayer et al., 1969
Salmonella	-	Herbold, 1981; Herbold and Lorke, 1980
	-	J. McCann, Univ. of Calif., Berkeley, personal communication, 1976
	-	Purchase et al., 1978

Assay System	Result	Reference
Host-mediated assay		
Salmonella/mouse	-	Buselmaier *et al.*, 1972
Drosophila		
Chromosome mutations	-	Browning, 1972
Chromosome mutations and nondisjunctions	-	Felix and de la Rosa, 1971b
Chromosome mutations	-	Knaap *et al.*, 1973
Recessive lethals	-	Knaap *et al.*, 1973
Recessive lethals	-	Vogel and Chandler, 1974
Gene mutation		
Chinese hamster	-	Chu and Bailiff, 1970
In vitro cytogenetics		
Human leukocytes	+	Stoltz *et al.*, 1970
	-	Brewen *et al.*, 1971
Chinese hamster fibroblasts	+	Dixon, 1973
Rat kangaroo	+	Green *et al.*, 1970
In vivo cytogenetics		
Rat bone marrow	-	Dick *et al.*, 1974
	-	Ford *et al.*, 1971
	+	Legator *et al.*, 1969
	-	Petersen and Figge, 1970
Chinese hamster bone marrow	-	Brewen *et al.*, 1971
Lamb (fetal) bone marrow	-	Turner and Hutchinson, 1974
Rat leukocytes	-	Mostardi *et al.*, 1972
Chinese hamster lymphocytes	+	van Went-de Vries *et al.*, 1975a,b
Chinese hamster HMA/human leukocytes	-	Brewen, 1977
Lamb (fetal) leukocytes	+	Turner and Hutchinson, 1974
Rat spermatogonia	+	Legator *et al.*, 1969
	-	Ford *et al.*, 1971
	-	Mizutani *et al.*, 1970
Mouse spermatogonia	-	Cattanach and Pollard, 1971
	-	Mitzutani *et al.*, 1970
Chinese hamster spermatogonia	-	Machemer and Lorke, 1976
Mouse spermatocytes	-	Mitzutani *et al.*, 1970
Mouse spot test	+ (weak)	Fahrig, 1982

Assay System	Result	Reference
Dominant lethal test		
Rat	-	Green et al., 1972
Mouse	-	Cattanach and Pollard, 1971
	-	Chauhan et al., 1975
	-	Ford et al., 1971
	-	Lorke and Machemer, 1974; Machemer and Lorke, 1975b
	+	Petersen et al., 1970; Petersen et al., 1972; Petersen and Figge, 1970
Cell transformation		
BHK21/C13	-	Styles, 1977; Styles, 1979
Virus-enhanced transformation	+	Casto, 1981
N-Hydroxycyclohexylamine		
Bacteria		
Bacterophage induction	-	Mayer et al., 1969
Drosophila		
Chromosome mutation	-	Browning, 1972
Chromosome mutation	-	Knaap et al., 1973
Recessive lethal	-	Browning, 1972
	-	Knaap et al., 1973
Gene mutation		
Chinese hamster cells	+ (weak)	Chu and Bailiff, 1970
In vitro cytogenetics		
Human leukocytes	-	Brewen et al., 1971
	+	Stoltz et al., 1970
In vivo cytogenetics		
Chinese hamster bone marrow	-	Brewen et al., 1971
Dominant lethal test		
Mouse	-	Petersen et al., 1970

APPENDIX 4B

THE CARCINOGENICITY PREDICTION AND BATTERY SELECTION METHOD (CPBS)

The Carcinogenicity Prediction and Battery Selection (CPBS) method uses Bayes' decision theory (Kendall and Stuart, 1967) to calculate the probabilities θ^+ and θ^- that a test chemical is or is not a carcinogen by taking advantage of the known performance characteristics of the various assays, i.e., their sensitivity [$\alpha^+ = Pr(+/CA)$] and specificity [$\alpha^- = Pr(-/NC)$], where Pr, CA, and NC signify probability, carcinogen, and noncarcinogen, respectively.

If assay A_i gives a positive result:

$$\theta_i^+ = Pr(CA/A_i = +)$$

$$\theta_i^+ = \frac{\theta_{i-1}^+ \, Pr(A_i = +/CA)}{\theta_{i-1}^+ \, Pr(A_i = +/CA) + (1 - \theta_{i-1}^+) \, Pr(A_i = +/NC)} \quad \text{and}$$

$$\theta_i^- = Pr(NC/A_i = +) = 1 - \theta_i^+ .$$

If assay A_i gives a negative result:

$$\theta_i^+ = Pr(CA/A_i = -)$$

$$\theta_i^+ = \frac{\theta_{i-1}^+ \, Pr(A_i = -/CA)}{\theta_{i-1}^+ \, Pr(A_i = -/CA) + (1 - \theta_{i-1}^+) \, Pr(A_i = -/NC)} \quad \text{and}$$

$$\theta_i^- = Pr(NC/A_i = -) = 1 - \theta_i^+, \text{ and we define}$$

$$\theta_o^+ = Pr(CA), \text{ and } \theta_o^- = Pr(NC),$$

where A_i is an assay and Pr(CA) and Pr(NC) refer to the expert's preknowledge regarding the possible carcinogenicity or noncarcinogenicity of the chemical under consideration. If on the basis of structural features or known metabolic transformation the expert believes that the chemical is likely to be a carcinogen or a noncarcinogen, the appropriate term can be increased to exceed 0.5. If, on the other hand, there is no preknowledge regarding the carcinogenicity, or lack thereof, of a test chemical, then a value of 0.5 is used. With cyclamate and cyclohexylamine, no such preknowledge is assumed and a value of 0.5 is used.

The value of α^+ (sensitivity) is the ratio of the number of carcinogens found to be positive in an assay to the number of carcinogens tested, and for α^- (specificity), it is the ratio of the number of noncarcinogens found to be negative in an assay to the number of noncarcinogens tested. These are estimated from the reported performance of the assay in the appropriate peer-reviewed Gene-Tox report (Palajda and Rosenkranz, 1985). There is an implied assumption that the chemicals used to estimate α^+ and α^- are representative of the class of chemicals for which θ^+ and θ^- are being computed. Moreover, simulations show that varying one of the α values in a battery of four tests had only a minimal effect on the values of θ. When there are not enough statistically significant test results for either an α^+ or α^- to be estimated (as for α^- in this report), a noninformative assumption is made, i.e., a lack of discrimination for noncarcinogens is assumed and a value of 0.5 is assigned (Pet-Edwards et al., 1985 a,b). A number of studies have shown that this will affect the estimated probabilities θ^+ and θ^- by less than 4% (on the conservative side). As demonstrated below for cyclamate and its derivatives, the effects are far smaller due to the unambiguous nature of the estimated values of θ^+ and θ^-.

Application of CPBS Method to Gene-Tox Data Base

Because the Gene-Tox data base consists of an unbiased, peered-reviewed compilation, it is of interest to estimate θ^+ and θ^- probabilities for these data and to compare them to the values obtained when the total data base is used. The Gene-Tox results for cyclamate and cyclohexylamine as well as the pertinent α^+ and α^- values derived from an analysis of the Gene-Tox data base (Palajda and Rosenkranz, 1985; Rosenkranz et al., 1984a) are listed in Table 4-2. To illustrate the CPBS method, a few detailed examples are given below.

Cyclohexylamine

The first example uses only data from assays for which the values of both α^+ and α^- are known. Using the Bayes equation (see above) and assigning values of 0.5 to Pr(CA) and Pr(NC) in the absence of preknowledge, one can calculate the following probabilities for *Drosophila* recessive lethal mutations (DRL):

$$\theta_1^+ = \frac{(0.5)\ (1 - 0.836)}{(0.5)\ (1 - 0.836) + (1 - 0.5)\ (0.806)} = 0.169. \qquad (1)$$

That is, based on the result of the DRL test only, there is a 17% probability that cyclohexylamine is a carcinogen, or an 83% probability of noncarcinogenicity.

TABLE 4-2. Summary of Gene-Tox Results on Cyclamate and Cyclohexylamine[a]

Test Name	Cyclamate	Cyclohexylamine	α^+	α^-	Class[b]
Drosophila chromosome mutation		-	0.781	? (0.5)	?[c]
Drosophila recessive lethal mutation		-	0.836	0.806	I
In vivo bone marrow cytogenetics	+	-	0.836	? (0.5)	?
In vitro mammalian cytogenetics		+	0.890	0.667	II
In vivo spermatogonia cytogenetics		+	0.650	? (0.5)	?
In vitro spermatocyte cytogenetics	-		0.333	? (.05)	IV[d]
In vivo leukocyte cytogenetics		-	0.836	? (0.5)	?
Host-mediated assay: *Salmonella*/mouse	-	-	0.757	0.800	I
BHK transformation assay	-	-	0.906	0.800	I
Virus-enhanced transformation		+	0.890	0.444	II

[a]Test results summarized from Table 4-1. The results for sodium cyclamate and calcium cyclamate were combined.
[b]Class indicates ranges for $\alpha+$ and $\alpha-$:
Class I: $\alpha+ \geq 0.8$, and $\alpha- \geq 0.8$
Class II: $\alpha+ \geq 0.8$, and $\alpha- \leq 0.8$
Class III: $\alpha+ \leq 0.8$, and $\alpha- \geq 0.8$
Class IV: $\alpha+ \leq 0.8$, and $\alpha- \leq 0.8$
[c]Cannot be evaluated due to lack of α value.
[d]Class IV assays such as this should not be used. Accordingly, results of this test are not be included in the estimations of θ.

Iterative use of the Bayes equation gives for the combination of DRL and *in vivo* mammalian cytogenetics (Cvt):

$$\theta_2^+ = \frac{(0.169)\ (0.890)}{(0.169)\ (0.890) + (1 - 0.169)\ (1 - 0.667)} = 0.353. \qquad (2)$$

For the combination DRL, Cvt, and host-mediated assay (HMA), it is

$$\theta_3^+ = \frac{(0.353)\ (1 - 0.757)}{(0.353)\ (1 - 0.757) + (1 - 0.353)\ (0.800)} = 0.159. \qquad (3)$$

For DRL, Cvt, HMA, and transformation in BHK21/C13 cells (BHK), it is

$$\theta_4^+ = \frac{(0.159)\ (1 - 0.906)}{(0.159)\ (1 - 0.906) + (1 - 0.159)\ (0.800)} = 0.0217. \qquad (4)$$

Finally, for the battery of DRL, Cvt, HMA, BHK, and virus-enhanced transformation (VET), it is

$$\theta_5^+ = \frac{(0.0217)\ (0.890)}{(0.0217)\ (0.890) + (1 - 0.0217)\ (1 - 0.444)} = 0.0343.^* \qquad (5)$$

In other words, by the above battery of five assays with known α^+ and α^- values, two of which give positive responses and three negative responses, it can be estimated that cyclohexylamine has a 97% (i.e., 100 - 3.43) probability of not being a carcinogen.

By assigning α^- values of 0.5 (i.e., nondiscrimination) to the other four assays--*Drosophila* chromosome mutation (DCM), *in vivo* bone marrow cytogenetics (Cbm), *in vivo* spermatogonia cytogenetics (Csg), and *in vivo* leukocyte cytogenetics (Cle)--and including them in these calculations beginning with DCM,

$$\theta_6^+ = \frac{(0.0343)\ (1 - 0.781)}{(0.0343)\ (1 - 0.781) + (1 - 0.0343)\ (0.5)} = 0.0153, \qquad (6)$$

and continuing for Cbm, Csg, and Cle, $\theta = 0.0022$ for all assays. That is, there is a 100% probability that cyclohexylamine is not carcinogenic, even though three of the nine assays gave positive responses.

The CPBS methodology permits a further refinement: tests that give essentially identical results should not be included in the same battery, since mechanistically they may be identical and hence may not provide additional probabilistic information. Cluster analaysis can be used to

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*The computation procedure using Bayes equation iteratively is numerically symmetrical, i.e., the same result would be obtained irrespective of the order in which the test results are computed.

identify groups of assays that give similar results (Pet-Edwards _et al_., 1985a).

Cluster analysis indicates that DRL and Cbm give 100% similar results (according to the Gene-Tox data). Therefore, when all the test results are used, it may be advisable to delete Cbm, since the other eight assays give a θ^+ value of 0.00658 (as opposed to $\theta^+ = 0.0022$ for all nine assays), or a 99% probability of noncarcinogenicity.

The CPBS method also includes established criteria for characteristics that constitute an ideal battery: an odd number of Class I assays, an equal number of Class II and Class III assays, and no Class IV assays (see footnote to Tables 4-2 and 4-3). (Of course, at this time assays with unknown α^- values are specifically excluded from the test battery. As more data become available, and better estimations of α^- can be made, it will be possible to increase the number of assays that can be included in a battery.) For cyclohexylamine, there are no Class III assays; hence, Class II assay results cannot be used. Therefore, the battery can consist of the three Class I assays: DRL, HMA, and BHK, which yield a θ^+ value of 0.007 or a 98% probability of noncarcinogenicity. The analyses are based on the assumption that the assay results are statistically independent.

Cyclamate

The results are similar for cyclamate. By first using the results of assays with known α^+ and α^- values (i.e., HMA and BHK), $\theta^+ = 0.0345$, or a 97% probability of noncarcinogenicity. By including Cbm (but not Csp, which is a Class IV assay), $\theta^+ = 0.0564$, or a 94% probability of noncarcinogenicity.

CPBS Method Applied to the Consensus Data Base

There is not always concordance between the results of the selective Gene-Tox data base and those from the total data base (Appendix 4A), which is summarized in Table 4-3. Since the committee included abstracts and unpublished laboratory reports and evaluated very recent publications, the total data base is more extensive than the Gene-Tox compilation.

As shown in Appendix 4A, there were some differences in some test results obtained in different laboratories with seemingly identical assays. When this occurred, the consensus result was obtained by majority, i.e., if more than 50% of the results were negative, the test was designated as negative. The same procedure was followed for positive results. (The panel recognized, of course, that there may be a bias in reporting the positive results rather than negative results

of short-term assays.) When an equal number of positive and negative results were obtained, the consensus result was listed as positive (Garrett et al., 1984). This is noted in a footnote to Table 4-3, which summarizes the results of the studies on cyclamate, cyclohexylamine, and N-hydroxycyclohexylamine. In most cases, test results were either predominantly positive or negative (see Appendix 4A). Table 4-3 also notes where an entry in the data base resulted from data originating from a single laboratory.

Cyclamate

By initially using only the results of tests with known values of α^+ and α^-, i.e., HMA, BHK, Sty, DRL, Cvt, and SCE, and using the Bayes equation iteratively, one can calculate a θ^+ value of 0.031, or a 97% probability that cyclamate is not a carcinogen, even though two of the six test results were positive. By including in the analyses the results obtained from the other assays, i.e., HMA, BHK, *Salmonella* mutagenicity (Sty), DRL, Cvt, and sister chromatid exchange (SCE) plus *Drosophila* aneuploidy (Dan), *Drosophila* crossing-over (DCM), Cle, Cbm, Csg, and micronucleus test (Mnt) and assigning a value of 0.5 to α^-, one obtains a θ^+ value of 0.0014, or a 100% probability that cyclamate is not carcinogenic, even though 4 of the 12 assays produced positive responses. By including only those assays that would constitute an ideal battery (as defined above), we could have two batteries: HMA + BHK + DRL + Sty + Cvt and HMA + BHK + DRL + Sty + SCE. The former gives a θ^+ value of 0.164, whereas the θ^+ for the latter is 0.012, or 84% and 99% probabilities of noncarcinogenicity, respectively.

Cyclohexylamine

In the same manner, the results of HMA, BHK, Sty, DRL, Cvt, and VET assays (i.e., tests with known α^- values) indicate that θ^+ is 0.0148, or a 98.52% probability of noncarcinogenicity. Inclusion of the results from the other assays, i.e., Dan, DCM, Cle, Cbm, Csg, mouse spot test (Msp), and CHO, gives an θ^+ of 0.0004, or a 100% probability of noncarcinogenicity, even though 4 of the 13 tests yielded positive responses. Using the rules for ideal batteries, one can have two batteries consisting of either HMA, BHK, DRL, Cvt, and Sty or HMA, BHK, DRL, Cvt, and VET. The former yields an estimate of 0.007 for θ^+, whereas the latter gives θ^+ as 0.0232, or 99.3% and 97.7% probabilities of noncarcinogenicity, respectively.

N-Hydroxycyclohexylamine

The results of assays with known α^+ and α^- values (i.e., DRL and Cvt) indicate that θ^+ is 0.352, or a 64.8% probability of noncarcinogenicity, which is higher than all the other estimates. Inclusion of results from all the other tests, i.e., DCM, Cle, Cbm, and Chinese hamster ovary (CHO), leads to a θ^+ value of 0.169, or an 83.1% chance of noncarcinogenicity.

TABLE 4-3. Summary of Total Data Base Results on Cyclamate, Cyclohexylamine, and N-Hydroxycyclohexylamine[a]

Type of Test	Cyclamate	Cyclohexyl-amine	N-Hydroxy-cyclohexylamine	α^+	α^-	Class[b]
Salmonella mutagenicity	-	-		0.612	0.806	III
Host-mediated assay: Salmonella/mouse	-[a]	-[a]		0.757	0.800	I
Drosophila recessive lethal	+[b]	-	-	0.836	0.806	I
Drosophila aneuploidy	-	-[a]		0.890	? (0.5)	I?[c]
Drosophila chromosome mutation	+	-	-	0.781	? (0.5)	?
In vitro mammalian cytogenetics	+	+	+[d]	0.890	0.667	II
In vivo leukocyte cytogenetics	-[a]	+	+[a]	0.836	? (.05)	?
In vivo bone marrow cytogenetics	+	-	-[a]	0.836	? (.05)	?
In vivo spermatogonia cytogenetics	-	-		0.650	? (.05)	?
In vitro spermatocyte cytogenetics	-[a]	-[a]		0.333	? (0.5)	IV[e]
Mouse spot test		+[a]		0.757	? (0.5)	?
Micronucleus test	-[a]			0.836	? (0.5)	?
Sister chromatid exchanges	-[a]			0.890	0.667	II
Transformation of BHK21 cells	-[a]	-[a]		0.906	0.800	I
Specific gene mutation in Chinese hamster ovary cells		-[a]	+[a]	0.781	? (0.5)	?
Viral-enhanced transformation		+[a]		0.890	0.444	III
Sperm abnormality		-				
Dominant lethal in rodents	-	-	-[a]			

[a]Indicates that the reported data originated from a single laboratory; for details see Appendix 4-A.
[b]Class indicates ranges for $\alpha+$ and $\alpha-$:
Class I: $\alpha+ \geq 0.8$, and $\alpha- \geq 0.8$
Class II: $\alpha+ \geq 0.8$, and $\alpha- \leq 0.8$
Class III: $\alpha+ \leq 0.8$, and $\alpha- \geq 0.8$
Class IV: $\alpha+ \leq 0.8$, and $\alpha- \leq 0.8$
[c]Cannot be evaluated due to lack of α^- value.
[d]Indicates a consensus positive result, but an equal number of laboratories reported the test chemical negative and positive; for details see Appendix 4-A.
[e]Class IV assay, not included in estimations.

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Evaluation of Cyclamate for Carcinogenicity
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Chapter 5

Animal Bioassays for Carcinogenicity

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Animal Bioassays for Carcinogenicity

Experiments in animals to determine the carcinogenicity of cyclamate have been conducted for more than 30 years. During this period standards for animal bioassays underwent considerable changes. The evaluations in this chapter are based on contemporary international standards (IARC, 1980; OSTP, 1984).

By carcinogenicity the committee means the capacity of cyclamate to increase the risk of cancer. The measures of increased risk for carcinogenicity in animal experiments include the induction of neoplasms that are not usually observed, the earlier induction of neoplasms that are usually observed, and the induction of more neoplasms than are usually found.

For convenience, the bioassays for cyclamate (CHS), for cyclamate-saccharin mixtures, and for the major metabolite of cyclamate, cyclohexylamine (CHA), are discussed separately. In each of these categories, the studies are further subdivided according to the species of animal used and the route of exposure to emphasize the extent of testing in various species and the comparative effects on different species. A separate section of this chapter addresses possible interactions of CHS with other substances in enhancing, suppressing, or otherwise modifying carcinogenesis.

Enhancement of cancer is used in this chapter as a general term to describe carcinogenic effects such as increases in the incidence, decreases in the time to appearance or detection, increases in multiplicity, or increases in the degree of malignancy when insufficient information is available to categorize the effects more narrowly as tumor initiation, promotion, progression, syncarcinogenesis, or cocarcinogenesis.

A table is included with each section as a guide to the various studies. A complete tabular summary of the animal studies is presented in Appendix A.

In addition to the final charge given to the committee, the Food and Drug Administration (FDA) asked a number of specific questions related to animal studies. These questions and the committee's responses are presented in Appendix B.

EVIDENCE FOR SODIUM AND CALCIUM CYCLAMATE

Mice

Single Generation Studies. In a study by Rudali et al. (1969), groups of 20 to 40 male or female mice of four strains (RIII, C3H, and XVII/G, or F_1 hybrids of C3H x RIII) were given drinking water containing sodium cyclamate (NaCHS) at 6 g/liter. There were equal numbers of untreated controls. Many of the treated mice were arbitrarily eliminated from the study (i.e., those that died prior to visible tumor development). Only 9 of 20 treated C3H males, 10 of 20 treated C3H females, 22 of 30 RIII males, 20 of 30 XVII/G females, and 34 of 40 treated and 28 of 40 control hybrid mice were "valid", i.e., animals that survived until tumors developed and thus are included in the statistical evaluation. Survival data were not given.

The authors concluded that the liver and lung tumors had increased in incidence and multiplicity in the exposed mice--a result they considered evidence of an acceleration or accentuation of spontaneous tumors. These conclusions were based on the following results. In XVII/G female mice, 3 of 16 (18.7%) controls had lung tumors, whereas 16 of 20 (80%) CHS-treated mice had multiple lung tumors. In the male F_1 hybrids, 12 of 28 (42.8%) controls and 22 of 34 (64.7%) CHS-treated mice had hepatomas. The incidence of these tumors in all other treated groups of the other mouse strains tested did not differ significantly from those in controls.

The dose level used in this study (6 g/liter of drinking water) is much lower than doses used in most other bioassays of NaCHS. Survival data were not given in relationship to tumor development. It is not known if earlier deaths in one treated group given CHS (XVII/G) were due to tumors or to other causes. The pathology protocols and methods of tumor diagnoses were not described. There was no report that urinary bladders were sectioned.

In this brief report, Rudali et al. (1969) stated, "Microscopically, all tumors (at least in the treated animals) were hepatocellular adenocarcinomas"; however, adenocarcinomas are rare tumors in the mouse liver. The authors did not note whether all control mice were examined or if all tumors were examined histologically. Even if they were, the difference in liver tumor incidence in male mice only in one of four experiments in small groups of mice is marginally significant and could well have occurred by chance (unrelated to treatment). According to a site visit report by a 1976 National

Cancer Institute (NCI) temporary committee (NCI, 1976), slide quality was poor and diagnoses of many lung and liver tumors were based on their gross appearance. Pulmonary abcesses and other inflammatory lesions may resemble tumors upon gross examination. In other papers from the same laboratory, Muranyi-Kovacs et al. (1975, 1976) reported that 20 of 40 (50%) female control XVII/G strain mice had developed lung tumors. In a 1972 paper, Muranyi-Kovacs and Rudali reported lung tumor incidence in similar strain of mice. These findings contrast with the 3 of 16 (18.7%) lung tumor incidence among controls in the 1969 study and the 16 of 20 (80%) incidence in CHS-treated mice of this strain. It is not clear whether the wide variations in reported lung tumor incidence are due to differences in the methods and thoroughness of diagnosis, to genetic drift (unlikely), or to some other unidentified variable.

The inconsistent findings of liver and lung tumors in controls and treated mice of various strains, the low dose levels used, the loss of many mice in both control and treated groups (suggesting intercurrent infectious diseases), and the questions concerning the adequacy of the pathology protocol seriously limit the interpretability of this study and its results.

As part of another study to test the carcinogenicity of NaCHS in combination with known oncogenic agents such as radiation and 2-acetylaminofluorene (AAF), Muranyi-Kovacs et al. (1975) exposed male or female C3Hf or XVII/G mice to drinking water containing NaCHS concentrations of 1 g/liter and C57BL male mice to water containing 6 g/liter. The initial number of mice used in each group was not clearly stated. It appears that 28 male or female XVII/G mice were started on the experiment and that groups of 14 C3Hf mice were used.

Complete autopsies were performed on all animals, and all gross lesions were fixed. Bladders were distended with fixative when sacrificed mice were suspected of having a hyperplastic bladder.

No survival data were given. It appears that 5 of 28 (18%) treated XVII/G females and 10 of 28 (36%) treated males died prior to the diagnosis of the first tumor. For strain XVII/G females, lung tumors were found in 20 of 40 (50%) control mice and in 18 of 23 (78%) CHS-treated mice. For males, total tumors were significantly decreased in treated males, i.e., 7 of 18 (38.8%) in the treated group had tumors, whereas 25 of 32 (78%) of the control males had them. For C3Hf mice, the tumor incidence was apparently presented for both sexes combined. Total tumor incidence was 2 of 26 (7.6%) in controls of both sexes and 9 of 28 (32.1%) in treated mice--a significant difference. But when sexes were evaluated individually, no significant increase was reported. Lung tumors were found in 4 of 28 (14.2%) CHS-treated mice and in none of 26 controls. Two subcutaneous fibrosarcomas were found in treated C3Hf females; none were observed

in controls. For C57BL male mice treated with CHS, there was no increase in tumor incidence.

The only significant finding noted by the authors is the increase of lung tumors in XVII/G female mice. The increase in frequency (from 20% to 50% of spontaneous tumors in the untreated controls) was regarded as a less significant finding than that in the 1969 report. They reported, "This is probably due to a selection in breeding during the 4-year interval that elapsed between the start of the initial and present experiments." They failed to note that the dose used in this experiment was one-sixth of the dose used in the earlier study.

The same limitations found in the 1969 study also apply to this study. The committee concluded that this study was not sufficiently well conducted or reported to evaluate the possible carcinogenicity of NaCHS to mice.

In a study by Roe et al. (1970), groups of 50 female Swiss mice were fed daily NaCHS doses of 6.7 g/kg bw (5% of diet) for up to 18 months. All mice also received one intragastric dose of polyethylene glycol as controls for another experiment. Weight gain was similar in all groups. At 18 months, 68% of the dosed mice were alive, compared with 61% to 65% of the controls. At autopsy the urinary bladders were examined macroscopically, not microscopically, whereas all grossly visible lesions were apparently examined microscopically. There was no increased incidence of any tumors.

Although the experiment provided no evidence for CHS carcinogenicity within the 18-month study period, the lack of bladder histopathology makes it impossible to evaluate the CHS potential for bladder carcinogenesis.

Brantom et al. (1973) gave groups of 30 male or female ASH-CS1 strain (Scientific Products Farm) mice daily NaCHS doses of 0.9 g/kg body weight (bw) (0.7% of diet), 2.3 g/kg bw (1.75%), 4.6 g/kg bw (3.5%), or 9.3 g/kg bw (7%) for 80 weeks. Sixty untreated control mice of each sex were used. Bladders of all mice killed at 80 weeks were inflated, bisected, and examined grossly. The bladders of only 21 to 27 mice in the four different groups of 30 treated males and 17 to 21 mice in the four different groups of 30 treated females were examined microscopically. Of the 60 control mice of each sex, only 44 or 45 bladders were examined microscopically.

There was no dose effect on body weight gain for males. For female mice, there was no effect until week 52 when treated mice had an effect not related to any dose. The authors stated that dose had no effect on survival. However, 14 of 30 (46.6%) of the female mice receiving the 9.3 g/kg/day dose (7% CHS diet) died by 80 weeks, whereas only 15 of 60 (25%) controls died. Eight of the 30 (26.6%) male mice on the 9.3 g/kg/day dose (7% diet) died, whereas 11 of 60 (18.3%) controls died by 80 weeks. Low erythrocyte counts and

hemoglobin levels were seen in male mice at the 9.3 g/kg/day (7% CHS) dose only from the 14th to 28th week of the study. Bladder lesions were not dose-related, but among mice on the 9.3 g/kg/day (7% CHS) dose, bladders of 8 of 30 (26.6%) males and 13 of 30 (43.3%) females were not examined microscopically. Four kidney adenocarcinomas were found in dosed males from one of three dosed groups. Lymphosarcomas and reticulum cell sarcomas were found in 6 of 25 (24%) of the 9.3 g/kg/day (7% CHS) treated females and in 3 of 45 (6.6%) of the untreated controls. No evidence of early mortality in these mice was given. More lymphoreticular tumors (8 of 46, or 17.4%) were found in control males than in high dose males (0 of 24). In this bioassay, there was no clear evidence for the carcinogenicity of NaCHS, although the survival of the mice and the examination of the urinary bladder of dosed mice were less than optimal. The reported increased incidence of lymphoreticular tumors in the high dose female mice is within the range of variation expected for this type of tumor in groups as small as 25 mice. There was no supporting evidence from the other treatment groups, and there was a negative trend for lymphoreticular tumors in male mice.

In a study by Homburger et al. (1973), groups of 25 Crl:Icr mice were fed diets containing 1.3 g/kg bw per day (1% NaCHS) or 6.7 g/kg bw per day (5% NaCHS) for up to 24 months. Control groups were of similar size. Duplicate treatment groups were used for each dose level, but there was only one control group for all four treated groups of each sex. Mice that died before 6 months were discarded. Histological examinations were performed on gross lesions of all animals and on all vital organs of at least 12 mice in each group. Male and female mice at both dose levels had depressed body weight gains as compared with controls, but there were no dose-related effects in survival and overall survival was 50% to 70% by 20 months. No bladder lesions were associated with CHS exposure, although many bladders were not examined histologically. The number of mice examined in each group varied from 11 to 22 for males and from 15 to 20 for females. Mice that died during the first 6 months were discarded without autopsy, and apparently incomplete autopsies were performed on mice that lived longer than 6 months but that were too autolyzed when found to permit histopathological examination.

The authors reported a treatment-related but not a dose-related increase in the incidence of lung and liver tumors in male mice. Lung tumors were found in 2 of 19 (11%) control male mice; in 3 of 11 (27%) and 5 of 18 (28%) of the 1.3 g/kg/day (1% CHS) treated males; and in 3 of 16 (19%) and 7 of 22 (32%) of the 6.7 g/kg/day (5% CHS) treated males. Hepatomas were found in 1 of 19 (5%) of the control males; 3 of 11 (27%) and 2 of 18 (11%) of the 1.3 g/kg/day (1% CHS) treated males; and in none of 22 and 2 of 16 (13%) of the 6.7 g/kg/day (5% CHS) treated males. No statistical evaluations were given, but the authors did not attribute the induction of these tumors to CHS. For females, no tumor increases were apparent. The authors stated,

"On the basis of these experiments, it cannot be concluded that cyclamate is carcinogenic."

Many autolyzed mice were not examined histologically. Thus, the true incidence of lung and liver tumors is not known. These findings along with the small group sizes make interpretation of the study difficult.

Single and Multigeneration Studies. Kroes et al. (1975, 1977) studied groups of 50 male or female Swiss mice given 2.7 g/kg bw per day (2% NaCHS) or 6.7 g/kg bw per day (5% NaCHS) in their diet for up to 21 months. Untreated groups of 50 male or female mice served as controls. The parental, third, and sixth generations were used for long-term and multigeneration studies. During pathological examination, the bladders were injected with formalin and all major tissues were fixed for histological examination. Mice that were severely autolyzed were examined only grossly. Generally, between 27 and 36 of the 50 mice in each group survived past 18 months. Survival was best in the parental generation. No effect on body weight gain was reported.

There was no evidence for the carcinogenicity of CHS, but from 8% to 40% of the mice in the different groups were found autolyzed and thus not examined histologically. In parental generation females fed 2.7 g/kg day (2% CHS), for example, 20 of 50 (40%) were not examined histologically, including 11 of 34 (35.4%) that died or were sacrificed after 18 months. Of the parental generation females given 6.7 g/kg/day (5% CHS), only 4 of 50 (8%) were autolyzed. One bladder tumor was found in each treatment group but none of them was statistically significant (one was found in a control female). Bladder calculi or hyperplasia that were apparently carefully evaluated were not associated with exposure to CHS.

These studies provided no evidence for the carcinogenicity of CHS. Although they seem to have been well conducted, a few deficiencies were noted. The studies were conducted over only 21 months, and significant numbers of bladders in a few treatment groups were not examined histologically. The fact that bladder calculi, hyperplasias, or dysplasias were not associated with chemical exposure in mice examined histologically provides reasonably good supportive evidence that CHS probably did not and would not cause bladder tumors in these mice under the conditions of bioassays. A summary of all these bioassays on orally administered CHS in mice is presented in Table 5-1.

Rats

Single Generation Studies. In a study by Fitzhugh et al. (1951), groups of 20 male and 20 female Osborne-Mendel rats approximately 21 days old were fed commercial laboratory chow containing NaCHS at the

TABLE 5-1. Oral Bioassays of Sodium Cyclamate in Mice

Route	Strain	Dose[a] Level	Duration	Possible Tumor End Points	Reference
Drinking water	RIII C3H XVII/G F_1(C3H x RIII)	6 g/liter	Lifetime	Lung and liver	Rudali et al., 1969
Drinking water	C3Hf XVII/G C57BL	1 g/liter 1 g/liter 6 g/liter	Lifetime	Lung and liver	Muranyi-Kovacs et al., 1975
Feed	Swiss	6.7 g/kg/d (5% diet)	18 months	None	Roe et al., 1970
Feed	ASH-CS1	0.9 g/kg/d (0.7% diet)	80-84 weeks	Lymphoreticular	Brantom et al., 1973
		2.3 g/kg/d (1.75% diet)			
		4.6 g/kg/d (3.5% diet)			
		9.3 g/kg/d (7.0% diet)			
Feed	Charles River CD	1.3 g/kg/d (1% diet)	24 months	Lung and liver	Homburger et al., 1973
		6.7 g/kg/d (5% diet)			
Feed	Swiss	2.7 g/kg/d (2% diet)	21-month multi-generation study	None	Kroes et al., 1975, 1977
		6.7 g/kg/d (5% diet)			

[a] Assumptions of ratios of body weight, food intake, and water intake used to obtained an estimate of dosage in terms of mg/kg bw/d were as follows:

Body weight (g)	Food intake (g/d)	Water intake (ml/d)
30	4	5

following levels: 0.005 (0.01%), 0.05 (0.1%), 0.25 (0.5%), 0.50 (1.0%), or 2 g/kg bw per day (5%). The purity of the compound was not specified. The experiment was conducted for 2 years, after which the surviving animals were sacrificed and examined pathologically. No overt toxic effects were observed during the experiment, but slight inanition was noted in the group given 2.5 g/kg bw per day (5%) group. Several animals developed inner ear infections and had to be sacrificed, but the incidence of infections appeared to be similar in treated and control animals. No increased incidence of tumors was reported for treated rats as compared to controls.

This was the first published chronic animal study on CHS. It is highly unlikely that sufficient animals survived long enough to detect any increased risk from the exposure, since only small numbers of rats were started on test and there was a high rate of mortality during the test. There is no indication that each animal received a pathological examination.

Friedman et al. (1972) conducted two chronic studies. In the first, groups of male weanling Holtzman rats were fed a semisynthetic diet containing either 10% or 20% casein and calcium cyclamate (CaCHS) at daily dose levels of 0, 0.5 g/kg bw (1%), or 1.0 g/kg bw (2%). There were 20 males in the control group fed a diet of 10% or 20% casein but no CaCHS; 20 males in a group fed a diet of 20% casein and CaCHS at 1.0 g/kg bw per day (2%); and 28 males in a group fed a diet containing 10% casein and CaCHS at 1.0 g/kg bw per day (2%). The animals were fed from the age of 21 days to 75 weeks. The test substance was obtained from City Chemical Corporation in New York City, but the purity was not specified.

Approximately half the animals died before the experiment was completed. The cause was apparently pneumonia, since the disease was reported in about one-third of animals on the test. The bladders were carefully examined grossly for the presence of tumors. Several bladder stones and calculi were noted in treated animals, possibly as a result of the increased calcium load in the diet. One papilloma was found in the bladder of an animal that received CaCHS at 1.0 g/kg bw per day (2%) and the 20% casein diet.

In the second study, control groups of 14 male and 14 female Osborne-Mendel rats and treated groups of 7 male and 7 female rats were fed a standard chow diet containing NaCHS or CaCHS at 0, 0.2 (0.4%), 1.0 (2.0%), or 5 g/kg bw per day (10%) for 23 months. The test substances were obtained from Abbott Laboratories in North Chicago, Ill. Approximately 40% of the animals died before the end of the test, apparently from intercurrent disease. The bladders of dead and sacrificed animals were carefully examined. No carcinomas were found in the bladders of rats fed NaCHS. However, bladder papillomas were noted in two animals fed NaCHS doses of 5 g/kg/day (10%). One of these was associated with the bladder parasite *Trichosomoides crassicauda*. No tumors were observed in the other dose groups. In

rats fed CaCHS at 5 g/kg/day (10%), one carcinoma and one papilloma were found before the 88th week. At the 0.2 g/kg/day (0.4%) level, however, two carcinomas and one papilloma were noted. No tumors were observed at the 1.0 g/kg/day (2%) dose level or in controls. Extensive calcification of the urinary system had occurred in the animals fed the CaCHS-containing diet. No tumors developed in CaCHS-treated rats between 88 and 101 weeks.

Both of these studies suffered from the fact that too few animals were used and there was a high rate of intercurrent disease and early deaths. In addition, the majority of the animals appeared to be infected with _T. crassicauda_, which further detracts from the usefulness of the study.

Schmähl (1973) fed groups of 52 male and 52 female Sprague-Dawley rats basal chow containing NaCHS at 0, 1 g/kg bw per day (2%), or 2.5 g/kg bw per day (5%). The NaCHS, obtained from Bayer Werke, contained 4 mg of CHA per kilogram of diet. The animals were approximately 11 weeks old when first exposed, and the test was continued for 30 months. The bladders and other organs were carefully examined histologically, and occasional bladder calculi were noted. One animal in the 1 g/kg/day (2%) group developed a transitional cell carcinoma and also had a large bladder calculus. In the 1 g/kg/day (2%) group, one animal also developed a bladder papilloma. No tumors were found in the control group or in the 2.5 g/kg (5%) dose group. _T. crassicauda_ was found in 16% of the animals examined histologically. This was a well-designed and well-conducted study that did not demonstrate evidence of carcinogenicity.

In a study by Hicks _et al._ (1975), 55 male and 50 female Wistar control rats were fed laboratory chow, and 95 rats divided almost equally by sex were given chow containing NaCHS at levels providing 1 g/kg bw per day. A group of 150 rats, again divided almost equally by sex, were fed the same diet containing NaCHS at levels providing a daily dose of 2 g/kg bw per day. These investigators used NaCHS from Abbott Laboratories that contained 13 mg of CHA per kilogram of diet. The animals were fed the diets from 6 weeks of age to 2 years of age, at which time survivors were sacrificed. No tumors were noted in the control group. In the 1-g dose group, one animal (sex not specified) of 84 examined histologically had a leiomyosarcoma of the bladder; at the 2-g dose level, two animals (sex not specified) of 144 examined had bladder carcinomas. The first tumor appeared during the 87th week. An increased incidence of nephrocalcinosis and bladder calculi noted in the treated animals was apparently not related to tumor development. Two animals at the low dose group developed transitional cell carcinoma of the kidney, but no tumors of this type were observed in the high dose group or in controls.

This was a well-conducted study in which the major tissues, including the bladders of most animals, were examined histologically with currently accepted practices. _T. crassicauda_ was apparently

absent in these rats. The incidence of bladder tumors was in the range normally found in the strain of rat studied.

In an unpublished study by Ikeda et al. (1975), 56 male Wistar rats were given lab chow containing NaCHS that provided a daily dose of 2.5 g/kg bw per day. A control group of 54 male rats were fed chow only. At 12 months, 10 to 16 animals per group were sacrificed for interim examination; at 24 months, an additional 11 per group were sacrificed. Terminal sacrifice appeared to have been carried out in the 28th month. Autopsies were conducted on all animals, with particular attention to bladder pathology. No tumors were found in the bladders of these animals. At 24 months, there was a statistically significant reduction in absolute, but not relative, weight of the testes in NaCHS-treated animals compared to controls. By 28 months no significant differences were apparent.

This appears to be a well-conducted study, except that histopathological evaluation was confined to the bladders. A final report of this study has not been published.

In a study by Homburger et al. (1973), groups of 25 male Holtzman rats, produced at the Charles River Breeding Laboratories, were given NaCHS at 0, 0.5 (1%), or 2.5 g/kg bw per day (5%) levels in Purina Lab Chow for 2 years. The NaCHS was obtained from Abbott Laboratories. Only one control group was used, but there were duplicate treatment groups. Therefore, 50 male rats were given each of the dietary levels. Approximately one-quarter of the animals was lost to early deaths and autolysis. Ova of the parasite T. crassicauda were noted frequently in the urine of both control and treated animals, but did not correlate with bladder tumors findings. In one of the NaCHS-treated replicates, no bladder tumors were noted at either dose level. In the other replicate, one carcinoma in situ and one papilloma were observed at the 2.5 g/kg (5%) level and one low grade carcinoma was noted at the 0.5 g/kg (1%) dose level. The bladder of one control animal contained a noninvasive carcinoma.

Although reasonably adequate in its initial design, this bioassay suffered from the loss of several animals to intercurrent infection or autolysis, thus reducing its usefulness. Nonetheless, there was no evidence of increased bladder tumor incidence related to treatment.

Bär and Griepentrog (1974) fed laboratory rats diets providing NaCHS at levels of 0, 0.15, 0.30, or 0.45 g/kg bw for 2 years. In a very brief report, the authors claimed that no bladder tumors were noted, but pathological examination of the animals was incomplete.

This study never has been published in its entirety. Moreover, the doses used are well below those tested in other studies that produced negative results. Thus, the usefulness of this study is markedly restricted.

As part of a study on physicochemical factors that affect local sarcoma production, Grasso et al. (1971) injected NaCHS or CaCHS solutions subcutaneously into albino rats of the Shell or Carworth Farm E strains 3 times per week at the same site for up to 107 weeks. Administration of the sodium salt (0.5 ml of a 15% solution) produced no tumors, but injections of CaCHS (1 ml of a 5% solution or 0.5 ml of a 15% solution) produced local sarcomas in 4 of 10 and 14 of 24 rats, respectively.

After subcutaneous injection, both NaCHS and CaCHS are rapidly removed from the site. (None was detectable after 4 hours.) Repeated injections of NaCHS produced almost no tissue destruction and no tumors, but repeated injections of CaCHS resulted in extensive granuloma formation around calcium deposits followed by local fibrosarcoma formation. It seems clear that the sarcoma formation was associated with the calcium rather than with the cyclamate. This report is described herein for completeness only.

Multigeneration Studies. In the study by Taylor et al. (1980) groups of 48 male and 48 female Holtzman rats produced by the Charles River Breeding Laboratories were fed CaCHS at a dose level of 2.5 g/kg bw per day (5% of diet) for 28 months. The animals had been randomly selected from litters whose parents had been exposed during gestation and postnatally to a 2.5 g/kg bw per day dose (5% CaCHS diet) in the typical in utero exposure model described by the U.S. Food and Drug Administration (FDA). A similar number of untreated controls selected in an identical fashion were also included in the study. Interim pathological and clinical studies were conducted on four animals of each sex per group at 14 months and on five animals of each sex per group at 18 months, leaving 39 animals of each sex per group for the main study. The bladders and other organs of animals that either died during the study or were killed terminally were subjected to careful pathological examination with currently accepted practices. During necropsy the bladders were inflated with formalin. No tumors (papillomas or carcinomas) were noted in the bladder of the CaCHS-treated animals.

This was a well-conducted in utero exposure study. It did not demonstrate any evidence of CaCHS carcinogenicity.

Schmähl and Habs (1980) treated groups of five to seven pregnant Sprague-Dawley rats with NaCHS orally at levels of 0, 0.2, 1.0, or 5.0 g/kg bw per day on days 14, 17, and 20 of gestation. Groups of approximately 30 (22 to 41) male and 30 (25 to 39) female offspring representing each of the parental treatment groups were observed over their lifespan. No bladder tumors were noted, and the incidence of other tumors was similar in the treated and control animals.

Due to the short treatment period, this study is considered inadequate for the assessment of carcinogenicity.

Hamsters

Althoff et al. (1975a,b) administered either NaCHS (containing 10 mg of CHA per kilogram of diet) or CaCHS (containing a trace of CHA) in drinking water to eight groups of 30 Syrian golden hamsters (Mesocricetus auratus) of both sexes for the lifetime of the animals. On the basis of weight loss observed in subacute toxicity tests conducted for 8 weeks, 3.8 g/kg bw per day (1.25%) in the drinking water was selected for the high dose level along with three other levels: 1.9 (0.625%), 1.0 (0.312%), and 0.5 g/kg bw per day (0.156%). At the higher levels, there were no appreciable differences in body weight from control levels. The hamsters may have tolerated slightly larger doses than given. Survival was poor. The average survival at the high dose level for NaCHS was 41 weeks; for CaCHS, it was 29 weeks. The average survivals for the control groups were 62 and 55 weeks, respectively. No differences in tumor incidences from untreated control groups were found for either substance, but statistical evaluation was not reported. No urinary tract neoplasms were found in step sections.

This study is considered inadequate for assesssing carcinogenicity because too few animals were at risk long enough for evaluation.

Nonhuman Primates

In an ongoing study by Coulston et al. (1975), one male and two female rhesus monkeys (Macaca mulatta) have been given NaCHS orally in doses of 0.20 g/kg bw per day, 6 days a week, for 6 and 7 years. Clinical examinations, including urine cytology, have revealed no evidence of neoplasia. Little or no information can be obtained about carcinogenicity from a study of this size. A later report on this study has not been found.

In another study in progress, Adamson and Sieber (1983) have given NaCHS per os at two dose levels: 0.50 g/kg bw per day (11 monkeys) and 0.10 g/kg bw per day (12 monkeys), 5 days a week for 10.5 years. Two monkeys have died at each dose level without evidence of tumor. According to S. Sieber (National Cancer Institute, personal communication, 1983), the four monkeys that died had been dosed for 84 and 24 months at 0.50 g/kg bw per day and for 3.5 months and 7 weeks at 0.10 g/kg bw per day. Bladder lesions were not reported. The remaining animals are still alive after 12 years and include at the higher dose level five male cynomolgus monkeys and two male and two female rhesus monkeys. At the lower dose level, there are two male and two female cynomolgus monkeys, two male and two female rhesus monkeys, and one male and one female African green monkeys.

The design of the experiment is considered insufficient for an adequate evaluation of carcinogenicity. The maximum tolerated dose has not been established, and the group sizes are too small. Most of the animals are still alive and have only been evaluated clinically.

Summary and Conclusions

NaCHS has been tested for carcinogenicity in mice by administration of the compounds in drinking water (two reports) and in the diet (four reports). No reports were found on bioassays of CaCHS in mice.

The two bioassays of NaCHS in drinking water were conducted by the same group of investigators, who used four strains of mice and a hybrid cross of two strains. The dose levels were much lower than those used by others in dietary studies. The results were mostly negative, but the authors reported an increase in lung tumors in one sex of one strain of mice (female XVII/G) in one experiment. This effect was not found when the experiment was repeated in the same strain at a lower dose level. They also reported an increased incidence of liver tumors in the male hybrid mice (C3H x RIII)F_1 but not in the females or in either parental strain.

The committee did not find these data convincing. The reported incidences of lung tumor in control mice of the strain studied in this laboratory changed considerably within a few years, and the incidence of lung tumors in mice receiving CHS in drinking water were within the range of some groups of control mice. Relatively few data on liver tumors were reported, and the methods used to examine the livers of dosed and control mice did not appear to be comparable. Moreover, the committee noted a number of deficiencies in the studies, including low dose levels, small group sizes, short survival, and limited pathological examinations. It concluded that these two reports did not provide evidence for the carcinogenicity of CHS when administered in the drinking water.

NaCHS was used in all four reports on dietary administration of CHS to mice. In the aggregate, the bioassays were well designed and used multiple dose levels with either 6.7 g/kg bw per day (5% diet) or 9.3 g/kg bw per day (7% in the feed) as the highest dose level. Among these reports is one multigeneration study. The duration of the experiments, from 18 to 24 months, meets contemporary requirements but is slightly less than ideal for most tumor end points. As indicated in Table 5-1, a few equivocal tumor end points (e.g., lymphoreticular tumors in one study and lung and liver tumors in another) were critically examined by the committee. The incidence of these tumors was within the background range expected for spontaneous lesions of this type. None were dose-related. Although each of the studies had some limitations, as indicated in the text, collectively they led the committee to conclude that NaCHS has been tested marginally but adequately in mice by the dietary route without convincing evidence for carcinogenicity.

In the rat, all the reported studies involved dietary administration. The test substances included NaCHS and CaCHS, and both single and multiple generation experiments were conducted (Table 5-2). Of

TABLE 5-2. Dietary Bioassays of Cyclamate in Rats

Form of Cyclamate	Strain	Generation	Dose,[a] g/kg/d	% of Diet	Duration	Reference
Sodium	Osborne-Mendel	Single	0.005 0.05 0.25 0.5 2.5	0.01 0.1 0.5 1.0 5.0	2 years	Fitzhugh et al., 1951
Calcium	Holtzman	Single	0.5 1.0	1.0 2.0	75 weeks	Friedman et al., 1972
Sodium	Osborne-Mendel	Single	0.20 1.0 5.0	0.4 2.0 10.0	23 months	Friedman et al., 1972
Sodium	Sprague-Dawley	Single	1.0 2.5	2.0 5.0	30 months	Schmähl, 1973
Sodium	Wistar	Single	1.0 2.0	2.0 4.0	2 years	Hicks et al., 1975; Hicks and Chowaniec, 1977
Sodium	Wistar	Single	2.5	5.0	28 months	Ikeda et al., 1975
Sodium	Sprague-Dawley	Single	0.5 2.5	1.0 5.0	2 years	Homburger et al., 1973
Sodium	Unknown	Single	0.15 0.30 0.45	0.3 0.6 0.9	2 years	Bär and Griepentrog, 1974
Calcium	Sprague-Dawley	Two generation	2.5	5.0	28 months	Taylor et al., 1980
Sodium	Sprague-Dawley	Two generation, only parents treated	0.2 1.0 5.0	0.4 2.0 10.0	Lifespan	Schmähl and Habs, 1980

[a] Assumption of ratios of body weight and food intake used to obtain an estimate of dosage in terms of mg/kg bw/d were as follows:

Body Weight (g)	Food Intake (g/d)
400	20

the 11 experiments in which rats were given NaCHS or CaCHS by the dietary route, no significant evidence for carcinogenicity was found. Although some of the studies were limited in design or execution, three were considered by the committee to be well done (Hicks et al., 1975; Schmähl, 1973; Taylor et al., 1980). Two additional studies, directed specifically at the urinary bladder, were also well done, and they too provided no evidence for carcinogenicity.

Because of limited design and conduct, the one study of CHS in Syrian golden hamsters and the two studies in progress in nonhuman primates are considered inadequate at present to evaluate the carcinogenicity of CHS in these species.

EVIDENCE FOR CARCINOGENICITY OF CYCLAMATE-SACCHARIN MIXTURES

Mice

Single and Multigeneration Studies. In a multigeneration study by Kroes et al. (1975), groups of 20 Swiss mice of both sexes were given diets containing a NaCHS-saccharin mixture for up to 21 months in one of two formulations: NaCHS at 7.3 g/kg bw per day (5% diet) and saccharin at 0.73 g/kg bw per day (0.5% diet) or NaCHS at 2.9 g/kg bw per day (2%) and saccharin at 0.29 g/kg bw per day (0.2%). Groups of 50 untreated mice of either sex served as controls. In addition to the parental generation, treated mice in the third and sixth generations were also evaluated. No evidence for carcinogenicity was found.

These studies were generally well conducted, although some animals were discarded because of autolysis and the duration of the experiment (21 months) may have been too short.

Rats

Single Generation Studies. In this study reported by Price et al. (1970) and Oser et al. (1975), groups of 35 male and 45 female FDRL-Strain (Wistar-derived) rats were fed diets providing a 10 to 1 mixture of NaCHS and sodium saccharin at levels of 0, 0.50, 1.12 or 2.50 g/kg bw per day. The animals were 28 to 35 days old at the start of the experiment, which was conducted for 2 years. At 78 weeks, the remaining animals in each group (except the controls) were subdivided into groups that received either no additional treatment or were given daily doses of CHA hydrochloride at 0.025, 0.056, or 0.125 g/kg bw per day for the remainder of the test. No bladder tumors were noted in the control or in the 1.12 g/kg bw per day dose group, including those that received CHA. In the 0.50 g/kg group, two rats had developed benign urinary bladder papillomas, but it is not clear whether these animals received CHA. In the 2.5 g/kg dose group, nine male and three female rats developed malignant bladder lesions (diagnosis made by two pathologists). The incidence of tumors was similar in rats that received CHA and in those that did not. This suggests that CHA did not have any modifying effect on tumor development. An increased incidence of nonmalignant proliferative lesions was found in the bladders of rats at the highest dose, but this was not noted at the other dose levels. Several animals on the test displayed nephrocalcinosis, urolithiasis, or bladder calculi; some animals were infected with *T. crassicauda*; but none of these findings was correlated with the incidence or severity of bladder tumors.

This is the only study demonstrating a statistically increased incidence of tumors in CHS-treated rats. Several factors have an impact on the interpretation of this study. First, the animals were given saccharin, a known bladder carcinogen for animals, along with the CHS, although the relatively low dose of saccharin and study design used would not have been expected to result in the development of saccharin-induced tumors (NRC, 1978). The committee is aware of an unpublished report (Toxicology Forum, Inc., 1983) that provides evidence that saccharin alone produces bladder tumors in rats at a dose of 0.5 g/kg bw per day (1% of the diet). Second, the animal colony was apparently infected with bladder parasites (T. crassicauda) that may have influenced tumor development. Third, extensive urolithiasis was noted in this study--a phenomenon not generally seen when the sodium salts (as opposed to calcium salts) are tested. Fourth, the rat strain used was different from the animal strains used in all other CHS studies. Finally, there may have been an interplay between various factors that led to the increased incidence of tumors at the highest dose level. In summary these findings are not readily explainable. It is noteworthy that no dose-response relationship was observed.

Schmähl (1973) fed groups of 52 male and 52 female Sprague-Dawley rats laboratory chow diets containing a 10:1 mixture of NaCHS and sodium saccharin at 0, 1 (2%), or 2.5 g/kg bw per day (5%) for 2 years beginning when the animals were 80 days old. The bladders were carefully examined for the presence of lesions. No tumors were found, but 16% of the bladders contained the adult form of T. crassicauda. Other organs were not systematically examined.

This was a well-conducted study that showed no evidence of CHS-saccharin bladder carcinogensis.

In a study by Ikeda et al. (1975), groups of 54 male Wistar rats were given a lab chow diet containing a 10 to 1 NaCHS-sodium saccharin mixture that provided a dose level of 0 or 2.5 g/kg bw per day. Other details of the study are the same as those described above for the group of rats fed CHS only. No tumors were found in the bladders of the treated animals.

This was a well-conducted study in which the bladders of all animals were carefully examined. Apparently, other organs were not examined histologically. There was no evidence of bladder parasites or extensive calculus formation.

Multigeneration Studies. Schmähl and Habs (1984) studied groups of approximately 35 male and 35 female Sprague-Dawley rats fed diets containing a 10:1 mixture of NaCHS and sodium saccharin at 0, 1 (2%), or 2.5 g/kg bw per day (5%) for their lifetime. The test and control animals were the offspring of parents who had been fed the same diets from weaning through gestation in the typical two-generation study. Animals were carefully observed during the entire study. The urinary

bladders and kidneys of all animals and organs that had macroscopically detectable changes were examined histologically. Only one benign bladder lesion was noted--a papilloma in a female rat treated with 1 g/kg bw per day (2% diet). The incidence of other tumors fell within the range normally expected.

This was a well-designed study, although some of the animals, both control and treated, suffered from pneumonia and had to be sacrificed. This loss somewhat reduced the power of this test. Nonetheless, the study showed no evidence that the mixture of CHS and saccharin induced carcinogenesis.

The results of dietary bioassays of NaCHS-saccharin mixtures in single and multigeneration studies are presented in Table 5-3.

TABLE 5-3. Dietary Bioassays of Sodium Cyclamate-Saccharin Mixtures

Type of Study	Test	Strain	Dose, g/kg/d	% of Diet	Duration	Reference
Single and multigeneration	Mouse	Swiss	2.7 6.7	2.0 5.0	21 months	Kroes *et al.*, 1975, 1977
Single generation	Rat	FDRL	0.5 1.12 2.5	1.0 2.5 5.0	2 years (half got CHA only from 18 months to 2 years)	Oser *et al.*, 1975; Price *et al.*, 1970
Single generation	Rat	Sprague-Dawley	1.0 2.5	2.0 5.0	2 years	Schmähl, 1973
Single generation	Rat	Wistar	2.5	5.0	28 months	Ikeda *et al.*, 1975
Two generations	Rat	Sprague-Dawley	1.0 2.5	2.0 5.0	Lifetime	Schmähl and Habs, 1984

Summary and Conclusions

Of the five dietary bioassays of a CHS-saccharin mixture (one in mice and four in rats), one in rats was reported to have shown an increased incidence of bladder tumors in the animals receiving the highest concentration of the test substance. The committee considered the results of that study equivocal for CHS, however, because the test material contained saccharin, a known bladder carcinogen for rats (NRC, 1978), and because two subsequent well-conducted replicate bioassays and a multigeneration study by other investigators failed to confirm the results.

EVIDENCE FOR CARCINOGENICITY OF CYCLOHEXYLAMINE

Mice

Single Generation Studies. Hardy et al. (1974, 1976) fed groups of 48 male and 50 female ASH-CS1 strain mice diets containing CHA hydrochloride at 0, 0.04 (300 ppm), 0.135 (1,000 ppm), or 0.4 (3,000 ppm) g/kg bw per day for 80 weeks. Body weights, food and water consumption, and hematological indices were determined at regular intervals. Mice were sacrificed at 80 to 84 weeks and subjected to gross and histopathological examinations. Bladders were distended with fixative and bisected. No effects on mortality, food or water intake, hematology, or tumor incidence for mice of both sexes or on rate of body weight gain for female mice were attributable to treatment. However, male mice had a dose-related decrease in body weight gain. The only histopathological changes that might be related to treatment were nonneoplastic and not related to dose, except for an increased incidence of minor hepatic changes in the females given 0.4 g/kg bw per day (3,000 ppm). There were, however, unexplained discrepancies in the numbers of mice examined by various types of measurements and observations.

This appears to be a reasonably well-conducted study. The number of test groups, group sizes, and dose selection are sufficient to identify carcinogenic activity, although the duration of the study was too short and the lack of histopathology in 10% to 20% of the mice weakened the study. The results gave no evidence that CHA hydrochloride is carcinogenic.

Multigeneration Studies. In a series of studies by Kroes et al. (1975, 1977), groups of male and female Swiss mice were given CHA in their diet at concentrations of 0 or 0.065 g/kg bw per day (0.5%). (The toxicity of CHS and saccharin, alone and in combination, were also investigated in this study.) The experiment consisted of six generations--the parental generation originally consisting of groups of 50 animals of each sex, and subsequent generations consisting of 20 females and 10 males per group. In five short-term studies, test groups were evaluated for reproductive performance, and all sacrificed animals underwent gross and histopathological examination. The histopathological examinations were focused on kidneys, liver, bladder, and grossly abnormal tissues. In the perinatal study, mice from selected generations were studied at weaning and at day 20 in utero. Fetuses were removed and examined for skeletal and soft tissue abnormalities. In a long-term study, groups of 50 male and 50 female mice from the parental, third, and sixth generations were maintained on the test diets for 21 months. Body weights, mortality, food intake, and hematological indices were recorded at regular intervals. All mice were presumably subjected to gross and histopathological examinations; however, only in approximately 50% of the animals were both the bladder and kidneys examined at the end of the study.

In the short-term studies, CHA induced growth retardation, which was most pronounced in the earlier generations and in the females. No histopathological abnormalities were observed in the dosed groups. In the reproduction studies, CHA was embryotoxic. Dosed groups showed a significant decrease in number of liveborn fetuses, an increase in postnatal mortality, and decrease in postnatal body weight on days 5 and 20. There were no reported teratogenic effects. In the long-term studies of three generations of mice, CHA caused reduced body weight gain in comparison to controls. No other significant clinical or histopathological differences, including tumor incidence, were observed between the dosed and control groups.

This study appears to be have been adequately conducted. Because only one dose group given CHA was included in the study design, conclusions regarding the carcinogenicity of the compound are somewhat limited, but it appears that the dose level used approached the maximum tolerated dose. The results indicate that 0.5% CHA in the diet leads to growth retardation and embryotoxicity; however, no dose-response relationship has been established.

Since dicyclohexylamine (diCHA) is an important industrial chemical that is fairly easily absorbed through undamaged skin, Pliss (1958) investigated the effects of subcutaneous injections in strain D mice obtained by crossing mice of strain C57 white with C57 black. Of 15 mice injected subcutaneously daily with 0.05 ml of 2.6% diCHA in sunflower oil (amounting to a dose of 0.0793 g per animal) and surviving 12 months, four developed sarcomas at the injection sites. This author did not indicate whether there were controls that received sunflower oil without diCHA.

The injections produced lipid granulomas. It is likely that the sarcoma formation was related to the vehicle rather than to the diCHA. Pliss (1958) also injected a substance identified as dicyclohexylamine nitrite [diCHAN]--the nitrous acid salt of diCHA-- subcutaneously into 54 mice. Daily injections of a 0.1% aqueous solution for 12 to 13 months produced no tumors that could be attributed to the injections, although the author concluded from this study and from the studies in rats (described below) that diCHAN had some carcinogenic activity.

Rats

Single Generation Studies. The potential carcinogenicity of CHA, diCHA, and diCHA nitrite were investigated in a study by Pliss (1958). One group of 50 rats (strain and sex not specified) was given CHA 6 days a week "in their food at a rate of 0.5 ml of a 5.0% oily solution" for 12 months, yielding a total dose of 8.925 g per animal. The control group consisted of 130 rats, which had also been injected subcutaneously for 10 months with octadecylamine and methyl stearylamine. All animals that died or were sacrificed were submitted for

gross and histopathological examination. The tissues examined were not specified.

Twenty-eight rats (56% of the animals) survived for more than 12 months, and 20 rats (40%) survived for more than 18 months. No tumors were found, although the liver and kidneys reportedly showed "albuminous dystrophic changes."

This study is inadequate for assessing the carcinogenic potential of CHA because only one dose group was studied, and the duration of dosing was less than lifetime. Moreover, the control group was not comparable to the experimental group, because the animals were injected with different test materials. The extent and completeness of the histopathological examinations were not characterized. Finally, there was relatively high mortality (almost 50%) after 12 months, with the result that the test group may not have been sufficiently large to detect a low incidence of tumors.

Pliss (1958) started an experiment in which rats were injected subcutaneously with diCHA. Because of tissue necrosis after 2 months, the injections were discontinued and the rats were then fed 0.5 ml of a 5% solution of diCHA in oil six times a week. Survival was poor because of pneumonia. Of 22 rats surviving 18 months or more, two developed tumors: a "liver tumor" and an omental sarcoma. Pliss also conducted two experiments in rats with diCHAN: one with the subcutaneous route (0.5 ml of a 2% aqueous solution, once per week) and another with diet (1 ml of a 3% aqueous solution, 6 days per week). Both studies suffered from the same limitations as the CHA and diCHA studies: small groups of animals, poor survival, and limited reporting of the data, especially for control animals. Although a few of the dosed rats had tumors, they were scattered in type and location and did not appear related to the treatment. These experiments are considered inadequate to evaluate the possible carcinogenicity of diCHA or diCHAN. These reports are included here only for completeness.

In a chronic oral toxicity study by Plank (1970), groups of 25 male and 25 female Charles River albino rats were given CHA sulfate in their diet for 2 years at dose levels equivalent to 0, 0.00015, 0.0015, and 0.015 g/kg bw per day. Food consumption (10 rats of each sex per group), body weight, mortality, behavioral reactions, hematology, clinical chemistry, and urine analyses (five rats of each sex per group) were determined at various intervals throughout the study. Gross pathological examinations were conducted on all animals that died during the study. Interim sacrifices were performed at 12 months on two rats of each sex per group, and gross and histopathological examinations were conducted on these animals. After 2 years on study, all surviving animals were given gross pathological examinations. Histopathological examinations were also conducted on all rats that received high doses (0.015 g/kg bw per day) and on five

rats of each sex from the other dose groups. Slight reductions in body weight gain were observed in male rats, primarily in those receiving high doses during the first 13 months of testing. At 24 months, the differences were not significantly different from the controls. No differences attributable to compound administration were observed with regard to food consumption, mortality, behavioral reactions, hematology, clinical chemistry, urine analyses, gross lesions, or histopathological examinations.

The study is inadequate for assessing the carcinogenic potential of CHA. The initial group size of 25 rats of each sex per group does not have sufficient power for satisfactory analysis. Furthermore, only five rats of each sex from the control groups and two low-dose groups and 15 rats of each sex from the high-dose group were examined histopathologically. Because of an infection that spread throughout the colony at 19 months causing deaths in all groups, fewer of the original animals were available for final analysis.

Schmähl (1973) gave diets containing CHA hydrochloride at 0.4%--a dose equivalent to 0.20 g/kg bw per day to 52 male and 52 female Sprague-Dawley rats. The doses were administered throughout the life-time of the animals. Body weights and hematological indices (five rats per groups) were examined periodically, and systolic blood pressure was checked after 2 years. Gross pathological examinations were conducted on all animals at the time of death, and all bladders and any grossly abnormal tissues were examined histopathologically. After 20 months, the hearts and heart vessels of selected animals were examined histopathologically. Additional test groups were included in this study to evaluate the effects of CHS and saccharin.

No significant effects were seen in the CHA-treated groups with regard to body weight, hematology, systolic blood pressure, lifespan, heart or bladder morphology, or tumor incidence. No bladder tumors were observed in any treated or control animals.

This study gave no evidence that CHA induces bladder tumors in rats when administered for a lifetime at a high dietary dose. Conclusions from the study are limited, however, principally because only one dose level was tested and because histopathological examinations of all animals were limited to the bladder.

In a study by Gaunt _et al_. (1974 a,b), groups of 48 Wistar rats of each sex were given diets containing CHA hydrochloride at 0, 0.03 (600 ppm), 0.10 (2,000 ppm), or 0.30 g/kg bw per day (6,000 ppm) for 2 years. Body weight, food and water consumption, hematological indices (in the 0, 0.10, and 0.30 g/kg/day groups), and urine analyses (in the control and 0.30 g/kg/day groups) were measured at various intervals. Complete gross pathological examinations were conducted on all animals that died during the study and on those sacrificed at 2 years; histo-pathological examinations were conducted on all major organs, including urinary bladders.

The following dose-related findings were observed: slight anemia, a failure to produce normally concentrated urine, and an increase in the number of animals with foamy macrophages in the pulmonary alveoli among the rats given CHA hydrochloride at 0.30 g/kg bw per day. Testicular changes (atrophy of tubules with few spermatids) were found in rats given the test material at 0.10 or 0.30 g/kg bw per day. Decreased body weight gain, food intake, and water intake; alterations in organ weights; lowered concentrations of serum urea; higher concentrations of serum albumin; and reduced incidences of certain histopathological alterations were observed in treated rats as compared to controls. The authors attributed these effects to dose-related lowered body weight resulting from decreased food consumption. No increase in tumors was observed in any treated group.

This appears to be a well-conducted chronic toxicity study. Questions about the adequacy of the carcinogenicity data arise, however, because the thoroughness of the histopathological examinations was not clearly characterized. Tissues were preserved in buffered formalin, and histopathological changes were reported in several tissues, including bladders; however, the extent of the histopathological examinations was not clearly defined. Despite this concern, the overall decrease in tumors in rats given 0.30 g/kg bw per day compared to controls did not suggest that CHA is carcinogenic at dose levels up to 0.30 g/kg daily under the conditions of study.

Multigeneration Studies. CHA hydrochloride toxicity was studied in a 2-year multigeneration feeding study reported by Carson and Vogin (1972) and Oser et al. (1976). Groups of 30 Wistar rats of each sex per group were fed the compound in their diets at levels equivalent to doses of 0.015, 0.05, 0.10, and 0.15 g/kg bw per day. Observations included growth, feed efficiency, clinical and hematological tests, reproduction, teratology, mortality, gross pathology, and histopathology. Pathological examinations were limited to F_0 generation rats (10 to 20 rats of each sex per group) on study for 2 years and to animals that died or were sacrificed in moribund condition during the study. Rats from the first litters of each generation from F_0 through F_4 were mated to produce succeeding generations. Offspring from the second litters of F_1 through F_4 were also mated. Fetuses from half the dams were delivered before birth (gestation day 20) for teratological examination, and litters from the other half were raised to maturity.

Physical and clinical observations in dosed groups were not significantly different from those of the controls, except for some nonprogressive growth retardation at 0.10 g/kg bw per day and above in the males and at 0.05 g/kg bw per day and above in the females. This growth retardation was attributed to lower food consumption. Reproduction rates were normal in all groups, although the size of the litters and their weaning weights were slightly lower than normal at

the two highest dose levels. No significant increase in soft tissue or skeletal malformations could be attributed to treatment.

Histopathological examination of the F_0 rats included all major organs and revealed, according to the investigators, a slightly increased incidence of mucosal thickening of the bladder walls at 50 and 150 mg/kg bw per day, evidence of renal calcification at 50 mg/kg bw per day and above, and an increased incidence of testicular atrophy in males at 50 and 150 mg/kg; however, there was no dose-related response and the differences were not statistically significant. The F_0 males continued to be fertile despite testicular abnormalities. No increase in the incidence of tumors was observed in any dosed groups.

This appears to be a thorough chronic toxicity study of CHA hydrochloride. Results of the pathological examination are limited, however, because the examinations were restricted to approximately half the controls and the highest dose group.

Dogs

Mastalski (1973, 1981) studied the effects of CHA sulfate administered orally to beagle dogs starting at 4 months of age. The test material was blended with lactose and given daily in gelatin capsules 6 days/week for 496 weeks (9.5 years). Groups of three male and three female dogs were given 0.015, 0.0015, or 0.00015 g/kg bw per day for 193 weeks (3.7 years). The doses were then adjusted upward to 0.15, 0.10, and 0.05 g/kg bw per day for the remainder of the experiment. Lowered body weights in dosed dogs were associated with the increase in dose level. Control dogs (three of each sex) received lactose only. One male and one female in each treated group were sacrificed after 52 weeks, and no significant lesions were found. Nine dogs (two high dose, one medium dose, three low dose, and three controls) died during the experiment. Two tumors were found: a salivary liposarcoma in a female dog in the low-dose group and a thyroid carcinoma in a male control dog. No lesions were attributed to CHA administration.

Noting the small group sizes of two dogs each, the absence of a stated rationale for dose selection, the adjustment of dose in mid-experiment, and the lack of publication, the committee concluded that the data were inadequate to assess the carcinogenicity of CHA sulfate for dogs.

Summary and Conclusions

CHA, the major metabolite of NaCHS or CaCHS, has been assayed for carcinogenicity in dietary studies of mice, rats, and dogs (Table 5-4). The two bioassays in mice, one of which was a multigeneration

study, appear to have been well conducted and give no evidence of carcinogenicity. The dose levels used approximated the maximum tolerated dose for mice. In rats, the five studies available for review all have some limitations, as noted above, but when considered together, the committee concluded that CHA has been reasonably well evaluated in the rat without revealing clear evidence of carcinogenicity and that additional bioassays would probably not add sufficient new information to warrant the time, expense, and use of animals. Toxic effects on the blood, testis, and fetus, however, are noteworthy and may be worthy of additional investigation. The one published bioassay in the dog is inadequate in design to evaluate the carcinogenicity of CHA for dogs.

TABLE 5-4. Dietary Bioassays of Cyclohexylamine for Carcinogenicity

Test Animal	Strain or Breed	Single or Multigeneration	Dose, g/kg/d	Portion of Diet, ppm	Duration	Reference
Mouse	ASH-CS1	Single	0.04 0.135 0.40	300 1,000 3,000	80-84 weeks	Hardy *et al.*, 1974, 1976
Mouse	Swiss	Multi-generation	0.065	5,000	21 months	Kroes *et al.*, 1975, 1977
Rat	Unknown	Single	Unclear	--	Unclear	Pliss, 1958
Rat	Albino	Single	0.00015 0.0015 0.015	3 30 300	2 years	Plank, 1970
Rat	Sprague-Dawley	Single	0.20	4,000	Lifetime	Schmähl, 1973
Rat	Wistar	Single	0.03 0.10 0.30	600 2,000 6,000	2 years	Gaunt *et al.*, 1974 a,b
Rat	Wistar	Multi-generation	0.015 0.050 0.10 0.15	300 1,000 2,000 3,000	110 weeks	Carson and Vogin, 1972
Dog	Beagle	Single	For 1 to 193 wks: 0.00015 0.0015 0.0150 For 194 to 496 wks: 0.050 0.10 0.15	 -- -- -- -- -- --	9.5 years	Mastalski, 1973, 1981

COMBINED EFFECTS OF CYCLAMATE WITH OTHER SUBSTANCES

Enhancement of Carcinogenesis

Mouse

• Cholesterol and cyclamate: Bryan and Ertürk (1970) surgically implanted pellets containing cholesterol or cholesterol and CHS into the bladders of 60- to 90-day-old Swiss mice (sex not specified). The pure cholesterol pellets had been made in a die by compressing recrystallized cholesterol into spheroids. Mixed cholesterol and CHS pellets were made by grinding NaCHS to a fine powder, mixing with 4 times its weight of cholesterol, and then compressing the mixture into spheroids as was done to form cholesterol pellets. The pellets weighed between 20 and 24 mg; the CHS content of each mixed pellet was estimated by the committee to be approximately 4 to 5 mg. In separate experiments to measure the elution rates of CHS from cholesterol pellets implanted in the bladder, the investigators found that 50% of the CHS was eluted within 1 hour and 99% by 6.6 hours. Information was not provided on the formation or persistence of metabolites. Duplicate implantation experiments were performed in four groups of 100 mice each with either cholesterol or cholesterol-CHS pellets, and the experiment was terminated after 13 months. Control groups without pellets or sham-operated experiments were not reported. Of the mice implanted with pellets composed entirely of cholesterol and surviving more than 175 days after implantation, 8 of 63 (13%) and 5 of 43 (12%) developed carcinomas of the bladder. Of those implanted with cholesterol pellets containing CHS, the corresponding incidences of bladder carcinomas were 45 of 58 (78%) and 30 of 49 (61%) in the duplicate groups. The investigators reported that the carcinomas in the CHS-treated groups were more frequently multiple and more invasive with higher mitotic activity. In no tissues other than the bladder were tumor incidences observed to be different in CHS-treated mice as compared to control mice, but data were not presented.

Two aspects of this research are noteworthy. First, after only 13 months, pellets themselves were associated with carcinoma formation in the bladder at an incidence of at least an order of magnitude greater than historic background levels. Second and more puzzling is that a short burst of CHS exposure for 7 hours or so could exert any significant effect at all. The experiment appears to have been well done and the results must be considered seriously, but intuitively one would not expect a response as great as that reported in this experiment, even if a potent carcinogen had been applied for a few hours. It is remarkable that this experiment appears not to have been repeated in the 14 years since publication.

• 2-Acetylaminofluorene (AAF) and cyclamate: Muranyi-Kovacs et al. (1975) gave each of four groups of 28 C3Hf mice either 60 mg of AAF per kilogram of diet, 1 g of NaCHS per liter of drinking water, the same doses of AAF and CHS concurrently, or neither treatment

(untreated control mice) until the mice died. The C3Hf strain was selected because it is predisposed to liver tumors, and the purpose of this experiment was to learn whether CHS would enhance hepatic carcinogenesis by AAF in this sensitive strain of mouse. The pathology protocol is uncertain, but the incidences of liver tumors reported for the different exposure groups were 19 of 27 (70%) with AAF alone, 3 of 28 (11%) with CHS alone, 27 of 27 (100%) with AAF plus CHS, and 2 of 26 (8%) in untreated control mice. The authors concluded that the additive effect of CHS and AAF could not be assessed, since the frequency of tumors in the group treated with AAF alone was too high.

The study is included here only for completeness. The experiment was not sufficiently sensitive to fulfill the objective.

- Whole body radiation and cyclamate: Muranyi-Kovacs *et al.* (1975) reported a study in which two groups of 35 male C57BL mice were given 250 rads of total body radiation. One of the groups also received 6 g of NaCHS per liter of drinking water until death. Apparently, the CHS was administered after the radiation. A third group received only CHS at a lower dose of 0.6 g/kg bw per day. Leukemia was found in 12 of 30 (40%) mice receiving radiation alone, in 3 of 28 (11%) of the control mice receiving CHS alone, and in 5 of 21 (24%) of the mice receiving both radiation and CHS. The investigators concluded that CHS did not significantly modify the carcinogenic action of whole body radiation.

The small experiment was part of a larger series of experiments on the combined effects of NaCHS with oncogenic agents. To be definitive, a larger, more complete series of experiments would have to be conducted with several combinations of dose levels, mouse strains with different sensitivities to radiation, larger groups, and comprehensive pathological examinations.

Rat

- N-methyl-N-nitrosourea and cyclamate: Hicks and her associates investigated the possible synergy between topical application of *N*-methyl-*N*-nitrosourea (MNU) to the urinary bladder and dietary administration of NaCHS (Hicks *et al.*, 1975; Hicks and Chowaniec, 1977). Groups of 6- to 8-week-old female Wistar rats were given single 1.5 or 2.0 mg instillations of MNU into the urinary bladder (see comment below). They were then randomly placed in treatment groups (R. M. Hicks, Middlesex Hospital Medical School, personal communication, 1984). Two different concentrations of NaCHS were fed to the animals in pelleted feed. The average intakes were 1.0 and 2.0 g/kg bw per day. Control groups of rats received either CHS alone, MNU alone, or neither treatment. The experiment was terminated after 2 years, and the bladder, kidneys, ovaries, uterus, liver, spleen, pancreas, and lung were examined microscopically, but only data for

the bladder were reported. Approximately 16% of the MNU-treated rats and 7% of the other rats were lost from the study through cannibalism, respiratory disease, and other causes.

In 98 untreated control rats of both sexes, no bladder tumors were found. Neither were there bladder tumors in 124 female rats given MNU alone, indicating that under the conditions of the experiment the single administration of MNU, a known carcinogen, was not carcinogenic. In the groups of male and female rats fed CHS without exposure to MNU, two bladder tumors were found: one among 144 (0.69%) rats at the higher dose level and one among 84 (1.2%) at the lower dose level. The combined incidence of bladder tumors in CHS-treated rats--3 of 228 (1.3%)--is higher than in untreated controls (0 of 98) and in historic control Wistar rats in the same laboratory (none found in 12 years), but the investigators reported that the differences from control values were not statistically significant. Later, in the same laboratory, bladder tumors were found in 1 of 51 (2%) control Wistar rats after exposure to MNU (Severs et al., 1982). In contrast, 20 of 45 (44%) MNU-treated, CHS-fed female rats had bladder tumors at the higher CHS dose level as did 14 of 24 (58%) given MNU and the lower CHS level. Moreover, there was considerable difference in the time until the first bladder tumor was detected: 87 weeks in rats receiving CHS alone but only 8 and 9 weeks in the two MNU-plus-CHS groups. The histological appearance of the tumors was reported to be of a lower grade of malignancy in the control group receiving CHS without MNU, but no details were given.

The effects on the bladder resulting from combined exposure to topically administered MNU and dietary NaCHS appeared to be much greater than additive in producing bladder tumors in Wistar rats. In this seminal study, the experiments generally seem to have been well designed and conducted, although some considerations that would have been helpful for assessment were not reported, for example, food consumption (CHS doses), the extent of bladder examination, the histological types and multiplicity of tumors found, findings in other organs, numerical separation of the sexes, and survival data for dosed and control rats. In addition, there seems to be a discrepancy in the nominal MNU doses reported. In the first report, Hicks et al. (1975) stated that the MNU dose was 2.0 mg; in the later report, which appears from the tabular data to be the same study, Hicks and Chowaniec (1977) stated that the MNU dose was 1.5 mg. Moreover, an attempt by Mohr et al. (1979) to repeat the study with 2.0 mg MNU doses failed, because the MNU alone produced such a high incidence of bladder tumors that the possible enhancing effects of CHS would have been difficult to detect (see below). It appears that the actual MNU dose used by Hicks and colleagues did not approach the nominal dose. The experiment is still valid, however, because whatever dose was used, the evidence indicates that under the conditions of the experiment it was not carcinogenic by itself but was synergistic with CHS for bladder carcinogenesis. The committee presumes that the same supply of MNU was used throughout and that the groups were treated concurrently,

since MNU tends not to be stable under some laboratory storage conditions.

The specific pathogen-free (SPF) rats used were free of the bladder nematode _T. crassicauda_, but at autopsy most (30 of 34) of the tumor-bearing rats in the MNU-plus-CHS groups were found to have bladder calculi, which may have contributed to the carcinogenic effect. On the other hand, 13 of 24 rats given MNU alone had bladder calculi but no bladder tumors. Thus, it appears that calculus formation alone is either not causative or not very effective in producing bladder tumors. The data also indicate that calculus formation is not obligatory for bladder carcinogenesis.

In an attempted replication of the Hicks experiment, Mohr, Green, and colleagues exposed 50 female Wistar rats to 2 mg of freshly prepared MNU by a single instillation into the bladder, followed the same day by dietary administration of 1% NaCHS (raised to 4% on week 10 of the experiment) (Green _et al._, 1980; Mohr _et al._, 1979; Soudah _et al._, 1981). The exposure was continued for the lifetime of the animals. Food intake was measured. The average dose levels of CHS consumed were, for example, 1.4 g/kg bw per day during the first week and 2.5 g/kg bw per day during week 52. At necropsy, special attention was given to the urinary system: 12 sections from the kidneys, ureters, and bladder of each rat were examined microscopically. Serving as controls were three groups of 50 female rats given either MNU alone, MNU plus 3% calcium carbonate in the diet, or water, which was instilled into their bladders instead of MNU. An additional group of 100 female rats served as untreated controls. A group receiving CHS only was not included.

No significant differences among the groups were found in food and water consumption, body weight, or survival. All three groups treated with MNU had increased incidences of urinary tumors compared to those not receiving MNU: untreated controls, 1 of 100; water-instilled controls, 1 of 50 (2%); MNU, 28 of 49 (57%); MNU plus calcium carbonate, 32 of 49 (65%); and MNU plus CHS, 35 of 50 (70%), but the incidence and average latency among the MNU-treated groups were not significantly different from one another. MNU produced tumors of the renal pelvis and ureter as well as the bladder, but a synergistic effect between dietary CHS and topical MNU instilled in the bladder was not shown.

As stated above, this attempt at replication failed because the 2 mg of MNU [the nominal subcarcinogenic dose reported by Hicks _et al._ (1975) and Hicks and Chowaniec (1977)] by itself produced a very high incidence of urinary tumors (57%) in this study. Among the possible explanations are differences in the actual amounts of MNU administered and differences in the substrains of rats, differences in technique, and differences in the histological sampling of the bladders. The authors concluded, "The unexpectedly high response to the initiating

dose of MNU makes it impossible to evaluate tumor promotion by ... cyclamate in the urinary tract" (Green et al., 1980).

The committee attempted to evaluate this study further on the basis of tumor location within the urinary tract, histological type, and degree of malignancy. Unfortunately, the tabular data are somewhat inconsistently and incompletely reported in the three papers cited above. This is presumably due in part to the additional histopathological sectioning and review after the conclusions were drawn by the investigators. For example, the two tumors in control rats are reported as papillomas in one paper and as transitional carcinomas in a later paper. In general, however, there do not appear to be any noteworthy differences in any of the factors reviewed between the group exposed to MNU alone and the group exposed to MNU plus CHS.

- N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN): Using another nitroso compound, BBN, Schmähl and Kruger (1972) investigated the possible enhancing effects of NaCHS on bladder tumors in rats. Groups of 40 male Sprague-Dawley rats approximately 100 days of age were either untreated, given BBN at 0.01 g/kg bw in their drinking water for life, or given BBN in drinking water concurrently with dietary CHS to give a CHS dose of 2.5 g/kg bw per day.

All the rats given BBN alone developed squamous cell carcinoma of the urinary bladder. Within 400 days, 50% of the animals had died. All but two of the rats given the BBN and CHS combined developed carcinoma of the bladder. The two rats that did not show carcinoma had papillomatosis of the bladder. There was no evidence of change in the latent period or in the degree of malignancy related to concomitant exposure to CHS.

Although the dose of BBN given to the rats was one-half to one-fourth of that customarily used in the early 1970s to produce bladder tumors in these animals, the effect of BBN under the conditions of the experiment was so great that it is very difficult to detect possible enhancement of carcinogenesis by CHS. Moreover, the overwhelming response to BBN could easily have obscured possible inhibition of carcinogenesis by CHS. Consequently, this study provides little information on the carcinogenicity of CHS.

Fukushima et al. (1982) compared the effects of partial cystectomy with and without NaCHS on the induction of preneoplastic lesions in the bladders of rats previously exposed to limited amounts of BBN. Groups of 10 male Fischer 344 rats were exposed to 0.05% BBN in their drinking water for 2 weeks followed by either 75% partial cystectomy, by 2.5% dietary NaCHS for 10 weeks, or by both. The animals were killed at the end of the 12-week period, and their bladders were examined for lesions described as papillary or nodular hyperplasia.

In other experiments in the same laboratory, Ito et al. (1983) demonstrated that a higher concentration, 0.5% BBN, in the drinking water of Fischer 344 rats induced so-called papillary or nodular hyperplasia in 100% of their bladders in 4 weeks. Four to 8 weeks later, while compound administration was continued, the formation of papillomas and carcinomas was observed, indicating that the hyperplastic lesions were predictors of later carcinoma formation.

Under the conditions of the experiment by Fukushima et al. (1982), BBN alone had little effect on the bladders of rats: 1 of 10 (10%) had papillary or nodular hyperplasia. The combined treatment of BBN and partial cystectomy produced no detectable papillary or nodular hyperplasia. Exposure to NaCHS did not alter the results significantly. The incidences of papillary or nodular hyperplasia were 1 of 10 (10%) in the group receiving BBN plus CHS, 0 of 7 in the group with partial cystectomy and CHS but no BBN, and 1 of 8 (12.5%) in the group receiving all three treatments (BBN, partial cystectomy, and CHS).

CHS had no demonstrable enhancing effect on lesions induced by a known carcinogen and considered by the authors to be preneoplastic. The experiment provides little evidence against a nonenhancing effect, however, since the dose rate selected for BBN administration may not have been optimal, the group sizes were small, the duration of the experiment was short, association between the hyperplastic end point measured and carcinogenesis is somewhat uncertain, and the partial cystectomies appear to have decreased urine retention times in the bladder as well as the amount of bladder mucosa at risk of transformation.

In a similar experiment by Ito et al. (1983), 36 6-week-old male Fischer 344 rats were given 0.01% BBN in their drinking water for 4 weeks, followed by dietary administration of 2.5% NaCHS for an additional 32 weeks. The rats were then killed, and their bladders were examined for papillary or nodular hyperplasia. Twenty-eight control rats received BBN but not CHS. Another control group of 60 male Fischer 344 rats received BBN without CHS but were killed 2 weeks later after a total experimental period of 38 weeks rather than 36 weeks. The incidences of papillary or nodular hyperplasias, not significantly different among the three groups, was 17 of 30 (57%) in the group receiving BBN followed by CHS and 11 of 28 (39%) and 27 of 60 (45%) in the two groups receiving BBN for 36 and 38 weeks, respectively. Measurements of the hyperplastic portions of the urothelium indicated that there were no significant differences among the three groups.

In this experiment, a lower concentration of BBN was administered for a longer period than in the previous study. Dose-range finding experiments (Ito et al., 1983) had correctly predicted that this dose rate would produce a slightly less than 50% incidence of papillary and nodular hyperplasias. Subsequent administration of CHS at the dose level selected had no apparent effect on the hyperplastic lesions

induced by BBN. The experiment provides no support for an enhancing effect of CHS on carcinogen-induced preneoplastic lesions but was limited by the relatively low dietary concentration of CHS.

Inhibition of Carcinogenesis

Mouse

• Benzo[a]pyrene and cyclamate: In a study designed to investigate the possible tumor-promoting activity of NaCHS for the stomach, Roe et al. (1970) pretreated a group of 50 female Swiss mice with a single 50 mg intragastric instillation of benzo[a]pyrene (BaP) in polyethylene glycol (PEG) in the morning after fasting from the previous midnight. Seven weeks later the mice were started on a diet containing 5% NaCHS. One control group of 50 mice received the polyethylene glycol vehicle without BaP followed by dietary CHS, and another control group of 100 mice received BaP but no CHS. Eighteen months after pretreatment with BaP or polyethylene glycol, the mice were killed and all grossly visible lesions suspected of being neoplasms were examined microscopically. No significant differences in survival or body weight were found among the groups. BaP produced tumors in the forestomachs of 21 of 61 (34%) surviving mice given BaP alone (20 papillomas, 1 carcinoma). Of 34 surviving mice that received CHS but not BaP, one (3%) had a papilloma of the forestomach. In 41 mice receiving BaP followed by dietary CHS, 4 (10%) had forestomach papillomas. The bladders with no grossly visible tumors were not examined microscopically.

There was no evidence that BaP-induced forestomach tumors were enhanced by NaCHS. If anything, the data showed an inhibitory effect under the specific conditions of this experiment. The difference between the 34% prevalence in mice receiving BaP alone and the 10% prevalence in those receiving BaP plus CHS has considerable weight because this study in effect was a single sacrifice experiment (all other data were censored or not collected), and the proportion in each group surviving to terminal sacrifice was quite good (61% for BaP alone, 68% for CHS alone, and 82% for BaP plus CHS). The results of this study must be regarded cautiously, however, because of the limited histopathology and nonrandom allocation of mice to test groups.

Rat

• 2-Acetylaminoflurene and cyclamate: Ershoff and Bajwa (1974) fed a group of 12 female Horton-Sprague-Dawley rats AAF and NaCHS concurrently in the diet for 40 weeks. The dose of AAF was 0.30 g/kg diet; NaCHS was administered at a concentration of 2.5 g/kg bw per day (5% of the diet). One control group of 12 female rats received AAF alone, and another control group of 12 female rats was untreated. Two sets of end points were measured: (1) palpation and macroscopic

observation of mammary and ear duct tumors, and (2) microscopic evaluation of liver and urinary bladder tumors.

Both groups of rats fed AAF for 40 weeks had retarded body weight gain, and after 20 weeks of treatment the rats fed CHS had lost a slight additional amount of weight. Only 2 of the 12 rats fed AAF and CHS had developed palpable tumors in contrast to 11 of 12 rats that had such tumors in the group that was fed AAF alone. Of the 24 palpable tumors in the AAF-treated rats, 3 were ear duct tumors and 21 were mammary tumors. In the AAF-plus-CHS group, each of the two affected rats had single tumors: one was an ear duct tumor and one was a mammary tumor. No tumors were found in the untreated control group. All rats fed AAF had liver tumors, but the tumors in the group also receiving CHS were smaller and were judged to be of a lower grade of malignancy microscopically. In the urinary bladder, the mucosal lining was hyperplastic in rats fed AAF but no tumors were found.

Failure to measure food consumption is one of the limitations of this study. The authors recognized that the animals fed AAF and CHS may have ingested fewer calories and thus less AAF. This study is also limited because the group sizes were smaller and only one dose level of each substance was used. It does suggest, however, that CHS might inhibit AAF tumorigenicity.

Data summarizing the combined effects of NaCHS with other substances in rodents are summarized in Table 5-5.

Summary and Conclusions

The effects of CHS in modifying carcinogenesis by other chemical substances have received relatively little study in experimental animals. Evidence for the enhancement of carcinogenicity (tumor promotion or cocarcinogenicity) by CHS comes from two experiments of different types. In one, a single application of MNU to the bladder followed by dietary CHS resulted in approximately 50% bladder tumors in female rats (Hicks and Chowaniec, 1977; Hicks _et al._, 1975). In the other, pellets containing both cholesterol and CHS produced a sevenfold increase in bladder tumors over cholesterol alone when implanted into the bladders of mice (Bryan and Ertürk, 1970). In another type of experiment, rats given BBN in drinking water followed by dietary CHS showed no enhancement, but the end point measured was an early preneoplastic change rather than later developing cancer (Ito _et al._, 1983).

Neither of the two studies reporting enhancement of bladder carcinogenicity have been satisfactorily repeated. The committee found no reports on the combined effects of CHS and other substances on carcinogenicity in parts of the body other than bladder, and no reports in which the capabilities of CHS to initiate or promote

TABLE 5-5. Combined Effects of Sodium Cyclamate With Other Substances

Test Animal	Strain	Cyclamate Route	Other Agent	Route	Reference
Experiments to determine enhancement:					
Mouse	Swiss	Pellet implanted in bladder	Cholesterol	Pellet implanted in bladder	Bryan and Ertürk, 1970
Mouse	C3Hf	Drinking water	AAF	Feed	Muranyi-Kovacs et al., 1975
Mouse	C57BL	Drinking water	X-rays	Whole body	Muranyi-Kovacs et al., 1975
Rat	Wistar	Feed	MNU	Bladder instillation	Hicks et al., 1975; Hicks and Chowaniec, 1977
Rat	Wistar	Feed	MNU	Bladder	Mohr et al., 1979; Green et al., 1980; Soudah et al., 1981
Rat	Sprague-Dawley	Feed	BBN	Drinking water	Schmähl and Kruger, 1972
Rat	Fischer 344	Feed	BBN ± cystectomy	Drinking water	Fukushima et al., 1982
Rat	Fischer 344	Feed	BBN	Drinking water	Ito et al., 1983
Experiments to determine inhibition:					
Mouse	Swiss	Feed	BaP in PEG	Intragastric	Roe et al., 1970
Rat	Sprague-Dawley	Feed	AAF	Diet	Ershoff and Bajwa, 1974

cancer were fully explored according to currently accepted criteria (OSTP, 1984).

Only two studies suggested tumor inhibition or suppression by CHS when the CHS was fed concurrently with AAF to rats (Ershoff and Bajwa, 1974) or when fed after gastric instillation of BaP (Roe et al., 1970), but these studies by themselves are not definitive.

COMPLICATING FACTORS IN BLADDER CARCINOGENESIS

While evaluating the experiments described in the preceding pages, the committee kept in mind the different factors that may have had confounding effects on the results. Of particular interest were predisposing events leading to the development of "spontaneous" bladder tumors. Relatively little is known about this subject, however, despite the fact that bladder carcinogenesis has been studied extensively in rats and other rodents.

Recent data on control rats indicate that the spontaneous incidence of benign urinary bladder papillomas in most commonly used strains ranges from 0.5% to 1% in rats allowed to live most of their expected lifetimes. The incidence of spontaneous transitional cell carcinoma, although not well established, seems to range from 0.05% to 0.1%. Some attempts have been made to determine whether urolithiasis (bladder stones) and the parasite _Trichosomoides crassicauda_ in the urinary system of rats are associated with the development of spontaneous bladder tumors or with chemically induced tumors as predisposing factors or as tumor promotors.

Trichosomoides crassicauda

There is no clear evidence that the nematode _T. crassicauda_ is involved in the development of bladder carcinoma in untreated control rats. Although infestation with this parasite may lead to nodular and papillary hyperplastic lesions in the urothelium, there is no documented evidence that these lesions progress to carcinomas (Cohen, 1983).

In the 1960s, Chapman (1964, 1969) expressed the view that bladder tumors may be causally related to the presence of this parasite; however, more recent evidence indicates that both hyperplastic lesions and papillomas occur spontaneously in colonies of untreated animals that are free of the parasite.

The effect that the parasite may have on the development of chemically induced tumors is less clear, mainly because few experiments have been conducted on this subject. Chapman (1969) did not find any exacerbating effect of the parasite on 2-AAF-induced tumors in rats. Because this experiment suffered from numerous shortcomings, no firm conclusions can be drawn. Although _T. crassicauda_ has occasionally been observed in other studies of chemical carcinogenesis (Ito _et al._, 1975), the data are generally insufficient to draw any conclusions with regard to its influence, if any, on chemically induced bladder tumors. This parasite has been observed in approximately one-half of the chronic animal studies conducted on CHS salts, but there is no evidence to suggest any interaction between parasite infestation and the administration of CHS that would contribute to the development of bladder tumors. In fact, with the exception of a study of a CHS-saccharin mixture by Price _et al._ (1970), the incidence of bladder tumors in CHS-treated rats derived from infected colonies has not been statistically elevated over controls in any of the studies reviewed by the committee.

Urinary Calculi

There does appear to be an association between the presence of bladder stones or foreign objects and the development of tumors in

rodents. Studies have demonstrated that implantation of pure cholesterol or paraffin pellets into the bladder of mice results in the development of papillomas and low grade carcinomas (Bonsor et al., 1953; Boyland et al., 1964; Clayson et al., 1958; Jull, 1975). In rats, bladder stones also have been causally related to the development of bladder tumors. Melnick et al. (1984) noted a statistically significant association between stone formation and bladder tumor induction in melamine-treated rats. Gross (1974) observed that urolithiasis was causally related to bladder tumor induction in rats fed a diet containing 5% terephthalic acid (Gross, 1974). Anver and Cohen (1979) reported that renal calculi and bladder stones appear to occur spontaneously in several strains of rats and that they may be partly responsible for the development of spontaneous bladder lesions.

In studies on CHS, bladder stones have been observed to different degrees. In general they have been found more often in studies with the calcium salt than with the sodium salt. Of all the studies reviewed by the committee, however, the most extensive degree of urinary tract calcification was reported by Price et al. (1970), even though the sodium salt was fed. If an association among CHS administration, bladder stones, and tumor formation were postulated, one would expect to see more tumors in CaCHS-treated rats than in those exposed to NaCHS, but this was not the case. The cause of the extensive calcification observed by Price and colleagues is not known but may have been related to the diet fed to the animals or to the unique strain of animals used in this test. Overall there is no convincing evidence of an association between stone formation and bladder tumor development in CHS-treated rats.

OVERALL SUMMARY AND CONCLUSIONS

Animal bioassays to test for the possible carcinogenicity of CHS have been extensive. Both sexes of a variety of strains of rats and mice have been used in experiments that collectively meet contemporary practices for carcinogenicity bioassays. The most relevant route of exposure for humans, the oral route, has been used in studies of rodents given CHS in drinking water or mixed with food. In the judgment of the committee, these studies and limited studies in hamsters and nonhuman primates have not provided convincing evidence for CHS carcinogenicity. In mice there were several equivocal findings of tumors at sites known to have high incidences of spontaneous tumors. None of these findings were confirmed in later, more extensive tests. Early studies in rats had raised suspicion that CHS might cause bladder cancer. The overall evidence from more recent and better studies capable of identifying such effects, however, indicates that CHS has not increased the incidence of bladder cancer in rats.

In animal bioassays, NaCHS has been investigated more often than CaCHS. The committee concluded, however, that both compounds have been adequately tested in rats.

In one bioassay, rats dosed with a 10-to-1 CHS-saccharin mixture were reported to have developed bladder tumors. The results suggested that CHS might have a synergistic effect when administered with the known bladder carcinogen saccharin. Two attempts by others to reproduce those findings (in a different strain of rat) failed to do so as did a multigeneration study, and the committee was unable to find any other evidence from animal studies to support this hypothesis. In all the studies of mixtures available for review, the ratio of cyclamate to saccharin was 10 to 1, and the dose levels of saccharin in the mixtures were lower than those expected to produce carcinogenic effects if saccharin were administered by itself.

CHA--the major metabolite of CHS--has been adequately tested in the mouse and less well but more often in the rat. The dose levels used in the bioassays of CHA were restricted by the toxicity and the unpalatability of the compound, but under the conditions of the experiments there was no evidence of carcinogenicity.

The committee concluded that both CHS and CHA have been adequately tested in animals by themselves without providing convincing evidence for carcinogenicity. When CHS was tested in combination with other substances, however, some evidence that was obtained suggests a tumor-promoting or cocarcinogenic[1] effect in the rodent bladder.

In one study, Hicks and her colleagues demonstrated that the single instillation of MNU (a known and direct-acting bladder carcinogen) into the bladders of Wistar rats at noncarcinogenic doses followed by dietary exposure to NaCHS resulted in very high incidences of bladder tumors in only a few months (Hicks and Chowaniec, 1977; Hicks _et al_., 1975). The results are so striking that they are unlikely to represent false-positive results. Two attempts to repeat their procedure and reproduce their findings have failed (Green _et al_., 1980; Soudah _et al_., 1981). However, the effective dose of MNU used by Hicks and her colleagues was not duplicated, and the follow-up studies therefore neither support nor refute their findings. The Hicks study strongly suggests that CHS has tumor-promoting activity.

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[1] By cocarcinogenesis, the committee means augmentation of the neoplastic response brought about by a noncarcinogenic factor operating in conjunction with a carcinogen. Operationally, cocarcinogenesis is defined herein as the enhancement of carcinogenicity resulting from administration of the noncarcinogenic modifying factor (cyclamate) either just before or together with the carcinogenic agent (cholesterol pellet). Tumor promotion, on the other hand, is used operationally to refer to enhancement of the tumorigenic response to an agent (MNU) when the second agent (cyclamate) is administered later.

In the second study (Bryan and Ertürk, 1970), pellets containing cholesterol and NaCHS were surgically implanted in the lumens of the urinary bladders of Swiss mice. In this study, cholesterol pellets alone caused bladder cancers, but the combination of cholesterol and CHS produced a much larger number of bladder tumors. This experiment suggests that CHS has cocarcinogenic activity with cholesterol. It could also be interpreted as demonstrating the tumor-initiating activity of CHS.

The interpretation of carcinogenesis studies involving implantation of foreign materials into the bladder lumen must be approached with caution. Clayson (1974) has discussed the limitations of this technique as a method of detecting bladder carcinogens. It is now well established that foreign materials, such as glass beads, paraffin and cholesterol pellets, calcium oxalate concretions, and even particles of wood, may themselves, with no further treatment, induce bladder tumors in rodents following surgical implantation into the bladder lumen. The incidence of tumors in animals containing such implants varies tremendously from study to study. In addition, Chapman _et al_. (1973) reported that tumor incidence appeared to be enhanced by natural calculi growing on implanted paraffin pellets. This may have resulted from the increased size of the pellets due to calculi or to the roughness of the foreign body produced by the growths. Thus, careful attention should be given to the size, configuration, and surface characteristics of any foreign body associated with tumors in studies involving bladder implantation.

Because chronic exposure to tumor promotors or cocarcinogens could represent potential public health risks, the committee strongly recommends that the experiment of Hicks _et al_. (1975) be repeated to seek confirmation or refutation. Some, but not all members of the committee also believe that the experiment of Bryan and Ertürk (1970) should be repeated. Those members who recommended repetition of this study believe that repetition could serve to confirm or exclude the possibility that the experiment was technically flawed. Other members have serious reservations about drawing any meaningful conclusions from repetition, even if the original findings are confirmed, in part because "the route of administration is inappropriate for assessing the carcinogenicity of a human dietary constituent" (NCI Temporary Committee, 1976). All members of the committee agreed that if the findings of these experiments are confirmed, uncertainty would still exist about the qualitative or quantitative assessment of human risk of cyclamate use because there are no data bases for extrapolating from the experimental model systems of promotion or cocarcinogenesis in the urinary bladder to natural human exposures. Therefore, it is not possible to state whether or not a positive outcome of these studies would indicate a risk of cyclamate to humans. Regardless of the outcome of these studies, the committee recommends that more research be carried out to gain an understanding of the predictive value for human health of such results. The committee recommends further that more generic research be carried out to develop properly

validated, relevant systems for the assessment of cancer-promoting agents.

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Chapter 6

Epidemiological Studies

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Epidemiological Studies

Studies to examine the potential carcinogenicity of cyclamate in humans have dealt almost exclusively with bladder cancer. This chapter therefore relates largely to cancer at that site.

Three types of epidemiological investigations have been conducted on bladder cancer in relation to artificial sweetener use: studies of time trends, case-control studies, and prospective studies. In time-trend studies, incidence or mortality rates of a disease are analyzed over time to determine if they have changed in association with changes in exposure to a putative causal agent. In case-control studies, a series of cases is identified, a suitable control group of unaffected individuals is selected, and the proportions of cases and controls with a history of exposure to factors of interest are compared. The relative risk (as estimated by the odds ratio), i.e., the ratio of risk in those exposed relative to that in those not exposed, is calculated in such studies and is the usual parameter used to indicate statistical associations between the factor of interest and the disease under study. In prospective studies, exposed persons are followed, and their incidence or mortality rates for diseases of interest are compared to those of unexposed persons or the general population.

EPIDEMIOLOGICAL STUDIES CONSIDERED

All published reports of epidemiological studies on artificial sweeteners in relation to human neoplasms were reviewed by the committee. In the different countries in which the studies were conducted, people had access to a variety of artificial sweeteners whose composition varied over time. Some studies were conducted in places where there was little or no opportunity for exposure to cyclamate. Often when there was exposure to cyclamate, no attempt was made to assess risk specifically in relation to cyclamate use, but only in relation to all types of artificial sweeteners together.

The epidemiological studies were therefore divided into three classes. Class 1 comprises studies that contain no information on

risks due to cyclamate use, because they were conducted where little or no exposure to cyclamate had occurred. Class 2 contains studies conducted where exposure to cyclamate had occurred, but in which data were presented only for artificial sweeteners in the aggregate. These studies provide information on mixed exposure to cyclamate and saccharin. Class 3 consists of studies in which some attempt was made to assess risk specifically in relation to cyclamate use.

Class 1 Studies

Kessler (1970) investigated the mortality experience of a cohort of more than 20,000 diabetics registered at a Boston diabetes clinic between 1930 and 1956. Since cyclamate consumption during the period of this study was practically nil, this study has been placed in Class 1.

In a time-trend analysis in the United Kingdom, Armstrong and Doll (1974) compared the mortality rates for bladder cancer in various birth cohorts with per capita saccharin consumption. According to the authors, cyclamate was first permitted in soft drinks in 1965 and in tablets in 1967. It was banned at the end of 1969. In the committee's opinion, the period during which cyclamate use was permitted in the United Kingdom was too short, and the study was conducted too soon after the first use of cyclamate, for any effect of cyclamate on risk to be observable in this investigation.

In another study, Armstrong and Doll (1975) studied causes of death among diabetics. This was also placed in Class 1 because of the low prevalence of cyclamate use.

Later, Armstrong et al. (1976) studied the mortality experience of a cohort consisting of 5,971 members of the British Diabetic Association (BDA) to determine whether their cancer mortality exceeded the expected rate, based on mortality in England and Wales. A survey indicated that more than half of the BDA members used saccharin tablets daily, but no data on cyclamate use were presented. Because the follow-up period was 5 to 8 years and the study was terminated in mid-1973, the exposure of this cohort to cyclamate was extremely limited. Thus, it has also been placed in Class 1.

Morrison et al. (1982) conducted a case-control study of artificial sweetener use in Manchester, United Kingdom, and in Nagoya, Japan. According to the authors, the sweetener in use at the time was primarily saccharin. The study was therefore designated as Class 1.

Cartwright et al. (1981) reported another case-control study in the United Kingdom in West Yorkshire. Throughout the paper, sacoharin is referred to explicitly, and for the time and population involved, cyclamate use would have been minimal.

In an effort to determine whether prenatal exposure to artificial sweeteners alters risk of bladder cancer, Moller-Jensen and Kamby investigated bladder cancer rates in cohorts of people born between 1941 and 1945, when sugar was scarce and artificial sweeteners were widely used (Jensen and Kamby, 1982). The artificial sweeteners available at that time contained no cyclamate.

Class 2 Studies

The eight studies in Class 2 are listed below. They are all case-control studies. Where more than one paper has been published pertaining to a particular study, each paper is cited.

Morgan and Jain, 1974

Wynder and Goldsmith, 1977

Howe *et al.*, 1977, 1980; Miller and Howe, 1977

Morrison and Buring, 1980

Hoover *et al.*, 1980; Hoover and Hartge, 1982;
Walker *et al.*, 1982; Silverman *et al.*, 1983

Najem *et al.*, 1982

Wynder and Stellman, 1980a,b

Mommsen *et al.*, 1982, 1983

These studies are described in detail in a subsequent section of this chapter.

Class 3 Studies

Four studies of bladder cancer have attempted to deal explicitly with exposure to cyclamate as distinct from saccharin. Three of them (Kessler and Clark, 1978; Moller-Jensen *et al.*, 1983; Simon *et al.*, 1975) are case-control studies similar in design to the Class 2 studies and also provide the same kind of information on mixed exposure to cyclamate and saccharin as Class 2 studies. Two of these were conducted in the United States; one (Moller-Jensen *et al.*, 1983) was conducted in Denmark. The fourth study (Burbank and Fraumeni, 1970) is a time-trend study. In addition, Barkin *et al.* (1977) reported three cases of bladder cancer in men who had regularly consumed unusually large quantities of cyclamate.

STUDIES OF MIXED ARTIFICIAL SWEETENER USE

Time-Trend Study

Burbank and Fraumeni (1970) examined the trends in bladder cancer mortality in relation to the time artificial sweeteners were introduced. They found that there was a steady increase in bladder cancer mortality in the United States from 1950 to 1967 but no discernible change in the rate of increase following the widespread introduction of cyclamate and saccharin mixtures during the early 1960s.

Case-Control Studies

The committee evaluated reports from case-control studies that provided estimates of relative risks for bladder cancer in relation to artificial sweetener use in the aggregate. All users of artificial sweeteners in those studies were exposed to sweetening agents at a time when at least some of them contained cyclamate. If such studies showed an increased risk in relation to use of artificial sweeteners, then this would suggest that either cyclamate, saccharin, or both are carcinogenic for the human bladder.

In a study by Morgan and Jain (1974), questionnaires were mailed to histologically confirmed bladder cancer patients and to matched controls with a previous diagnosis of benign prostatic hypertrophy (males) or stress incontinence (females). Controlling for smoking and alcohol consumption, the authors found that the relative risk for bladder cancer was 1.0 for men and 0.4 for women. There were several problems with this study, including a low response rate (69% of the cases, 57% of the controls) and missing data on artificial sweetener use (artificial sweetener data were available for only 64% of the cases and 49% of the controls). Moreover, the medical conditions of the control group may in themselves have influenced intake of fluids, including artificially sweetened beverages.

Wynder and Goldsmith (1977) added three brief questions concerning saccharin consumption to the end of a detailed questionnaire devoted primarily to tobacco use. The interviewers identified newly diagnosed cancer cases falling within certain specific tobacco-related categories (lung, larynx, mouth, esophagus, and bladder) in a network of hospitals throughout the United States. Unmatched controls were selected concurrently with the cases from among persons hospitalized for causes not related to tobacco use, e.g., fractures, benign prostatic hypertrophy, and gynecological disorders. Because the three questions on artificial sweeteners were introduced into the study questionnaire shortly before the analysis was begun, data are available only for 132 of the 574 men (23.0%) and for 31 of 158 women (19.6%). The actual number of artificial sweetener users was extremely small -- only 13 male and 4 female cases -- and there were 16 male and 5 female exposed controls. Relative risks were very close

to 1.0, but confidence limits were extremely broad.

Howe and colleagues were the first to demonstrate a positive association between cancer and artificial sweetener use, albeit in men only (Howe et al., 1977, 1980; Miller and Howe, 1977). Cases included 480 males and 152 females with newly diagnosed primary bladder cancer, as ascertained through provincial registries, pathologists, and urologists in British Columbia, Nova Scotia, and Newfoundland. Controls were selected from the general population of these areas and were matched to cases on age and neighborhood of residence. Among the females, no increase in bladder cancer risk was associated with the use of artificial sweeteners (relative risk = 0.6), whereas males using sweeteners had a significantly increased relative risk of bladder cancer (1.6). This increase was observed only in users of tabletop artificial sweeteners, but not in relation to dietetic beverage consumption. The risk estimates were not changed when controlled for occupational exposure, history of bladder or kidney infections, smoking combined with consumption of instant coffee, and use of a private water supply. Examination of three levels of tablet usage revealed an increase in risk with increased quantity and duration of use. For those who consumed more than 2,500 tablets per year for more than 3 years, the relative risk was 5.3. Strengths of this study are a high response rate among controls, random selection of neighborhood controls, and home interviews by trained interviewers. Although extensive multivariate analysis by Howe et al. (1980) confirmed the earlier findings obtained by stratification, similar detailed analyses of the data on females were not presented, and the restriction of the observed association to males presents problems in the interpretation of the findings.

Kessler and Clark (1978) conducted a case-control study in 19 Baltimore area hospitals. Largely because of deaths prior to interview, only 519 (45.4%) of 1,143 cases were interviewed. This percentage drops to 39.9% (519 of 1,300 cases) when 157 cases identified late in the study and not "processed" are also considered eligible for study. This low response rate renders any results from this study questionable. One hospitalized control was selected for each of the 519 interviewed cases. They were matched to the cases on sex, race, age, marital status, and hospital. Of the 519 controls initially selected, 390 (75.1%) were interviewed. Additional people were selected as controls for the cases whose initial control was not studied -- a questionable practice. For persons who ever used artificial sweeteners, the investigators reported nonsignificant relative risks: 1.1 for males and 0.8 for females.

Morrison and Buring (1980) conducted a population-based study of cancers of the lower urinary tract, consisting primarily of bladder cancer cases (94%). The remainder of the cases had malignancies of the ureter, urethra, renal pelvis, and other sites. Controls were drawn from a random sample of the 1976-1977 list of residents in the Boston Standard Metropolitan Statistical Area (SMSA) and were matched

for age and sex. A more detailed questionnaire than in any of the previous studies sought information about specific brands of diet soft drinks and sugar substitutes. The relative risks for lower urinary tract cancer showed no consistent increase in risk for either male or female users of artificial sweeteners. Strengths of this study include a relatively complete ascertainment of cases within the study population and supplementation of stratified analyses with logistic regression analysis to control simultaneously for current age, age at leaving school, marital status, religion, and cigarette-smoking. Potential problems with this study include the use of an unspecified number of proxy interviews and the inclusion of some telephone interviews rather than personal interviews.

Hoover et al. (1980) reported results from a population-based case-control study that had been conducted in response to the proposed ban on saccharin in 1977 and the subsequent publication of the study by Howe et al. (1977). The study was undertaken by the National Cancer Institute (NCI), which had been asked to provide a definitive answer to the question: Does saccharin consumption cause bladder cancer in humans? New cases with a first diagnosis of bladder cancer from December 1977 through November 1978 were ascertained through population-based cancer registries in the metropolitan areas of Atlanta, Detroit, New Orleans, San Francisco, and Seattle, and the states of Connecticut, Iowa, New Jersey, New Mexico, and Utah. Included were persons 21 to 84 years old with new diagnoses of histologically confirmed carcinoma of the urinary bladder (or papilloma not specified as benign) during that period. For each case, approximately two controls were drawn from an age- and sex-stratified random sample of the general populations in the areas in which the cases arose. Interviews were conducted in the homes of the respondents. The study is the largest case-control study of bladder cancer conducted to date. It included 2,258 male and 752 female cases and 4,337 male and 1,446 female controls. For people who ever used tabletop artificial sweeteners or diet beverages, the relative risks were approximately 1.0 for both sexes.

Hoover et al. (1980) concluded:

> The data from this study do not provide support for earlier reports of a relative risk as high as 1.6 for men who used table-top AS [artificial sweetener]. Thus, this study rules out a strong or moderate carcinogenic effect on the human bladder of artificial sweeteners as these have been used in the U.S.A. in the past. In the total study group, there was no evidence of increased risk to long-term users or to those first exposed decades ago.

Despite this interpretation that there is no overall increase in risk from artificial sweetener use, heavy long-term users did have the highest relative risks of bladder cancer, and an increase was observed

in several population subgroups. These findings will be considered in relation to other similar results in a subsequent section of this chapter.

In a case-control study of tobacco-related cancers, Wynder and Stellman (1980a) also looked at artificial sweetener use. They compared the distribution of artificial sweetener and diet beverage use among 302 male and 65 female bladder cases with the distribution in hospitalized controls. Matching criteria were age, sex, race, hospital of interview, and hospital status (private, semiprivate, or ward). Relative risks were not significantly different from 1 for either males or females. A lifetime consumption index (based on quantity times duration) showed no significant differences between cases and controls. Study strengths include histopathological verification of diagnoses, high response rate, rapid identification and interview of patients in the hospital, and control for important confounding variables, including history of diabetes, obesity, occupation, religion, education, and coffee and tea consumption.

Najem et al. (1982) conducted a case-control study in New Jersey. Cases were 75 persons seen in four private urology practice clinics during 1978. Two controls were selected for each case from among other patients treated in the same practices and were matched to the case on age, place of birth, sex, race, clinic, and census tract of residence. Information was obtained on ingestion of diet beverages and saccharin. Since some of the saccharin was consumed at a time when tabletop artificial sweeteners contained cyclamate, exposure to saccharin in this study probably also included exposure to cyclamate. The relative risks in ever-users of diet beverages and saccharin were 1.2 and 1.3, respectively, and the 95% confidence limits of these estimates both included 1.0.

Mommsen et al. (1982, 1983) conducted a case-control study in a predominantly rural area of Denmark. Their subjects were 165 male and 47 female newly diagnosed bladder cancer cases who had been consecutively admitted to one hospital from 1977 to 1980. Controls were selected from the national cancer registry and were matched to the cases on age, sex, and geographical area of residence. One male control was matched to each male case, and two female controls were matched to each female case. A number of etiological factors were investigated, including socioeconomic level, type of work, exposure to chemicals, alcohol and smoking habits, coffee-drinking, drugs, and "use of artificial sweeteners as a sugar substitute." Artificial sweetener use did not rank among the 13 factors that had the highest degree of association with bladder cancer for men. No further information on men was presented. When relative risks for artificial sweetener use among women were calculated by logistic regression, a strong association was found (the estimated relative risk was 6.7). However, this estimate is based on only six exposed cases and two exposed controls, and the confidence interval of the relative risk is

wide (1.5 - 30.2). The prevalence of artificial sweetener use among the controls was low in this study (2%).

Interviews were conducted with cases in the hospital, whereas controls were questioned either through mailed questionnaires or telephone interviews. The elevated relative risk could therefore be due to more complete ascertainment of information on artificial sweetener use from the cases than from the controls.

A study reported by Moller-Jensen et al. (1983) was conducted in the Greater Copenhagen area. Although they attempted to study all cases in the area during a defined period, the authors found that they had included only about two-thirds of the cases reported in the Danish Tumor Registry for that time. Since those studied did not differ from those not studied with respect to age, sex, place of residence, and occupation, selection bias is probably not a serious problem. The investigators interviewed 389 of the 412 cases (94.4%) that had been selected for study. Controls were obtained from the national register. A random sample was selected and stratified by age and sex to be comparable to the cases. No increase in risk of bladder cancer in relation to use of artificial sweeteners was found in either men or women.

Ever-Use of Artificial Sweeteners

Table 6-1 summarizes the relative risks of bladder cancer in persons who ever used tabletop artificial sweeteners, as reported in the 10 case-control studies described above. In all but two studies, there was no association between past use of artificial sweeteners and bladder cancer in either sex. In two studies, however, risk was significantly elevated in one sex only. Howe et al. (1977) reported a relative risk of 1.6 for males and 0.6 for females. Mommsen et al. (1983) found a relative risk of 6.2 for females; the risk in males was not reported but was said to be not significant (Mommsen et al., 1982).

Heavy Use of Nonnutritive Sweeteners

Ever-use of nonnutritive sweeteners may not be a sufficiently sensitive measure to detect an increased risk, especially if risk is increased only in persons who have had heavy or long-term exposure, or only after a long latent period.

Different investigators have defined heavy dosage levels differently by using different units (e.g., drops, tablets, or milligrams per day) or different distributions of the same units (e.g., 5 or more units per day vs. 10 or more). Another problem in assessing risk in relation to dose is that the average dose from a typical serving of a diet beverage is substantially greater than for a typical serving of

TABLE 6-1. Ever-Use of Tabletop Artificial Sweeteners

Study	Sex	Number Exposed/Total Number Cases	Controls	Relative Risk	Significance or 95% Confidence Interval
Morgan and Jain, 1974	M	30/158	30/158	1.0	NS[a]
	F	13/74	28/74	0.4	$p < .01$
Wynder and Goldsmith, 1977	M	13/132	16/124	0.7	NG
	F	4/31	5/29	0.7	NG
Howe et al., 1977	M	73/480	47/480	1.6	$p = .009$
	F	18/152	30/152	0.6	NS
Kessler and Clark, 1978[c]	M	29/365	126/365	1.1	NS
	F	79/154	76/154	0.8	NS
Morrison and Buring, 1980	M			0.8	0.6-1.1
	F	NG[b]	NG	1.6	0.9-2.7
NCI Collaborative Study (Hoover et al., 1980)[c]	M	909/2,258	1,723/3,977	1.0	0.9-1.1
	F	384/742	732/1,499	1.1	0.9-1.3
Wynder and Stellman, 1980a	M	76/302	77/299	0.9	0.7-1.3
	F	14/65	19/65	0.6	0.3-1.4
Najem et al., 1982	Both	12/74	19/142	1.3	0.6-2.8
Moller-Jensen et al., 1983	M	55/284	150/583	0.7	0.5-1.0
	F	26/96	50/193	1.1	0.6-1.9
Mommsen et al., 1982, 1983	M	NG	NG	NS	NG
	F	6/47	2/97	6.7	1.5-30.2

[a] NS = Not statistically significant.
[b] NG = Not given.
[c] All forms of nonnutritive sweeteners.

food sweetened with a tabletop artificial sweetener. Therefore, the average dose for subjects classified as heavy users of tabletop artificial sweeteners who did not consume diet beverages may actually be lower than that for consumers of moderate amounts of diet beverages.

Table 6-2 summarizes the studies that provide data on heavy use of tabletop artificial sweeteners, diet drinks, or both. In the NCI study (Hoover et al., 1980), heavy use of tabletop sweeteners was defined as greater than or equal to six uses per day. No significant increase in risk was seen for users of tabletop sweeteners at this dose level. In the study by Wynder and Stellman (1980a), in which heavy use of tabletop sweeteners was defined as use of five or more servings per day, no increase in bladder cancer risk was observed at this high dose. In the study by Moller-Jensen et al. (1983), there was a relative risk of 2.8 for females who used more than 15 servings per day, which was not statistically significant, and no alteration in

TABLE 6-2. Heavy Use of Artificial Sweeteners

Study	Sex	Definition of Heavy Use	Number Exposed/Total Number Cases	Controls	Relative Risk	Significance or 95% Confidence Interval	Dose Response
TABLETOP ARTIFICIAL SWEETENERS:							
NCI Collaborative Study (Hoover et al., 1980)	M	≥ 6 average uses per day	39/1,789	59/3,390	1.1	NS[a]	No
	F	≥ 6 average uses per day	16/547	20/1,131	1.4	NS	Possible
Moller-Jensen et al., 1983	M	≥ 15 uses per day	12/281	23/547	1.0	0.5-2.0	No
	F	≥ 15 uses per day	3/93	4/175	2.8	0.9-8.8	No
Wynder and Stellman, 1980a	M	≥ 5 uses per day	16/298	16/299	1.0	NS	No
	F	≥ 5 uses per day	2/65	4/65	0.5	NS	No
DIET DRINKS:							
NCI Collaborative Study (Hoover et al., 1980)	M	≥ 3 drinks per day	25/1,853	41/3,547	1.0	NS	No
	F	≥ 3 drinks per day	15/572	20/1,198	1.4	NS	Possible
Wynder and Stellman, 1980a	M	≥ 8 per week	10/302	11/302	0.9	NS	No
	F	≥ 8 per week	1/65	3/65	0.3	NS	No
BOTH DIET DRINKS AND TABLETOP ARTIFICIAL SWEETENERS:							
NCI Collaborative Study (Hoover et al., 1980	Both	≥ 6 tabletop uses and ≥ 2 diet drinks per day	7/2,707	8/5,232	1.6	NS	Yes

[a] NS = Not statistically significant.

risk for males. No dose response was observed in any of these studies, except possibly for women in the NCI study. Data on heavy use of diet drinks were included in published reports from the NCI study (Hoover et al., 1980) and the study by Wynder and Stellman (1980a). Neither of these studies demonstrated significantly elevated relative risks among the heaviest users. In the NCI data, however, there was a slight trend of increasing risk with dose among female subjects. Data from the NCI study (Hoover et al., 1980) on diet drinks and tabletop artificial sweeteners combined are shown in the last section of Table 6-2. A relative risk of 1.6 was observed for those with more than six uses of tabletop artificial sweeteners per day and two or more diet drinks per day. Data in the published report also seem to indicate a dose response. However, the number of heavy users was small, and these results could also be due to chance.

Long-Term Use of Nonnutritive Sweeteners

The data on long-term use of nonnutritive sweeteners are summarized in Table 6-3. Wynder and Goldsmith (1977) found no increase in relative risk for those with at least 5 years of use, and Howe et al. (1977) found no increase in males after 3 or more years of use. Morrison and Buring (1980) found no increase in relative risk of bladder cancer for males; however, females who had used diet beverages for more than 5 years had a relative risk of 3.7. In the NCI study, there was no increase in relative risk for people who had used artificial sweeteners for at least 20 years (Walker et al., 1982). Wynder and Stellman (1980a) and Moller-Jensen et al. (1983) similarly found no increase in relative risk for long-term users.

Interactions

Several authors have analyzed specially selected subgroups of their study populations. These analyses provide an alternative method of controlling confounding variables (by restricting analyses to subjects homogeneous on critical variables) and allow testing of hypotheses involving interaction of artificial sweetener-related effects with other carcinogenic effects (as, for example, smoking or occupational exposures). The drawback is that such analysis is based on a new subdivision of the same data. Because this leads to multiple statistical comparisons, the chance of a spurious finding is increased. This is largely avoided if the particular subdivision of the data is chosen in advance by proposing a biologically plausible hypothesis, as opposed to searching the data for significant results and rationalizing the use of a specific subdivision leading to such results after the fact.

If artificial sweeteners induce bladder cancer, and if this effect is biologically independent of other causes of bladder cancer, then it would be easier to detect this effect in people not exposed to other known causes of bladder cancer. For example, if artificial sweeteners induce 5 new cases per 100,000 person years of exposure, these cases would be more easily detected in a population where bladder cancer occurred in the absence of artificial sweeteners at a rate of 1 case per 100,000 person years than in a population where bladder cancer occurred under the same circumstances at a rate of 50 cases per 100,000 person years. In the first population, we would be dealing roughly with a rate of 6 cases per 100,000 users of artificial sweeteners and a rate of 1 per 100,000 in nonusers for a relative risk of 6 (6 ÷ 1). In the second population, rates would be approximately 55 and 50 per 100,000 in users and nonusers, respectively, and the relative risk would be 1.1 (55 ÷ 50). On the basis of this reasoning, if artificial sweeteners induce bladder cancer independent of other exposures, then one would expect to see higher relative risks for bladder cancer in nonsmokers than in smokers. This phenomenon might be more pronounced among women than among men, because men are

TABLE 6-3. Long-Term Use of Nonnutritive Sweeteners

Study	Sex	Definition of Heavy Use	Number Exposed/Total Number Cases	Controls	Relative Risk	Significance or 95% Confidence Interval	Dose Response
Wynder and Goldsmith, 1977	M	≥ 5 years	5/132	7/124	0.6	0.2-2.2	No
	F	≥ 5 years	2/31	2/29	0.9	0.1-15.4	No
Morrison and Buring, 1980	M	≥ 10 years,					
		diet drinks	17/330	21/307	0.7	NS[b]	No
		NNS[a]	17/317	14/298	1.0	NS	No
	F	≥ 5 years,					
		diet drinks	22/123	6/123	3.7	1.8-10.3	Yes
		NNS	22/124	17/117	1.3	NS	No
Howe *et al.*, 1977	M	≥ 3 years,					
		diet drinks	NG[c]	NG	NS	NG	No
		NNS	28/455	14/455	2.0	1.2-NG	No
NCI Collaborative Study (Walker *et al.*, 1982)	Both	≥ 20 years, NNS	124/2,807	299/5,481	0.8	0.7-1.0	No
Wynder and Stellman, 1980a	M	≥ 10 years,					
		diet drinks	22/301	16/301	1.3	NS	No
		NNS	26/302	20/302	1.3	NS	No
	F	≥ 10 years,					
		diet drinks	3/65	4/65	0.7	NS	No
		NNS	5/65	8/65	0.6	NS	No
Moller-Jensen *et al.*, 1983	M	≥ 10 years	10/276	34/558	0.5	0.2-1.0	No
	F	≥ 10 years	5/90	10/180	0.8	0.3-2.5	No

[a] NNS = Nonnutritive sweeteners.
[b] NS = Not statistically significant.
[c] NG = Not given in text.

more likely to be occupationally exposed to bladder carcinogens.

Table 6-4 summarizes data from four case-control studies that provided estimates of relative risks in relation to artificial sweetener use for smoking and nonsmoking women. All estimates are greater than 1.0, and in three of the four studies, the relative risks are greater for nonsmokers than for smokers (although the actual estimates for nonsmokers were not given in two of the studies).

The analysis from the NCI study was restricted to white women who, in addition to being nonsmokers, also had not been exposed in the workplace to dye, rubber, leather, ink, or paint (Hoover *et al.*, 1980). Among these women, the relative risk for bladder cancer increased significantly with both the amount of tabletop artificial sweeteners and diet drinks consumed daily and the duration of use of these products.

TABLE 6-4. Relative Risks of Bladder Cancer in Women Who Ever Used Nonnutritive Sweeteners from Various Sources, by Smoking Status

Study	Country	Definition of Smoking	Source of Artificial Sweeteners	Relative Risk	
				Nonsmoker	Smoker
Morrison and Buring, 1980	USA	Current smoker	Diet beverages	2.6	1.2
			Sugar substitutes	2.1	1.5
Moller-Jensen *et al.*, 1983	Denmark	≥ 15 cigarettes per day	Nonnutritive sweeteners	1.2	1.2
Mommsen *et al.*, 1983	Denmark	Ever smoked	Nonnutritive sweeteners	3.3	NG[a]
NCI Collaborative Study (Hoover *et al.*, 1980)	USA	Not applicable	Tabletop artificial sweeteners	1.2	NG
			Diet beverages	1.1	NG

[a]NG = not given.

It thus appears to be a reasonably consistent finding among the various studies that bladder cancer risk is moderately elevated in women who use artificial sweeteners and who are not exposed to other bladder carcinogens. As shown in Table 6-5, the findings for men do not have this consistency, although if the men in these studies were at increased risk for reasons other than smoking, such as occupational exposures, then these differences from the findings for females are not unreasonable.

If artificial sweeteners act as promoters of the effects of other carcinogens, as suggested by some animal experiments, rather than as independent initiators of bladder cancer, then one might expect to observe higher relative risks in users of artificial sweeteners who smoke than in nonsmokers. The data for women in Table 6-4 clearly do not support this hypothesized promotional effect. Also unsupportive are the findings for men in four of the six studies summarized in Table 6-5, as well as in the Class 1 study in the United Kingdom and Japan mentioned previously (Morrison and Buring, 1980). However, in the Canadian study of Miller and Howe (1977) and in the NCI study reported by Hoover *et al.* (1980), there were associations between the use of artificial sweeteners and bladder cancer in male smokers but

not in male nonsmokers. In the NCI study, the relative risk for bladder cancer in male smokers increased with the amount of both artificial sweeteners and diet drinks consumed daily. In the Canadian study, the relative risk associated with artificial sweetener use in male smokers was greater in relatively heavy smokers than in light smokers. This was observed in both current and former smokers.

Results from both the NCI and Canadian studies thus support the hypothesis that artificial sweeteners potentiate the carcinogenic effects of tobacco smoke on the bladder. The reasons for the discrepant findings from other studies are unclear. The NCI study was by far the largest and, therefore, the one with the most power to detect interactions with other risk factors, such as smoking. Because they were smaller, the studies that did not show such interactions in males could have had insufficient power to do so.

In the aggregate, however, results for the two sexes are not consistent, and the findings for males are not consistent among the different studies. To explain these findings biologically, one could hypothesize a weak initiating effect observable in the (low risk) nonsmoking females and a weak promoting effect observable only in smoking males in the larger, more powerful studies. This interpretation cannot be ruled out. Other nonbiological reasons for the findings summarized in Tables 6-4 and 6-5 are also possible, however. These include chance and unidentified sources of bias. If the associations that have been summarized are causal, they could be due to cyclamate, to saccharin, or to a combination of the two.

STUDIES OF CYCLAMATE USE

One case report specific to cyclamate use and three case-control studies have been published.

Case Report

In the single case report, Barkin et al. (1977) reported bladder cancer in three persons who had consumed large amounts of cyclamate (from 40 to 75 mg/kg body weight per day). Two of these people were diabetics, and two were smokers.

Case-Control Studies

Simon et al. (1975) studied 135 (62.5%) of 216 female cases of bladder cancer hospitalized in Massachusetts and Rhode Island, and 390 (60.2%) of 648 controls from the same hospitals. They matched three controls to each case on year of birth, type of residence (i.e., urban or rural), and hospital. The study subjects were mailed a self-administered questionnaire containing specific questions about

frequency of saccharin and cyclamate use for sweetening coffee and tea and for other reasons. The respondents were thus relied upon to recall the sweetening substance contained in the product that they had used. Only the proportions of cases and controls that reported ever using cyclamate in coffee or tea were presented and used in calculating relative risks. The data shown in the top row of Table 6-6 are for cyclamate use in coffee only. The data for use in tea in that study were virtually identical. The users of coffee and tea are not mutually exclusive. The estimates of the relative risks were not

TABLE 6-5. Relative Risks of Bladder Cancer in Men Who Ever Used Artificial Sweeteners from Various Sources, by Smoking Status

Study	Country	Definition of Smoking	Source of Artificial Sweeteners	Relative Risk[a]	
				Nonsmoker	Smoker
Morrison and Buring, 1980	USA	Current	Diet beverages	0.9	0.7
			Sugar substitutes	1.1	0.6
Moller-Jensen *et al.*, 1983	Denmark	≥ 25 cigarettes per day	Nonnutritive sweeteners	1.9	0.2
Kessler and Clark, 1978	USA	Ever smoked	Nonnutritive sweeteners	1.7[b]	NG[c]
Wynder and Stellman, 1980a	USA	Current and > 10 years	Nonnutritive sweeteners	NG	0.6
Miller and Howe, 1977	Canada	Ever smoked	Nonnutritive sweeteners	0.7	1.7[d]
NCI Collaborative Study (Hoover *et al.*, 1980)	USA	> 40 cigarettes per day	Nonnutritive sweeteners	NST[e]	1.7
			Diet beverages	NST	1.4

[a] Compared to nonusers in the same smoking category.
[b] The estimated relative risk after controlling for multiple potential confounders was reported to be 2.6, which was statistically significant.
[c] NG = Not given.
[d] p =.01.
[e] NST = No significant trend with amount of artificial sweetener consumed.

TABLE 6-6. Summary of Results from Three Case-Control Studies of Cyclamate and Bladder Cancer

Study	Sex	Number Exposed/Total Number Cases	Controls	Relative Risk	95% Confidence Interval
Simon *et al.*, 1975[a]	F	9/122	22/349	1.2	(0.5-2.6)
Kessler and Clark, 1978[b]	M	86/365	79/365	1.15	(0.78-1.70)
	F	45/154	55/154	0.61	(0.34-1.14)
	Both	131/519	134/519	0.97	(0.71-1.32
Moller-Jensen *et al.*, 1983[b]	M	[c]/290	[c]/592	0.72	(0.26-2.04)
	F	[c]/98	[c]/195	1.33	(0.22-8.13)

[a] For use of cyclamate in coffee.
[b] For use of cyclamate in tea.
[c] Not available from published report.

controlled for the potentially confounding effects of other variables. Because of the matching, however, the cases and controls were comparable with respect to age, hospital, and location of residence (urban or rural). The matching was not retained in the analysis, although this is unlikely to have had an appreciable influence on the results. The relative risk of 1.2 was not significantly different from unity.

In the Kessler and Clark (1978) study (described above), in-person interviews were conducted to obtain detailed information on use of all nonnutritive sweeteners, including brand names and frequency of use. From the brand names, subjects were classified according to whether they had ever used cyclamate. If they had, their years of use and number of servings per year were estimated. Unfortunately, the latter two quantitative estimates were not used to assess risk in relation to long-term or heavy use. Estimates of relative risks in ever-users of cyclamate, and the numbers on which these estimates are based, are shown in the second through fourth rows of Table 6-6. The relative risks were estimated using logistic regression and were controlled for the potentially confounding effects of smoking, age, occupation, race, diabetes, marital status, education, overweight, and dieting. None of the relative risks are significantly different from 1.

In the Moller-Jensen study (also described above), all subjects were interviewed in person and questioned about their use of artificial sweeteners in coffee, tea, or foods for 3 or more months, the reason for their use, their ages during use, brand names used, and amounts consumed. Use of cyclamate was determined from the brand names of the products. Unfortunately, the data on cyclamate were not

presented in detail in the report of the study. The authors reported that 10.7% of the study subjects (presumably both cases and controls combined) used artificial sweeteners. The relative risks for men and women who had ever used cyclamate are shown in the last two lines of Table 6-6. Neither of the estimates shown are significantly different from unity. The estimates were calculated using the Mantel-Haenszel method to control for the potentially confounding effects of age, sex, and smoking. Other variables were also considered but no controls were instituted for them because there were no differences between cases and controls.

In the aggregate, these three studies provide no evidence of an increased risk of bladder cancer in cyclamate users. None of the estimates of relative risk is significantly different from unity, and the results are not consistent between the two sexes or among the three studies. The variation in relative risks is most likely due to chance.

The chances of a true increase in bladder cancer risk in cyclamate users being detected by these studies are not high for several reasons. As shown in Table 6-7, the study by Simon _et al._ (1975) had an 80% chance of detecting a relative risk not lower than 2.8 in people who had ever used cyclamate. The other studies had an 80% chance of detecting relative risks less than 2, which seems adequate if all users were exposed to a carcinogenic dose. However, many of

TABLE 6-7. Minimal Relative Risks of Bladder Cancer in Users of Cyclamate Detectable from Three Case-Control Studies

Study	Sex	Number of Cases	Number of Controls per Case	Percentage of Controls That Used Cyclamates	Minimal Detectable Relative Risk[a]: Ever-Users	Minimal Detectable Relative Risk[a]: Users with One-Third Heaviest Use
Simon _et al._, 1975	F	122	3	6	2.8	4.8
Kessler and Clark, 1978	M	365	1	22	1.6	2.2
	F	154	1	36	1.8	2.5
	Both	519	1	26	1.4	1.8
Moller-Jensen _et al._, 1983	M	290	2	10	1.9	2.7
	F	98	2	10	2.7	4.7
	Both	388	2	10	1.7	2.4

[a] Assuming a two-sided test with $\alpha = .05$ and $\beta = .80$.

the persons in the ever-use category may have used cyclamate only occasionally or for short periods. If we assume that risk is elevated only in the one-third of the users who used the most cyclamate, then, as shown in the last column of Table 6-7, the power of the studies is much lower. By combining data from both sexes, only the Kessler and Clark (1978) study would have an 80% chance of detecting an actual relative risk of less than 2.

More importantly, although cyclamate was used in the United States from about 1950, consumption levels were low until about 1960, and they were banned in 1969. In Denmark, they were available only from 1960 to 1969. It is therefore possible that cyclamate, even if carcinogenic, was not ingested in sufficient amounts or for a sufficiently long period to alter risk in humans to an extent measurable by the epidemiological studies cited above.

The latency period between exposure to a carcinogen and the development of a detectable carcinoma must also be considered. In the two U.S. studies (Kessler and Clark, 1978; Simon et al., 1975), the last cases were diagnosed in 1975 and 1971, respectively. Although these diagnoses were 25 and 21 years since the first opportunity for exposure in 1950, it is unlikely that many study subjects were exposed before 1960, which is only 15 and 11 years prior to the diagnosis of the last cases studied. In the study by Moller-Jensen et al. (1983), the last case was diagnosed in 1981, which is 21 years after the first opportunity for exposure in 1960, but again most study subjects were probably exposed after 1960. It is therefore unlikely that any of the three studies could have detected an increase in risk if the latency period following exposure to cyclamate is greater than approximately 15 years. Many industrial bladder carcinogens have latency periods longer than this.

In summary, although the three epidemiological studies of cyclamate use do not show an increased risk of bladder cancer in persons who had ever ingested these substances, none of the studies could have detected anything but rather large increases in risk following a relatively short latency period after fairly low levels of exposure. The studies could not have detected small increases in risk in heavy users after a long latency period.

Because of flaws in the Kessler and Clark (1978) study (especially the low response rate), further analysis of the data from that investigation would be unlikely to provide additional useful information. Because of the low prevalence of use, and the short period during which cyclamate was available in Denmark, further analysis of the Moller-Jensen et al. (1983) study would probably also not be fruitful. The Simon et al. (1975) study lacks sufficient statistical power to warrant consideration for further data analysis and contains no information on duration.

STUDIES OF CANCERS OTHER THAN BLADDER

With the exception of the study by McLaughlin *et al.* (1984), all case-control studies performed to date have focused exclusively on cancer of the urinary tract, primarily the urinary bladder and to a lesser extent related cancers of other parts of the urinary tract, such as the ureter, urethra, and renal pelvis. The explanation for this comes from results of animal experiments. In 1977, when the Canadian Government made public the results of its saccharin feeding experiments in rats, which ultimately led to the proposed banning of saccharin in the United States (and its actual banning in Canada), the tumors observed in the rats involved the urinary bladder. The studies in humans that followed were, in effect, testing hypotheses based on a rat model. If the rat model is not the most appropriate for humans, and if the hypothesis that cyclamate causes human cancer is worthy of epidemiological testing, then cancers other than those of the bladder should also be investigated.

Case-Control Studies of Kidney Cancer

The only published study dealing primarily with kidney cancer was conducted by McLaughlin *et al.* (1984), who investigated all white residents of the Minneapolis-St. Paul SMSA who had a first diagnosis of renal cell carcinoma between January 1974 and June 1979. Controls were selected via age- and sex-stratified sampling from telephone listings (ages 30 through 64) or Health Care Financing Administration records (ages 65 through 85). Data were obtained for 495 of 506 identified cases, giving a nominal response rate of 98%. However, because of what the authors describe as the "retrospective nature of the case ascertainment and the case-fatality rate of renal cell carcinoma," nearly half the interviews were obtained from spouses, children, siblings, and friends. The influence of proxy interviews on the results is uncertain.

Regular use of diet soda or tabletop artificial sweeteners for 6 months or longer was associated with age- and smoking-adjusted relative risks of 1.8 for males and 1.0 for females, neither of which was statistically significant. There was no evidence of a dose response in either sex.

In their bladder cancer study, Wynder and Stellman (1980a) found obesity to be a major correlate of artificial sweetener use. They noted that although obesity is not a risk factor for bladder cancer, it is a risk factor for kidney cancer and predicted that kidney cancer patients would be heavy users of nonnutritive sweeteners. They confirmed this prediction in one group of 65 male kidney cancer patients but not in a second group of 88. This suggests that kidney cancer patients are likely to use greater than average amounts of nonnutritive sweeteners, and that obesity may be an important confounder that must be considered in determining whether cyclamate alters the risk of kidney cancer.

RESEARCH IN PROGRESS

Studies in progress or completed but not yet published will provide additional data on artificial sweetener risks. Fourteen studies were identified either from the listings in the IARC Directory of On-Going Research in Cancer Epidemiology (Muir and Wagner, 1984) or by canvassing the epidemiological community. The difficulty of separating cyclamate from saccharin exposures is likely to be as great for most of these studies as for the ones reviewed in this document. Two exceptions involve studies in Canada, where access to cyclamate in the recent past has been considerably greater than in the United States. Specific cyclamate exposure may therefore be more readily estimated in Canada than in most other studies reviewed.

Both Canadian studies are being conducted at the University of Toronto. Anthony B. Miller has undertaken a new case-control study in Southern Ontario and in part of Alberta. This investigation is modelled after his earlier study of 1977. Geoffrey Howe is directing a follow-up study of a cohort of approximately 13,000 Canadian diabetics, among whom artificial sweetener consumption has already been found to be very high. In both studies, primary consideration has been given to artificial sweeteners in the study design. Because of the relatively young age of the members of the diabetic cohort, sufficient data will not be available for several more years to produce statistically significant results.

Several of the ongoing studies are either continuations or direct outgrowths of studies already discussed. Others are entirely new and should provide fresh information from populations not previously investigated. One study, that of Oscar Auerbach and Lawrence Garfinkel, is of a design not encountered so far. It involves examination of sectioned bladders removed at autopsy and evaluation of atypia in epithelial tissue in relation to nonnutritive sweetener use as reported by relatives of the deceased.

Some of these studies extend to sites of cancer other than the bladder, including the kidney (renal cell and hypernephroma), liver, and lung. Prospective studies being conducted by the American Cancer Society and by the University of Toronto include all causes of death. The committee believes that some of the ongoing studies show promise of providing valuable information on the carcinogenicity of both cyclamate and saccharin in the future. However, decades must pass before sufficient data will have accrued to assess adequately the effects on risks of various cancers that would result from long-term exposure to high doses beginning at an early age.

CONCLUSIONS

All studies considered relevant by this committee were conducted in populations that had used both saccharin and cyclamate as

artificial sweeteners. In most of the studies, cyclamate had been used in combination with saccharin and for shorter periods than for saccharin alone. In many of them, results were reported for artificial sweeteners in the aggregate -- not specifically for cyclamate (or saccharin) alone. If the results of such studies were to show an association between bladder cancer and artificial sweeteners, it would not be possible to determine whether the increased risk, if not spurious, was due to cyclamate, saccharin, or both. Nonetheless, positive associations would raise legitimate questions as to the safety of cyclamate. These studies were therefore reviewed by the committee.

The results of studies of mixed artificial sweeteners show no significant overall increase in risk of bladder cancer in persons who have _ever_ used these substances. In four studies, however, the risk of bladder cancer was increased in nonsmoking women who used artificial sweeteners. The relative consistency among the four studies, although possibly due to chance, could also be interpreted as an indication of a true carcinogenic effect on the bladder that was more readily observable in this low risk subset of the population in the absence of the confounding or masking effects of other known carcinogens. Furthermore, in the largest study, and therefore the one with the best chance of assessing risk in long-term heavy users, Hoover _et al._ (1980) found the risk of bladder cancer in female nonsmokers to increase with the amount of nonnutritive sweeteners consumed.

In this same large study, the risk of bladder cancer was reported to be increased in persons who consumed unusually high doses of artificial sweeteners in the form of either food additives or diet drinks (Hoover _et al._, 1980). Although these findings are based on a small subset of the total data, and could represent a noncausal association, they could also suggest that use of artificial sweeteners in quantities not normally consumed in the past may increase the risk of bladder cancer. In the aggregate, the results of the studies on artificial sweeteners are therefore not totally reassuring.

Epidemiological studies distinguishing exposure to cyclamate from exposure to saccharin have not shown statistically significant associations between use of cyclamate and risk of bladder cancer. Such negative results may mean either that cyclamate does not increase the risk of bladder cancer or that the studies failed to detect a true increase in risk. Although there is no way to determine which of these interpretations is correct, there are several reasons why these studies could have failed to detect a true carcinogenic effect of cyclamate on the human bladder: (1) Prior use of cyclamate could have been inadequately ascertained from the respondents or inaccurately classified and distinguished from other artificial sweeteners by the investigators; (2) in the populations studied, cyclamate could have been used in insufficient quantities or for too short a period to cause an observable increase in bladder cancer risk; or (3) the studies could have been conducted too soon after exposure of the population to

cyclamate to detect an increase in risk after a long latent period.

On the basis of epidemiological information, the committee concluded that there is no firm evidence for an increase in risk of bladder cancer in relation to the combined use of cyclamate and saccharin as artificial sweeteners containing these substances have been used in the past. However, the data on such use are insufficient to rule out small increases in risk, elevated risk in heavy users, excess risk long after initial exposure, or increased risk in persons exposed to known bladder carcinogens. The information available is insufficient to assess risk of bladder cancer specifically in relation to cyclamate.

Data from epidemiological studies of cyclamate or saccharin and neoplasms other than bladder cancer are virtually nonexistent, except for some limited information on mixed artificial sweetener use in relation to cancer of the renal parenchyma. There is thus insufficient information to judge whether cyclamate alters the risk of neoplasms other than carcinoma of the bladder in humans.

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Appendixes

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APPENDIX A1: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CYCLAMATE CARCINOGENICITY BIOASSAY ON MICE [a]

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control, Sex and No./Group M	Control F	Treated, Sex and No./Group M	Treated F	Treatment, Duration, Dose, or Conc.	Comparable Dose g/kg/d [b]	Effective No. of Animals [c] C	T
Sodium Cyclamate	Rudali	NaCHS,	Oral-	Mice 30-d					Lifetime			
	et al.	Schuhardt-	Drinking	old					6 g/liter,	0.6	N.G.	
	(1969)	France	water						daily avg.	0.8		
	Foundation	99.5%		RIII,	30	--	30	--	3-4 ml			
	Curie, Inst.	(CHA		C3H,	20	20	20	20	(20-25 mg/kg/d)			
	du Radium,	unknown)										
	Paris,			XVII/C	--	30	--	30				
	France			F_1(C3H								
				x RIII)	40	--	40	--				
	Muranyi-Kovacs			Mice 4-6-					Lifetime			
	et al. (1975)			wk old								
	Foundation			XVII/C	56	56	28	28	1 g/liter/d	0.1	72	41
	Curie, Inst.											
	du Radium,			C57BL	35	--	35	--	6 g/liter/d	0.6	27	23
	Paris,				35	--	35	--	plus 250 rad/min		30	21
	France			C3Hf	14	14	14	14	1 g/liter/d	0.1	26	28
					14	14	14	14	plus 60 mg/kg AAF		27	27
									80 wk			
	Brantom	NaCHS,	Oral-Feed	Mice	60	60				0.0		
	et al. (1973)	Abbott Labs.,		ASH-CS1			30	30	0.7%	0.9	89	168
	Brit. Indust.	Imp. Chem.					30	30	1.75%	2.3		
	Biol. Res.	Ind.& Laporte					30	30	3.5%	4.6		
	Assoc.	Ind., 98%					30	30	7.0%	9.3		
	Surrey, U.K.	(< 100ppmCHA)										
	Homburger	NaCHS,	Oral-Feed	Mice					2 yr			
	et al.	Monsanto &		Charles	25	25				0.0		
	(1973)	Merck Co.		River			25	25	1%	1.3	36	138
	Bio-Research	Free of		CD			25	25	5%	6.7		
	Consultants,	Impur.					Duplicate					
	Cambridge,	> 99%					groups					
	Mass., USA						used					
	Kroes	NaCHS,	Oral-Feed	Mice-Swiss					21 mo			
	et al. (1975)	Bayer		outbred -								
	1977)	Farma,		SPF								
	Natl. Inst.	98.2%										
	Public Health,	(2.1 ppm		F_0 gener-	50	50			0%	0.0	81	371
	Bilthoven,	CHA)		ation			50	50	2%	2.7		
	Netherlands						50	50	5%	6.7		
				F3b gener-	50	50			0%	0.0	89	457
				ation			50	50	2%	2.7		
							50	50	5%	6.7		
				F6a gener-	50	50			0%	0.0	94	452
				ation			50	50	2%	2.7		
							50	50	5%	6.7		
Cycl:Sacc		Cycl: Sacch							21 mo			
		(Na) 10:1,		F_0 gener-			50	50	2%Cycl+0.2%Sach	2.9		
		Bayer,		ration			50	50	5%Cycl+0.5%Sach	7.3		
		Farma, 99.3%		F3b gener-			50	50	2%Cycl+0.2%Sach	2.9		
		(2.1 ppm		ration			50	50	5%Cycl+0.5%Sach	7.3		
		CHA		F6a gener-			50	50	2%Cycl+0.2%Sach	2.9		
				ration			50	50	5%Cycl+0.5%Sach	7.3		
Cyclohexylamine		CHA,							21 mo			
		Bayer		F_0 gen.			50	50	0.5%	0.065		
		Farma		F3b gen.			50	50	0.5%	0.065		
		Some		F6a gen.			50	50	0.5%	0.065		
		impurities										
	Pliss	diCHA,	Sub-	Mice	Number		22	35	24 mo	0.000-0.0793	N.G.	
	(1958)	Voroshilov	cutaneous	Strain D	and sex				Single daily	g/animal		
	Experimental	Sci.Res.	injection	[C_{57} white	not				Inj. of 0.05 ml of			
	Oncology	Inst.Org.		x C_{57} blk]	specified				2.6% oily sol. of di-			
	Lab., Acad.	By-Prod & Dyes							CHA for 11-12.5 mo			
	Med. Sci.,	diCHA-					31	23	Single daily inj.	0.051-0.059		
	Moscow,	nitrite							of 0.1 ml of 1%	g/animal		
	USSR	(Source as							aqueous sol. of			
		above)							diCHA-nitrite			
		Pure							for 12-13 mo			
	Hardy	CHA-HCl,	Oral-Feed	Mice					80 wk			
	et al. (1976)	Laporte		ASH-CS1	48	50				0.000		
	Brit. Indust.	Indust.		(SPF)			48	50	300 ppm	0.040	90	254
	Biol. Res.	N.G.					48	50	1000 ppm	0.135		
	Assoc.,						48	50	3000 ppm	0.400		
	Surrey, U.K.											

[a] Modified from original references, FDA, 1984; IARC, 1980; NCI, 1976.

[b] Assumption of ratios of body weight, food and water intake used to obtain an estimate of dosage in terms of mg/kg body weight per day were as follows:

Species	Body Weight (g)	Food Intake (g/d)	Water Intake (ml/d)
Mouse	30	4	5
Rat	400	20	30
Hamster	100	10	25

Interim or Terminal Survival	Extent of Bladder Exam.	No. with Tumors/No. Examined: Lymphoreticular [d] System		Liver		Lung		Comments on Study
No. valid animals & Avg surv.:	Mainly gross; bladders not opened							As compared to control mice, there were differences in lung tumor incidence for dosed XVII/G female mice and in hepatic tumor incidence for male C3H x RIII hybrid mice or for parental C3H or RIII strains. The study was limited by incomplete histological examinations and by unexplained losses of many dosed and control mice from the study.
22, 498d								
9M, 667d								
10F, 450d		C	T	C	T	C	T	
20, 430d		F:	—	0/16	1/20	3/16	16/20	
34, 592d		M: 2/28	0/34	12/28	22/34	2/28	7/34	
Survival range	Micro of macroscopically altered organs & suspected bladders	C	T	C	T	C	T	As compared to controls, differences in lung tumor incidence were observed in XVII/G females, but much less than in Rudali's study in the same strain. Cyclamates did not enhance leukemia incidence in C57BL treated males, whereas whole body irradiation alone enhanced it. Moreover, cyclamate decreased somewhat the leukemogenic effect of irradiation, but increased the incidence of liver and lung tumors in AAF-treated C3Hf mice. No bladder lesions were observed in any of the mouse strains.
300 d		F: 21/40	18/23	2/40	0/23	20/40	18/23	
		M: 25/32	7/18	4/32	0/18	22/32	7/18	
437-553 d		M: 4/27	3/28	0/27	0/28	1/27	2/28	
		M: 12/30	5/21	4/30	0/21	3/30	0/21	
393-454 d				2/26	3/28	0/26	4/28	
				19/27	27/27	4/27	1/27	
TS (80-84 wk)	Micro of several bladders at TS	M	F			M	F	Bladders of 8/30 males and 13/30 females fed 9.3 g/kg body weight per day (7.0%) cyclamate were not examined microscopically. The increases in lymphoreticular tumors in female mice were not dose-related and in male mice were inversely related to dose.
7M, 12F		8/46	3/45			15/46	6/45	
11M, 15F		3/21	4/19			5/21	2/19	
1M, 10F		3/27	4/18			6/27	6/18	
7M, 8F		0/23	5/21			1/23	6/21	
8M, 14F		0/24	6/25			7/24	3/25	
TS (24 mo) Survival not affected in any group by treatment	Micro of pathological & vital organs from at least 12 mice	M		M		M		No credible dose-response relationship in lymphoreticular and liver tumor incidence was observed. Only one transitional cell carcinoma together with a large bladder stone was found in a control male. Increased incidence of lung tumors in males could be due to unusually low incidence of tumors in the control group. Many mice were not examined microscopically and the group sizes were small.
		2/19		1/19		2/19		
		1/29		5/29		8/29		
		2/38		2/38		10/38		
18 mo. surv. for F_0 & F6a, 20 mo surv. for F3b gens	Complete micro, especially kidneys, livers & inflated bladders	M	F	M	F	M	F	This study essentially involved three studies, thus providing a triplicate trial for comparison. The studies lasted for only 21 months. The number of mice found autolysed in most groups was high (between 8 and 40%). These mice were not examined histologically. Seven bladder tumors were found, one in each of the seven groups. But these findings were not statistically significant. (One was found in a control female.) Bladder calculi were not associated with exposure.
34M, 30F		2/40	8/41	2/40	0/41	1/40	2/41	
27M, 26F		6/41	9/30	1/41	0/30	2/41	2/30	
34M, 33F		8/41	6/39	2/41	0/39	0/41	1/39	
28M, 26F		4/45	6/44	13/45	3/44	3/45	4/44	
30M, 25F		2/47	3/42	12/47	2/42	2/47	4/47	
22M, 20F		8/44	7/40	0/44	0/40	1/44	1/40	
38M, 29F		5/48	7/46	7/48	1/46	10/48	4/46	
30M, 19F		9/50	7/39	4/50	1/39	3/50	1/39	
31M, 31F		1/46	5/38	3/46	0/38	3/46	2/38	
		M	F	M	F	M	F	
32M, 29F		6/39	6/35	0/39	1/35	0/39	2/35	
30M, 21F		4/39	3/31	0/39	0/31	0/39	0/31	
31M, 28F		1/48	6/46	10/48	2/46	1/48	0/46	
31M, 27F		2/48	7/43	2/48	1/43	0/48	0/43	
39M, 27F		6/49	5/45	9/49	1/45	4/49	5/45	
36M, 35F		0/48	7/45	0/48	0/45	2/48	1/45	
		M	F	M	F	M	F	
37M, 29F		3/42	3/34	1/42	0/34	1/42	1/34	
46M, 34F		0/50	3/49	1/50	0/49	0/50	0/49	
39M, 37F		6/46	4/46	3/46	0/46	1/46	2/46	
After 12 mo 15 mice	None	No compound-related tumors were observed in the internal organs.						Sarcomas appeared at the site of diCHA injection in 4 of the 15 mice that were alive after 12 months or more. The oil vehicle caused granulomas. No vehicle controls were reported. There were no local tumors after aqueous diCHA-nitrite injection.
23 mice								
TS (80 wk)	Micro all bladders & abnormal tissues	M	F			M	F	Data provided no evidence for any effect of CHA-HCl on tumor induction.
8M, 8F		1/46	4/44			0/46	0/44	
7M, 11F		1/45	5/46			0/45	0/46	
7M, 15F		2/31	6/42			1/31	0/42	
10M, 9F		1/46	5/44			0/46	0/44	

[c] Effective number is the number examined histopathologically.
[d] Refers to leukemia, lymphoma, lymphosarcoma and reticulum cell sarcoma. Abbreviations: AAF - 2-acetylaminofluorene; BaP - Benzo[a]pyrene; BBN - N-butyl-N-(4-hydroxybutyl)-nitrosamine; C = Control; CHA - Cyclohexylamine; Cycl:Sacch - a mixture of 10:1 cyclamate to saccharin; F - Female; M - Male; Micro - Microscopic examination; MNU - N-methyl-N-nitrosourea; N.G. - Not given; PEG - Polyethylene glycol; T - Treated; SPF - Specific pathogen free; TS - Terminal sacrifice.

APPENDIX A 2: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CYCLAMATE CARCINOGENICITY BIOASSAY ON RATS [a]

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control Sex and No./Group M	Control Sex and No./Group F	Treated Sex and No./Group M	Treated Sex and No./Group F	Treatment, Duration, Dose, or Conc.	Comparable Dose [b] g/kg/d	Effective No. of Animals [c] C	Effective No. of Animals [c] T
Sodium or Calcium Cyclamate	Fitzhugh *et al.* (1951) FDA Wash., D.C. USA	NaCHS, Unknown N.G.	Oral-Feed	Rats Osborne Mendel	20	20			24 mo	0.0		
							20	20	0.01%	0.005	N.G.	
							20	20	0.1%	0.05		
							20	20	0.5%	0.25		
							20	20	1.0%	0.5		
							20	20	5.0%	2.5		
	Grasso *et al.* (1971) Brit. Indust. Biol. Res. Assoc., Surrey, U.K.	NaCHS, Abbott Labs., 99%	Sub-cutaneous injection	Rats Shell or Carworth Farm E strains			40	40	107 wk 0.5 ml of a 15% sol. 3 inj/wk		N.G.	
		-.-.-.-.- Calcium Abbott Labs., 99%.					30	30	-.-.-.-.- 107 wk 0.5 ml of a 15% sol. (3 inj/wk)			
							20	20	85 wk 1 ml of a 5% sol. (3 inj/wk)			
	Friedman *et al.* (1972) Div. Toxi., Bureau of Foods, FDA, Washington, D.C. USA	NaCHS, Abbott Labs., N.G.	Oral-Feed Chow diet	Rats Osborne Mendel	14	14			101 wk	0.0		
							7	7	0.4%	0.2	19	15
							7	7	2.0%	1.0		
							7	7	10.0%	5.0		
		-.-.-.-.-.- Calcium Cyclamate, Abbott Labs., N.G.			14	14			101 wk	0.0	19	17
							7	7	0.4%	0.2		
							7	7	2.0%	1.0		
							7	7	10.0%	5.0		
		CaCHS in casein diet, City Chem. Corp., Unknown	Oral-Feed Semi-synthetic diet	Rats Holtzman	20				75 wk	0.0		
							20		1% plus 20% (normal) casein	0.5	19	31
							20		2% plus 20% casein	1.0		
							28		2% plus 10% (low) casein	1.0		
	Homburger *et al.* (1973) Bio-Research Consultants, Cambridge, Mass., USA	NaCHS Abbott Labs., Free of Imp. >99%	Oral-Feed	Rats Charles River CD-1	25				24 mo	0.0		
							25 Dupl.		1%	0.5	16	71
							25 groups used		5%	2.5		
	Schmähl (1973) German Cancer Res. Ctr., Heidelberg W. Germany	NaCHS, Beyer-Werke, (CHA <4ppm)	Oral-Feed	Rats Sprague-Dawley	52	52			30 mo 0.0	0.0		
							52	52	2%	1.0	57	127
							52	52	5%	2.5		
	Bär & Griepentrog (1974) Fed. Health Dept., Berlin,W.Germ.	NaCHS, Unknown	Oral-Feed Dough cake	Rats lab-bred strain init. wt. 150-180 g	25	25	35	35	24 mo 150 mg/kg/d	0.15	N.G.	
					20	20	30	30	150 mg/kg/d	0.15		
					15	15	15	15	300 mg/kg/d	0.30		
					20	20	55	55	450 mg/kg/d	0.45		
	Hicks *et al.* (1975); Hicks & Chowaniec (1977) Middlesex Hospital Medical School, London, U.K.	NaCHS, Abbott Labs., (CHA 13 ppm)	Oral-Feed	Rats Wistar	55	50			24 mo	0.0		
							52	41	1 g/kg/d	1.0	98	228
							72	76	2 g/kg/d	2.0		
	Ikeda (1975) Natl. Inst. Hyg. Sci., Tokyo Japan	NaCHS, Ueno Seiyaku Co., 98%	Oral-Feed	Rats Wistar-5-wk old	54				28 mo 0.0	0.0		
							56		2.5 g/kg/d	2.5	33	37
	Schmähl & Habs (1980) German Cancer Res. Ctr., Heidelberg W. Germany	NaCHS, Drugofa Co., Unknown	Gavage	Rats (Offspring alive after 28d) Sprague-Dawley	25	33			14, 17, and 20 d of gestation	0.0	N.G	
							29	25	0.2 g/kg/d	0.2		
							41	39	1.0 g/kg/d	1.0		
							22	31	5.0 g/kg/d	5.0		
	Taylor *et al.* (1980) Div. of Tox. & Path., FDA, Wash., D.C., USA	CaCHS, Abbott Labs., 98% (CHA 12ppm)	Oral-Feed	Rats Sprague-Dawley-CD	48	48			114-121 wk	0.0		
							48	48	5%	2.5	49	53

Interim or Terminal Survival	Extent of Bladder Exam.	No. with Bladder Tumors/No. Examined	Comments on Study
T.S. at 24 mo	Only high dose micro examined	N.G.	No neoplasia was observed, but there is no indication that specific histopathological examination was performed on the bladder of individual rats.
N.G. At 49 wk 24 At 66 wk 10	None	N.G.	Repeated subcutaneous injection of 0.5 ml 15% and 1.0 ml 5% calcium cyclamate resulted in local deposition of calcium, fibroblastic proliferation, and sarcoma formation in 14/24 (58%) and 4/10 (40%) rats, respectively. No tumors were detected in sodium cyclamate-treated rats, thus demonstrating the potential of the calcium ion (not cyclamate) in inducing sarcoma production.
At 101 wk 6/14 4/14 5/14	Micro all bladders & kidneys	(see comments)	No carcinomas were observed in bladders. One and two bladder papillomas out of six and five animals examined were reported in the 0.4% and 10% levels, respectively (one of those in the 10% group was associated with bladder nematodes).
At 101 wk 9/14 9/14 9/14	Gross & micro	0/19 2/9(1M&1F) 0/6 1/8(M)	Transitional cell carcinomas of the bladder were observed in only one low and one high dose male, and in one low dose female. Extensive calcification of the urinary system was observed.
At 75 wk 7/20 8/20 13/20	Micro of all bladders & kidneys	(see comments)	Rats were given calcium cyclamate in casein. One out of 11 males in the 2% cyclamate plus 20% casein group had transitional cell papillomas with stones in the bladder and calcium deposits in the kidneys. All three studies suffered from the small number of animals used, high rate of intercurrent disease and early deaths, and infection with parasitic nematodes, which detract from their usefulness in the evaluation of the carcinogenicity of cyclamate.
TS at 24 mo Survival not affected in any group by treatment	Micro of pathological & vital organs from at least 12 animals/group	1/16 1/35 1/36	The relatively small number of animals remaining due to loss from intercurrent infection and autolysis decreased the sensitivity of the study. Ova of the _Trichosomoides crassicauda_ nematode parasite were noted frequently in the urine of both control and treated animals, but did not correlate with bladder tumor findings. No credible dose response in incidence of bladder carcinoma was evident.
N.G.	Micro of all bladders & abnormal tissues	0/60 1/88 0/80	Only one transitional cell carcinoma of the bladder was reported in a male rat fed 2% sodium cyclamate. The bladder of this rat also contained calculi. Parasitic nematodes were noted in 16% of all animals. This study did not show evidence of carcinogenicity. It is considered of limited value because histopathological examination was confined to the bladder.
TS at 24 mo Survival unknown	Micro of all bladders	(see comments)	Results from the high dose group (450 mg/kg/d) have not been published. No bladder tumors were reported from the lower dose levels. Therefore, few definitive conclusions can be drawn from this study.
TS at 24 mo	Micro of inflated bladders & other major organs in sacrificed animals	0/98 0/84 2/44	Two high dose male rats had transitional cell carcinoma of the bladder; they also had mineralization and bladder calculi. One low dose male rat had a leiomyosarcoma of the bladder. Two transitional cell carcinomas of the renal pelvis were also observed in a low dose male rat but none in the high dose or the control groups. _T. crassicauda_ were absent. No credible dose-response in incidence of bladder carcinoma was evident.
At TS (28 mo) 6 alive Interim Sac. at 12 & 24 mo	Micro of bladders; other tissues still being examined!	(see comments)	No bladder tumors were observed in any of the control or treated rats; however, no final report containing complete results of histopathology has been published.
Offspring observed for life	Micro of bladders organs with macro abnormal	(see comments)	In this study, only parents were treated. _In utero_ exposed rats did not show tumors of bladder, and the incidence of other tumors was similar in the treated and control animals. Due to the short exposure period, however, this study is considered limited for the assessment of carcinogenicity.
TS 28 mo 20% survival 35M, 32F at 18 mo	Micro of bladders	None	In this two-generation study, _in utero_ exposed rats had no tumors in urinary bladders or kidneys.

APPENDIX A3: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CYCLAMATE CARCINOGENICITY BIOASSAYS ON RATS [a]

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control Sex and No./Group M	Control Sex and No./Group F	Treated Sex and No./Group M	Treated Sex and No./Group F	Treatment, Duration, Dose, or Conc.	Comparable Dose g/kg/d [b]	Effective No. of Animals [c] C	Effective No. of Animals [c] T
Cyclohexylamine	Pliss (1958) Experimental Oncology Lab., Acad. Med. Sci. Moscow, USSR	CHA	Oral-Feed	Rats strain not specified	130 (sex not specified) injected subcutaneously for 10 mo. with octadecylamine and methyl stearylamine				24 mo		N.G.	
							22	28	6 days at a rate of 0.5 ml of	8.925 g/animal		
		diCHA					25	25	a 5% oily sol. for 12 mo	8.875 g/animal		
		di-CHA nitrite Voroshilov Sci. Res. Inst. Org. By-Prod. & Dyes					17	13	6 days at a rate of 1 ml of a 3% aqueous solution for 12 mo	9.180 g/animal		
		diCHA-nitrite (source as above) Pure	Sub-cutaneous injection				32	22	once a week at a dose of 0.5 ml of a 2% aqueous solution for 11-13 mo	0.48-1.195g/animal		
	Plank (1970) Indust. Bio-Test Lab., North Brook, Ill., USA	CHA-Sulfate Abbott Labs., N.G.	Oral-Feed	Rats Charles River CD Albino	25	25			2 yr 0.0			
							25	25	0.15 mg/kg/d	0.00015	13	46
							25	25	1.5 mg/kg/d	0.0015		
							25	25	15.0 mg/kg/d	0.015		
	Carson&Vogin (1972) Food & Drug Res. Lab., Maspeth, N.Y. or Oser et al. (1976) FDRL East Orange, N.J., USA	CHA-HCl, Baker, 99% pure	Oral-Feed	Wistar Rats	30	30			110 wk	0.000		
							30	30	15 mg/kg/d	0.015	47	204
							30	30	50 mg/kg/d	0.050		
							30	30	100 mg/kg/d	0.100		
							30	30	150 mg/kg/d	0.150		
	Schmähl (1973) Ger. Canc.Res.Ctr., Heidelberg, W. Germany	CHA, Beyer-Werke, (CHA <4ppm)	Oral-Feed	Rats Sprague-Dawley	52	52	52	52	30 mo 200 mg/kg/d	0.2	57	63
	Gaunt et al. (1976) Brit. Indust. Biol. Res. Association, Surrey, U.K.	CHA-HCl, Laporte Indust., N.G.	Oral-Feed	Rats Wistar	48	48			104 wk			
							48	48	600 ppm	0.03		
							48	48	2000 ppm	0.10	53	213
							48	48	6000 ppm	0.30		

Interim or Terminal Survival	Extent of Bladder Exam.	No. with Bladder Tumors/No. Examined	Comments on Study
After 18 mo 20 rats 22 rats After 12 mo 16 rats	Micro & macro exam on all animals that were killed	N.G.	No tumors were found in CHA-treated animals. In 16 diCHA-treated rats remaining alvie, one developed a liver tumor after 21 months and one a sarcoma originating in the omentum after 22.5 months. Many animals died of pneumonia. In di-CHA nitrite-treated rats after 17 mo, one rat was found to have a group of tumor nodules (polymorphocellular sarcomas) in the abdominal cavity that mestastasized into the mesenteric and thoracic lymph nodes.
After 12 mo 31 rats			Tumors of the internal organs were found in seven rats after 8, 14.5, 15, 21, 22, and 24 months, respectively, but were considered unrelated to treatment.
At TS (2 yr) 4M, 9F 6M, 10F 8M, 9F	Micro on all high-dose rats & 5 rats/ sex from other group at TS	 0/8 0/4 0/6 1/8	One high dose male rat hed a low grade carcinoma of the bladder. However, the study was considered insensitive because of the small number of the animals, the low dosages used, and an infection in the colony at 19 mo leading to deaths in all groups.
TS at 110 wk 14M, 14F 13M, 16F 12M, 16F 12M, 15F 17M, 20F	Micro of all bladders plus all organs of 10 cont. and high dose. Fewer organs at low doses	None	In this multigeneration feeding study, no increase in the incidence of tumors was observed at any dose level.
N.G.	Micro of all bladders and abnormal tissues	None	No tumors of the bladder were observed. Conclusions are limited, because histopathological examination was limited to bladders.
At TS (104 wk) 27M, 38F 30M, 44F 43, 41F	Micro of bladders, abnormal tissues, & vital organs	(see comments)	No increase in tumor incidence in any organ and no tumors or any other proliferative lesions of the bladder were observed. Because the extent of histopathological examination was not clearly defined, however, the study study is considered of somewhat limited value in the evaluation of the carcinogenicity of CHA.

APPENDIX A4: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CYCLAMATE CARCINOGENICITY BIOASSAYS ON RATS [a]

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control Sex and No./Group M	Control Sex and No./Group F	Treated Sex and No./Group M	Treated Sex and No./Group F	Treatment, Duration, Dose, or Conc.	Comparable Dose g/kg/d [b]	Effective No. of Animals [c] C	Effective No. of Animals [c] T
Cyclamate : Saccharin	Oser et al. (1975) Food and Drug Res. Lab., East Orange, N.J. or Price et al. (1970) FDRL Maspeth, N.Y., USA	Cycl.: Sacch (Na) 10:1 Abbott Labs. >99% No CHA detected	Oral-Feed	Rats FDRL strain Wistar-derived 28-35d-old	35	45			24 mo	0.0	38	
				Group 1			35	45	500 mg/kg/d	0.5		52
							GROUP DIVIDED AFTER 78 WK					
				1a			9	14	Continue above treatment	0.5		
				1b			9	14	As above: CHA-HCl 25 mg/kg/d	0.5+ 0.025 CHA		29
				Group 2			35	45	1,120 mg/kg/d	1.1		52
							GROUP DIVIDED AFTER 78 WK					
				2a			9	15	Continue above treatment	1.1		
				2b			10	15	as above: CHA-HCl 56 mg/kg/d	1.1+ 0.056 CHA		32
				Group 3			35	45	2,500 mg/kg/d	2.5		53
							GROUP DIVIDED AFTER 78 WK					
				3a			12	16	Continue above treatment	2.5		
				3b			11	14	As above: CHA-HCl 125 mg/kg/d	2.5+ 0.125 CHA		38
	Schmähl (1973), Ger. Canc.Res.Ctr, Heidelberg, W. Germany	Cycl:Sacch (Na) 10:1 Beyer-Werke (<4 ppm)	Oral-Feed	Rats Sprague-Dawley	52	52			30 mo	0.0		
							52	52	2% Diet	1.0	57	116
							52	52	5% Diet	2.5		
	Schmähl & Habs (1984) German Cancer Res. Ctr., Heidelberg, W. Germany	Cycl:Sacch (Na) 10:1 N.G.	Oral-Feed	Rats (F1 generation) Sprague-Dawley	36	34			Life-long two-generation study		N.G.	
							33	39	2%	1.0		
							34	37	5%	2.5		
	Ikeda et al. (1975), Natl. Inst. Hyg. Sci., Tokyo, Japan	Cycl:Sacch (Na) 10:1 98%	Oral-Feed	Rats Wistar 5-wk old	54				28 mo	0.0	33	36
							54		2.5 g/kg/d	2.5		

Interim or Terminal Survival	Extent of Bladder Exam.	No. with Bladder Tumors/No. Examined	Comments on Study
TS at 24 mo 5M, 5F each group sacr. at 15 and 53 wk	Micro all bladders plus all organs of high dose & controls. Lower dose select tissue only		In this multigeneration study, bladder carcinomas appeared in the high dose animals (250 g/kg/d)--9 in males and 3 in females. The occurrence of tumors was similar in rats that received CHA and in those that did not, suggesting that CHA did not have any modifying effect on tumor development. No malignant bladder tumors were found in the intermediate dose levels or in the control groups in both sexes. An increased incidence of malignant proliferative lesions was noted in the bladder of rats at the highest dose relative to controls, but this was not noted at the other dose levels. Several animals on test displayed nephrocalcinosis, urolithiasis, and/or bladder calculi and some animals were infected with T. crassicauda, but the incidence of these observations was not correlated with the incidence and severity of bladder tumors. The presence of saccharin was the major confounding factor in this study. Saccharin and cyclamate controls were lacking.
10M, 19F at 2 yr		M:0/15 F:0/31	
		M:0/14 F:0/26	
9M, 23F at 2 yr		M:0/16 F:0/25	
14M, 24F at 2 yr		F:1/23	
		M:3/15 F:1/23	
		M:6/15 F:1/23	
Observed for life	Micro all bladder & abnormal tissues	None	No tumors of the bladder were observed. Bladders of 16% of the animals contained the nematode parasites T. crassicauda.
Observed for life	Micro of kidneys, bladders, and organs with macro abnormality	(see comments)	In this two-generation study, only one benign bladder tumor (papilloma) was found in a female rat at the 2% dose level. The incidence of other tumors was within normal range.
8 at 28 mo	Micro all bladders	None	No tumors of the bladder were observed; other organs were not examined histopathologically. No evidence of bladder parasites or extensive calculus formation was found.

APPENDIX A5: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CYCLAMATE CARCINOGENICITY BIOASSAYS ON MICE AND RATS COMBINED EFFECTS WITH OTHER AGENTS

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control Sex and No./Group M	Control Sex and No./Group F	Treated Sex and No./Group M	Treated Sex and No./Group F	Treatment, Duration Dose, or Conc.	Comparable Dose g/kg/d [b]	Effective No. of Animals [c] C	Effective No. of Animals [c] T
											MICE	
Sodium Cyclamate	Roe et al. (1970) Chester Beatty Canc. Res. Inst., London, U.K.	NaCHS, Abbott Labs., N.G.	Oral-Feed plus pre-treatment with PEG or BaP	Mice Swiss albino		100			18 mo BaP only	0.0	65	34
								50	5% cycl. plus PEG pretreat.	6.7		
								50	5% cycl. plus BaP & PEG pre-treatment			
	Bryan & Ertürk (1970) Univ. Wisconsin Medical Sch., Madison, USA	NaCHS in Cholesterol(1:4) N.G.	Bladder implant. choles. pellet	Mice Swiss 60-90-d old		106		107	Once 50% in 1 hr 99% in 6.6 hrs	Can't be calculated	106	107
											RATS	
	Hicks et al. (1975); Hicks & Chowaniec (1977) Middlesex Hospital Medical Sch., London, U.K.	NaCHS, Abbott Labs., (CHA 13 ppm)	Oral-Feed	Rats Wistar		150			24 mo	0		
								80	1 g/kg/d 2 g/kg/d plus 1.5-2.0 mg MNU instilled once into bladders	1.0 2.0	124	69
	Green et al. (1980) Institute for Experimental Pathology, Hanover, W. Germany	NaCHS, Abbott Labs., N.G.	Oral-Feed	Rats Wistar AF-Han	Untreat 100F Water 50F Instilled				104 wk			
								50	2.0 mg MNU only			
								50	MNU+3% $CaCO_3$			
								50	MNU+4% NaCHS	2.0	298	300
	Schmähl & Kruger (1972) Germ. Canc.Res.Ctr., Heidelberg, W. Germany	NaCHS, Unknown	Oral-Feed	Rats Sprague-Dawley		40			Life			
								40	BBN (10 mg/kg/d) in drinking water		N.G.	
								40	BBN+2,500 mg/kg/d NaCHS	2.5		
	Fukushima et al. (1982) Dept.of Path., Nagoya City Univ. Med. School, Nagoya, Japan	NaCHS, Wako Pure Chem. Co., Osaka, Japan N.G.	Oral-Feed	Rats F344					12 wk			
								10	BBN(0.5%)in drink. water		10	
								10	BBN+75% Part. cystectomy		10	
								10	BBN+2.5% NaCHS	1.25	10	
								10	BBN+Partial cyst.+ NaCHS	1.25	8	
								10	Partial Cyst.+NaCHS	1.25	7	
	Ito et al. (1983) Dept. of Path., Nagoya City Univ. Med. School, Nagoya, Japan	NaCHS, Wako Pure Chem. Co., Osaka, Japan N.G.	Oral-Feed	Rats F344 6 wk old					32 wk			
								28	BBN(0.1%)drink.water 4 wk		28	
								60	BBN(kept 2 more wk)		60	
								30	BBN+2.5% NaCHS	1.25	30	
	Ershoff & Bajwa 1974) Inst. Nutr. Stu., Culver City, Calif.	NaCHS, Abbott Labs., N.G.	Oral-Feed	Rats Horton-Sprague Dawley		12			40 wk Untreated control		12	
								12	AAF (3 ppm)		12	
								12	AAF+5% NaCHS	2.5	12	

Interim or Terminal Survival	Extent of Bladder Exam.	No. with Bladder Tumors/No. Examined	Comments on Study
TS at 18 mo 34/50 at 18 mo 41/50 at 18 mo	Micro all neopl. & suspected lesions; bladders macro and not micro	Lack of bladder histopathology impedes evaluation	Mice in this study were not randomly allocated to treatment groups. The incidence of tumors was as follows: lymphoreticular system controls 8/91 (8.8%), treatment 3/45 (6.7%); liver: control 7/91 (7.7%), treatment 1/45 (2.2%); and lungs: controls 19/91 (20.8%), treatment 10/45 (22.2%); papilloma/carcinoma of the forestomach: in BaP-treated mice 21/61 (20 pap. & 1 carcinoma) (34.4%), PEG plus cycl 1/34 (2.9%), BaP plus cycl. 4/41 (9.8%). Data are suggestive of an inhibitory effect on BaP-induced tumors.
Av 371d Av 393d Sacr. at 13 mo	Micro all bladders and representative tissues	C_1:8/63, C_2:5/43 T_1:45/58, T_2:30/49	This study suggests enhancement of carcinogenicity, since the incidence of bladder carcinomas was 75/107 (70%) for experimental as compared to 13/106 (12%) for control animals with pellet implants without cyclamate.
	Micro of inflated bladders & other major organs in sacrificed animals	0/124 14/24 20/45	Statistically significant bladder tumors (34) and kidney tumors (18) were found in MNU+NaCHS-treated female rats. No tumors were found for rats treated with MNU only. The earliest tumor was observed 8 weeks after MNU plus cyclamate treatment as compared to 87 weeks for rats receiving cyclamate alone. An additional control group treated with MNU and cyclophosphamide yielded no bladder tumors out of 203 animals examined. These results suggest a promotional effect of cyclamates on MNU-induced bladder neoplasia.
At TS (24 mo) 59F 28F 13F 15F 14F	Micro of bladders, kidneys & ureters	0/100 1/50 19/50 19/50 20/50	Animals from all groups instilled with MNU displayed tumors of the urinary bladder, ureter, and renal pelvis (60-70% incidence). The incidence of bladder tumors only ranged from 39% to 42%. Mean latency period of bladder tumors for the MNU only, MNU-$CaCO_3$ and MNU-NaCHS was 69, 82, and 84 weeks, respectively, and the earliest bladder tumors were observed at 16, 26, and 37 weeks, respectively. This study sheds no light on possible carcinogenicity or enhancing effect of cyclamate because of the effect of MNU alone.
N.G.	Micro all bladders	(see comments)	All rats given BBN alone developed squamous cell carcinoma of the urinary bladder, and all animals but two given BBN and cyclamate developed carcinomas; these two animals had papillomatosis of the bladder. Because of the overwhelming response to BBN, this study contributes no relevant data to the evaluation of carcinogenicity of cyclamate.
10 10 10 8 7	Micro all bladders plus liver & kidneys	1/10 0/10 1/10 1/8 0/7 (all are hyperplasia)	Cyclamate had no enhancing effect on nodular hyperplasia of the bladder in this experiment. However, group sizes were small, the duration of experiment was short, the relation of the nodular hyperplasia to carcinogenesis is somewhat uncertain, and the partial cystectomies appear to have decreased urine retention time in the bladder as well as the amount of bladder mucosa at risk of transformation. This study is considered inadequate to evaluate the carcinogenicity of cyclamate.
28 60 30	Micro all bladders	11/28 27/60 17/30 (all are hyperplasia)	The incidence of papillary or nodular hyperplasia due to BBN treatment was apparently not affected by administration of cyclamate.
12 12 12	Micro all bladders & liver; macro mammary and ear duct tumors	(see comments)	No tumors were found in untreated control rats. Eleven rats fed AAF alone had 24 palpable tumors (3 were ear duct and 21 mammary). All rats treated had liver tumors. The mucosal lining of the bladders was hyperplastic, but no bladder tumors were observed. Those rats fed AAF plus cyclamate had single tumors (one ear duct & one mammary gland) in two affected rats and small liver tumors with a lower grade of malignancy. This study is suggestive of possible inhibitory effect of AAF on carcinogenicity.

APPENDIX A6: SUMMARY OF EXPERIMENTAL CONDITIONS AND ANALYSES OF CARCINOGENICITY BIOASSAYS OF CYCLAMATE ON HAMSTERS, DOGS, AND MONKEYS[a]

Agent	Principal Investigator, Year, Institute	Chemical, Source, Purity	Route of Admin.	Test Animal	Control Sex and No./Group M	Control Sex and No./Group F	Treated Sex and No./Group M	Treated Sex and No./Group F	Treatment, Duration, Dose, or Conc.	Comparable Dose g/kg/d [b]	Effective No. of Animals [c] C	Effective No. of Animals [c] T
										HAMSTERS		
Sod or Cal Cycl	Althoff et al. (1975 a,b) Eppley Inst. Canc. Res., Univ. Neb., Med. Ctr., Omaha, Neb., USA	NaCHS, Sigma Chem. Co., (10ppm CHA)	Oral-drinking water	Syrian Golden Hamsters	30	30	30	30	Lifetime 0.156%	0.5	24	70
							30	30	0.312%	1.0		
							30	30	0.625%	1.9		
							30	30	1.25%	3.8		
		Calcium Cyclamate Sigma Chem Co., 98% Trace of CHA			30	30	30	30	Lifetime 0.156%	0.38	15	60
							30	30	0.312%	0.80		
							30	30	0.625%	1.42		
							30	30	1.25%	3.11		
										DOGS		
Cyclohexylamine	Mastalski et al. (1973, 1981) Industrial Bio-Test Lab., North Brook, Ill., USA	CHA-Sulfate Abbott Labs., Blended as 25% mixture with lactose wk 1-193 and 50% mixture wk 194-496	Oral-gelatin capsules	Beagle dogs pure-bred	3	3			Admin. 6/day/wk 45 mg/kg lactose wk 1-193 300 mg/kg lactose wk 194-496		6	
							3	3	0.15 mg/kg wk 1-193 wk	0.00015		6
									50 mg/kg wk 194-496 wk	0.05		
							3	3	1.5 mg/kg wk 1/193 wk	0.0015		6
									100 mg/kg wk 194-496	0.10		
							3	3	15.0 mg/kg wk 1-193 wk	0.015		6
									150 mg/kg wk 194-496 wk	0.15		
										MONKEYS		
Sodium	Coulston et al. (1975) Albany Med. College, Albany, N.Y., USA	NaCHS, Abbott Labs., N.G.	Oral-intubation	Rhesus monkeys	3	3	1	2	6-7 yr 200 mg/kg/d 6 days/wk	0.2	6	3
Cyclamate	Adamson(1975); Adamson & Sieber (1983); Sieber, Pers. Comm. (1983) NCI, Bethesda, Md., USA	NaCHS, Abbott Labs., > 90%	Oral-vitamin sandwich	Monkeys: Cyno., African-Green, Rhesus	A control colony of 211 animals was used		7	5	95 mo 100 mg/kg/d Avg. Cum. dose 730 g 5 days/wk	0.1	Still ongoing	
							7	4	500 mg/kg/d Avg. Cum. dose 4714 g Animals observed for additional 5 years	0.5		

Interim or Terminal Survival	Extent of Bladder Exam.	Comments on Study
Avg. surv. 50-60 wk. Avg. surv. 41 wk -.-.-.-.-.- No Sacrifice	Gross and micro of all organs. Step sections of bladders	No tumors of any organ related to treatment, and no bladder tumors were observed at any dose level in either sex throughout the study. The highest level of cyclamate used was less than in most mice or rat studies and, together with low survival, limits the sensitivity of this study.
17 animals died/sacr.: 2 cont M wk 52 & 351 3 Cont F wk 52, 492, & 493 2 low M dose wk 52 & 273 3 Low dose F wk 52, 225 & 388 1 mid dose M 52 wk 2 mid F wk 52 & 372 2 high M wk 52 & 212 2 high F wk 52 & 273	Complete gross & histopathological examination	This study lasted for approximately 9.5 years. One dog of each sex per group was sacrificed at week 52. There were several deaths beginning at week 212, and only 7 dogs survived to week 496. A low dose level of cyclamate was used in this study.
3 alive 2F at 89 mo 1M at 86 mo	Special attention to bladders	No evidence of hyperplastic or neoplastic lesions in any organ, including the urinary bladder, was found in the three treated and six control animals. The value of this study is limited due to the relatively low dose levels, relatively short duration compared to animal longevity, and the small number of animals.
4 died 2 low dose M at mo 2 & at 4.5 mo 1 high dose M at 28 mo 1 high dose F at 54 mo	Complete histopathological examination	This is an ongoing study in its 14th year. No tumors of any organ, including the bladder, have been found in necropsied animals. However, definitive conclusions have to await final histopathological examination at termination of the study.

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APPENDIX B

RESPONSES TO SPECIFIC QUESTIONS ON ANIMAL STUDIES POSED TO THE COMMITTEE BY THE FOOD AND DRUG ADMINISTRATION

QUESTION: Do any of the mouse studies, e.g., the Brantom, Kroes, and Hardy studies, appropriately considered in terms of their biological and statistical significance, indicate that cyclamate causes lymphoreticular neoplasia?

RESPONSE: None of these studies indicate that cyclamate (CHS) causes lymphoreticular neoplasms. In the studies by Kroes _et al_. (1975, 1977) with sodium cyclamate (NaCHS), CHS:saccharin mixtures, and cyclohexylamine (CHA), and by Hardy _et al_. (1976) with CHA, the incidences of lymphoreticular neoplasms are essentially the same when animals in the various dose groups are compared with their respective control animals. In the study by Brantom _et al_. (1973) with NaCHS there was a dose-related decrease in lymphoreticular tumors in male mice (3 of 45 controls, 4 of 19 at 0.7% in the diet, 4 of 18 at the 1.75% level, 5 of 21 at the 3.5% level, and 6 of 25 at the 7.0% level). However, the incidences in female mice in the Brantom study do not show a dose-related effect and are within the range of variability expected in groups of mice of this size and age. The data reported in these three studies do not permit age-adjusted analysis, but there were no indications that significant numbers of premature deaths resulted from lymphoreticular neoplasms.

QUESTION: Is it reasonable to combine tumors from the three generations of mice in the Kroes study and compare them to the sum of their corresponding controls?

RESPONSE: Primary analysis in any bioassay, including the Kroes _et al_. studies (1975, 1977), is performed by comparing dosed animals with their respective concurrent matched controls, that is, those animals randomly assigned from the same stock at the beginning of the experiment and given the same diet under identical environmental conditions over the same period, but differing from the dosed animals only in that they are not exposed to the substance being tested. Where multiple bioassays are conducted at the same time in the same laboratory with the same stocks of mice and with similar protocols, as in the Kroes studies, it is possible to obtain more information on the variability to be expected in the incidences of tumors among multiple groups of control animals of similar size. That information may only add additional weight to the evidence for the adequacy of the experiments and for (or against) carcinogenicity.

QUESTION: Is it proper to combine tumors from different histogenic sites such as the mouse liver and lung tumors in the Rudali _et al_. (1969) study?

RESPONSE: Judgments by pathologists are required for combining or separating lesions of various sorts at different body sites for analysis. This subject was discussed in 1984 by an Ad Hoc Panel of the National Toxicology Program (DHHS, 1984). In general, lesions of histogenetically similar origin, such as tumors of vascular endothelium in the spleen and liver or of the epithelium lining the upper and lower intestine, can be considered together as can lesions known to be part of a series of related developmental stages of a single tumor type. Tumors arising in the hepatic parenchymal epithelium and in the respiratory epithelium are generally considered separately, unless there is some biological evidence that provides a rational basis for considering them together in a particular experiment.

QUESTION: Do any of the studies, e.g., the Rudali study, considered in terms of their biological and statistical significance, indicate that cyclamate causes lung or liver tumors? Does this study raise a significant degree of suspicion about the possible carcinogenicity of cyclamate? Should the evidence for the drifting historical controls in the XVII/G strain of mice be factored into the evaluation?

RESPONSE: The studies by Rudali _et al_. (1969) and Muranyi-Kovacs _et al_. (1975) were interpreted by the authors themselves as providing evidence only for acceleration or accentuation of spontaneous liver and lung tumors in mice by CHS. The data have limited value, however, because some of the tumors were diagnosed solely on the basis of macroscopic examination, and the microscopic examinations were performed only on grossly visible lesions. Moreover, with this sampling method an effect on the liver was reported only for one of seven experiments in which four strains of mice were used. For lung tumors, only female mice of one strain (XVII/G) were reported to be affected, and in that strain there was an inexplicable variation in the reported incidences of lung tumors in female control mice from 18.7% to 60% during the study period, which prevented evaluation of the tumorigenic effects of CHS on the lung. Subsequent studies by others in which mice were given much higher doses of CHS provided no significant evidence for CHS-associated increases in liver or lung tumors.

QUESTION: Do any of the studies, e.g., the Schmähl, Homberger, Taylor, Ikeda, and Hicks studies, considered in terms of their biological and statistical significance, indicate that cyclamate is a bladder carcinogen? Do any of these studies, considered individually or collectively, raise a significant degree of suspicion about the possible carcinogenicity of cyclamate?

RESPONSE: In none of the studies in which rats or mice were given NaCHS, CaCHS, or CHA were bladder tumors found in incidences exceeding the expected background. In one study (Oser _et al_., 1975) in rats given a mixture of CHS and saccharin, an excess of bladder tumors was found at the highest dose level, but two subsequent studies by others designed to confirm these results failed to do so. The only suspicion of a possible effect of CHS on the bladder comes from studies in which CHS was given

after pretreatment by a known carcinogen (N-methyl-N-nitrosourea) (Hicks and Chowaniec, 1977; Hicks et al., 1975), or given together with another substance (cholesterol) (Bryan and Ertürk, 1970). (See text of Chapter 5.)

QUESTION: Is it appropriate to combine the incidence of bladder cancers from different animal strains, e.g., from the studies by Schmähl (1973), Homberger et al. (1973), Taylor et al. (1980), Ikeda et al. (1975), Hicks et al. (1975), and Hicks and Chowaniec (1977)?

RESPONSE: Since the background levels of urinary bladder tumors vary with strain, age, and a variety of other factors, each experiment has to be evaluated separately. The weight of evidence from each study can then be used for a comprehensive assessment of all the data available.

QUESTION: Do the data from feeding studies with cyclamate and cyclohexylamine demonstrate cyclamate's noncarcinogenicity in the rat? In the mouse?

RESPONSE: Animal bioassays for carcinogenicity cannot prove lack of carcinogenicity. They are conducted under a limited set of circumstances and are limited in power by such factors as the group sizes used. The oral studies conducted thus far in rats and mice do not provide evidence for the carcinogenicity of CHS, but, as for all substances tested for carcinogenicity in animals, possible weak carcinogenic effects cannot be excluded.

QUESTION: Should the presence of bladder parasites (Trichosomoides crassicauda), bladder stones, or calculi weigh in the consideration of the significance of tumors that may be present in such bladders, e.g., the Hicks, Ikeda, and Friedman studies?

RESPONSE: The role of complicating factors in bladder carcinogenesis is discussed in some detail in the text of Chapter 5. At present there is no clear evidence that the bladder parasite T. crassicauda is involved in the development of bladder carcinoma in untreated rats. The parasite has been observed to be present in about half of the CHS bioassays [present in the study by Friedman et al. (1972), absent in the study by Hicks et al. (1975), and not reported to be present in the study by Ikeda et al. (1975)], but there is no evidence to suggest that there is any interaction between parasite infestation and CHS administration in the development of bladder tumors in rats. The presence of bladder calculi, however, has been associated with the development of bladder tumors in rodents. In the bioassays of CHS in the rat, bladder stone formation and urinary tract mineralization were observed to variable degrees [present in the studies by Hicks et al. (1975) and Friedman et al. (1972), but not reported in the studies by Ikeda et al. (1975)]. In general, they were present to a greater extent when the calcium salt was given than when the sodium salt was administered. Overall, however, there is no convincing evidence of an association between calculus formation and bladder tumor formation in CHS-treated rats.

QUESTION: In general, what considerations should guide the pooling of tumors for the determination of cancer incidence? For example, the lymphosarcomas with other lymphoreticular neoplasia in the Kroes study (Kroes *et al*. 1975, 1977).

RESPONSE: In addition to the discussion regarding the pooling of tumor data given in response to the third question, tumors, specifically those of the lymphoid tissues, are currently combined for analysis according to the cell type (B-cell, T-cell, histiocyte, etc.). Until recently, however, the cell of origin of most lymphoreticular tumors in mice was not known, and the lymphoreticular neoplastic diseases were characterized by tissue of origin and by histomorphology. Because the data indicate that chemical carcinogens may induce lymphoid tumors of specific types or of multiple types, it is customary to analyze them both combined and separated no matter what classification scheme is applied.

QUESTION: What considerations should guide the selection of a significance level, i.e., a *P*-value? Should the weight to be given a *P*-value be absolute, or should the weight accorded it and statistical, biological, or other factors be considered a matter of overall scientific judgment?

RESPONSE: A significance level (i.e., a *P*-value) is the probability of obtaining a result as extreme as that observed purely by chance. As such they are useful in detecting end points that should be evaluated and in judging the weight of the evidence for carcinogenicity. Statistical and biological evaluations are not independent: statistical evaluations rest on biological assumptions, and biological evaluations are made more quantitative by statistical methods. Therefore, one type does not have greater value than the other for evaluating carcinogenicity, but both are parts of the entire spectrum of information upon which judgments are based.

QUESTION: When you have a series of studies of different quality, sensitivity, and size, is it reasonable to conclude that a seemingly significant positive effect in a weaker study may be disregarded in the face of clearly negative findings in more powerful studies?

RESPONSE: All data must be considered. When conflicting conclusions are reached from multiple studies, attempts are made to reconcile them by detailed analysis of the similarities, differences, and limitations of the several studies. If rational explanations are not found, additional experiments may be required to resolve the differences.

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APPENDIX C

COMMITTEE ON EVALUATION OF CYCLAMATE FOR CARCINOGENICITY: AFFILIATIONS AND MAJOR RESEARCH INTERESTS

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