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BASIC MECHANISMS IN RADIOBIOLOGY

III. BIOCHEMICAL ASPECTS

Proceedings of an Informal Conference

Held At Highland Park, Illinois, May 13-15, 1954

Harvey M. Patt, Editor

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FOREWORD

The Committee on Nuclear Science of the National Academy-Research Council has given special attention to those phases of nuclear studies in which two or more of the older, well-established disciplines of scientific investigation have been drawn together. To accomplish this, the Committee has organized a number of subcommittees to provide the wide range of competence needed to consider and deal with these new and rapidly developing areas of science. This is well exemplified in the work of its Subcommittee on Radiobiology, which has originated a series of conferences on the basic mechanisms of radiobiology where physicists and chemists, as well as biologists, meet to exchange information and to test hypotheses of biological actions in the light of the combined experience of the group. This has resulted in free, informal and critical discussions of information available from several specialized fields of scientific study. At the same time, deficiencies in existing data have been revealed and suggestions developed for obtaining the missing information.

The organization of these conferences, arranged to encourage frank and detailed discussion in an informal atmosphere, has been a task of some magnitude. A vital part of the conference is the publication of the proceedings, so that all interested persons may have the benefit of these deliberations. It is obvious, even to a layman in biology who may read these pages, that the vital essence of the fundamental processes are being exposed in the reasonings and arguments which have been recorded. In many instances, it is also apparent that a first tentative approach is being made to the solution of a particular problem. Therefore, it is essential to continued progress that conferences of this kind be encouraged to continue to aid in plotting a course through the myriad of complicated reactions which occur when radiation interacts with living tissues.

The Subcommittee has thus far organized four conferences. The first was a symposium on radiobiology held at Oberlin College, June 14-18, 1950. It consisted of a series of formal papers and formal discussions; these were published in 1952 ("Symposium on Radiobiology", J. J. Nickson, Ed., John Wiley and Sons, New York, 1952). The second was a highly informal conference held in Highland Park, Illinois, May 31 - June 2, 1951, the proceedings of which were not published. The third considered specifically the Physical and Chemical Aspects of Basic Mechanisms in Radiobiology and has been published as publication No. 305 of the Academy-Research Council, Nuclear Science Series. The current conference considered the biochemical aspects of the subject, and again no attempt was made to make the discussions comprehensive; but rather topics of current interest were discussed and analyzed.

The expenses of the conference covered by this report were borne jointly by the Atomic Energy Commission, the National Science Foundation and the Office of Naval Research. It is a pleasure to thank them for this support, not only on behalf of the Subcommittee on Radiobiology, but also of all those who may receive inspiration from reading this publication.

L. F. Curtiss, Chairman
Committee on Nuclear Science

H. J. Curtis, Chairman
Conference Committee

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AUGUSTINE O. ALLEN
Brookhaven National Laboratory

E.S.G. BARRON
University of Chicago

EDWARD L. BENNETT
University of California

CHARLES E. CARTER
Yale University

ERWIN CHARGAFF
Columbia University

WALDO E. COHN
Oak Ridge National Laboratory

HOWARD J. CURTIS
Brookhaven National Laboratory

KENNETH P. DUBOIS
University of Chicago

ALEXANDER E. HOLLAENDER
Oak Ridge National Laboratory

HARDIN B. JONES
University of California

MARTIN D. KAMEN
Washington University

HENRY S. KAPLAN
Stanford University

JOHN L. MAGEE
Notre Dame University

DANIEL MAZIA
University of California

HARVEY M. PATT
Argonne National Laboratory

ROBERT L. PLATZMAN
Purdue University

ERNEST C. POLLARD
Yale University

VAN R. POTTER
University of Wisconsin

FREDERICK G. SHERMAN
Brown University

SOL SPIEGELMAN
University of Illinois

CORNELIUS A. TOBIAS

University of California

RAYMOND E. ZIRKLE

University of Chicago

JOSEPH BUTTS

Atomic Energy Commission

JOHN S. COLEMAN

*National Academy of Sciences—
National Research Council*

DOUGLAS WORF

Atomic Energy Commission

THE DIRECT EFFECT OF RADIATION ON PROTEINS, VIRUSES AND OTHER LARGE MOLECULES

Ernest C. Pollard

Well, I richly deserve what I am getting today. In reading through the very fine summary of last year's conference, I realize that I was operating with a needle most of the time and it is very fair and just recompense for having done this. Here I am in a position of putting up or shutting up and so I had better talk about it.

The work we have done at Yale had as its original interest the use of radiation to study structure. This follows directly along the pattern set by Lea, and we consider ourselves, if you like, descendants of Lea in our philosophy and outlook in the way in which we seek to use radiation. The use we have made of it has somewhat surprised us; some of the methods of using ionizing radiation to study viruses have been summarized in my book. (1)

Somewhat to my surprise and pleasure, one structure that we sort of postulated for Newcastle disease looks very much like the electron micrographs that are now being turned up in New York by Morgan et al (2). So there is evidently satisfactory validity to this method of using radiation to study structure. I am acutely sure it has limitations, and it would be very foolish to use it without knowing these limitations. One of the reasons for this discussion today is to send me away with a clearer idea of what the limitations are.

In this work there is one basic aim; i.e., to preserve the space relations of ionizing radiation. You use these space relations to tell you something about the nature of the system you are studying. There is a second feature to this: the ionizing events that occur must make some change in whatever you are looking at. This change is, at the present, rather too inclusive. It involves, as a rule, the removal of activity or some very vital change like that. It would be preferable if the change were more moderate and could be studied in some detail after it had occurred, because then we would get more information.

Because space relationships have to be preserved, we have operated almost entirely in the dry state. Everyone will ask, "How dry?", and the answer that I can give to that question is in the following terms:

First, the specimens are exposed in high vacuum, usually inside the vacuum chamber of a cyclotron, so that the total vapor pressure goes down to 10^{-5} mm. Hg.

Second, the thermal inactivation constants for these materials are completely different from those that are obtained in the wet state. If you heat-inactivate, under the condition that we usually use for work on a virus or an enzyme, you will find that the inactivation is characterized by a low entropy of activation, zero or negative. This seems to be characteristic of inactivation in the dry state, and you can show a very striking contrast between how the material behaves as far as heat is concerned. This we look on as an auxiliary sort of evidence. Whether any biological material can be considered as absolutely dry is doubtful; but that radiation can migrate via the medium of water under these conditions I would strenuously deny. I don't see how it can.

Now I should like to say a little about what I call a theoretical approach to radiobiology. Looking back, the strongest needle I put into the conference last year was in the form of a somewhat impassioned plea for recording of all the effects that radiation produces. I felt that we were relieved when we learned that radiation can produce an effect on water and that this in turn can produce an effect on the cell, and I tried to indicate that that relief was perhaps a little excessive, that there had to be a consideration of all the effects that radiation can bring about, and that among these effects, the direct action on the biological components of the system was most important.

I should like now to suggest that it is altogether possible to formulate a theoretical approach to radiobiology in the following terms. We can say what the parts of the cell are and list them, not just talk about them in absolute generalities but list them. Then we can inquire as to what the radiation action is on these parts separately. Having done that, we can inquire as to what function these parts have in the work of the cell and then we can synthesize a probable explanation for radiation action.

I am amazed, to tell the truth, that this is being done so little. We have tried it in what I thought was an amateurish way, and in the progress reports of our work we have, each year, written a sort of statement as to what we think this sort of theoretical radiobiology should be like. Among the things that we have discussed has been the relative proportion of direct and indirect action based solely on this theoretical approach.

The fact is that when you get into this theoretical approach you become acutely aware that a lot of data that you need are not only unknown, but are not even being sought. In the first place, it is urgently necessary to know the lifetimes of all the products of radiation action everywhere: not only lifetimes in water, but lifetimes in the solid state, and lifetimes of things such as HO_2 and, in fact, any agent that can be thought of as being involved in the response to radiation. The study of these lifetimes is at least not clearly visible in the literature.

In this connection, Dr. Smith, working at Yale during a leave of absence from the Department of Radiotherapeutics at Cambridge, conducted, I thought, a very nice experiment along the line of the one first used by Dr. Mazia. He made a measurement of the lifetime of radicals in water (3), and came out in two cases with a magnitude of about 3 microseconds. Just this one value alone completely modifies Lea's own speculations as to the relation between direct and indirect action. Lea adopted, without measurement, a figure of 0.3 microsecond. Clearly, when you have a lifetime as low as 0.3 microsecond, a radical formed far away from the important biological molecule would not be effective. It will hit something unimportant before it gets there, and the unimportant thing may even be the thing that causes it to recombine as measured in these experiments. A figure 10 times greater modifies this.

The conclusion we come to from the sort of broad theoretical approach in which we consider the effects of radiation on proteins, nucleic acid, and the microscopic cytoplasmic components is that direct and indirect actions are split about 50:50 but that they are both variable. You can, on occasion, get a cell in which only 25 percent of the action is direct, or the other way around. One must not consider either of these to be the functions of radiation that are constant. They depend on the condition of the cells and on the molecules that are in them, and we have to recognize that both of these can be variable. There is no question whatever about the variability of indirect action with respect to the nature of the cytoplasm.

I should like to suggest that this can also be said of direct action, and this is a somewhat bolder statement than I think anybody has made in the past.

One tremendous and absolutely vital datum is sitting, waiting to be found by somebody. No one knows the ionic yield for indirect action on large nucleic acid molecules. Large nucleic acid molecules obviously play a most dramatic role in cellular function, particularly in any function that takes time and requires the cell to develop. Yet I do not know of any work in which the ionic yield for indirect action on nucleic acid molecules is given. By a nucleic acid molecule, I mean one that has a biological function. I discount measurements in which the nucleic acid is either polymerized or depolymerized without a guarantee that at the same time biological function goes along with it.

CARTER: Do you mean transforming principle?

POLLARD: Transforming principle, for example, would be beautiful, but at the moment I don't think it is pure enough to measure.

CARTER: I think transforming principle is pure enough to measure and I am surprised that Dr. Chargaff does not have the data.

CHARGAFF: It is very difficult to say really what proportion of the transforming principle you have in any nucleic acid preparation. That is one of the great difficulties. We are now fractionating preparations. Maybe we will know soon a little more about that.

POLLARD: We know the yield for direct action. This has already been measured in three places and the agreement is good. But we do not know the efficiency for indirect action. It is not known, for example, whether it takes 10 ion pairs to inactivate one transforming principle molecule or, say, 1,000. Empirically, we would expect a figure of 1,000 but it might be as low as 1. If, indeed, it is 1, it would be well worthwhile to concentrate on that one subject as the probable single basic radiobiological action. But until we know that, the basis for this theoretical radiobiology is missing. It does not seem to be an impossible experiment.

CARTER: Actually the obstacle is that Dr. Chargaff has not purified the transforming principle.

CHARGAFF: You are faced with an almost philosophical dilemma. You may have between 1,000 and 10,000 different species of nucleic acid per nucleus, and if you equate the nucleic acids with the genes obviously you really don't know what you are measuring. If you are measuring one transformation feature, e.g., Hotchkiss' sulfonamide factor, you don't know what proportion of the total nucleic acid really corresponds to this particular activity. I think it will be a long time before we will be able to answer that question.

POLLARD: Well, you can answer it for direct action.

It is interesting that Fluke, Drew and I (4) measured the radiation sensitivity of the transformation of just rough to smooth. Fluke and Marmur (5) have measured the streptomycin and, I believe, one other transformation from Hotchkiss' laboratory with substantial agreement, although with a rather interesting extension that there may be two classes of molecules present.

I had a letter from Dr. Latarjet to the effect that he has found somewhat similar, though rather less, sensitivity. One can say that the apparent radiation sensitive volume for the direct action of radiation on nucleic acid corresponds to a molecule in the order of between 3 and 7 million molecular weight -- very sensitive. So that the temptation for me, as a direct action man, is to see this enormous figure and to want to say, "Well, that is the key to radiobiology." It is a great temptation because it is a huge figure. Nevertheless, it will be most unwise to take this attitude until we have the other data as to whether, by any chance, 1 ion pair can also inactivate such nucleic acid molecules by the medium of water. If that is true, than we have just as good, in fact, a better line of approach in terms of the action of water. So these are important data. Moreover, I think it is so important that it would be quite all right if we knew it only within a factor of 20. So if one had a purified transforming principle with only 40 percent inactivity it still would be worth working on.

BENNETT: I don't know if this is the type of thing you are thinking about, but Dr. Stent at the University of California is incorporating essentially carrier-free P^{32} phosphate into phage and determining how many disintegrations are required to inactivate them. It is a different type of phenomenon, I think.

POLLARD: There are two things there. In addition to the ionization, there is actually a change of atomic species plus a violent recoil and actual motion of a heavy atom, which is a very drastic thing indeed, in the case of a big molecule. Dr. Kamen knows about this because he did pioneer work on the subject. (6) The effect of incorporating P^{32} is some 30 times as great as the effect of radiation from the outside. So that there is clearly something else taking place that is not normally present in ordinary radiobiology. Radiobiology is not concerned with making nucleic acid radioactive. If that were so, the cross-section would be very small, I believe.

KAMEN: I wonder if you could tell us how the half-life was measured for radicals in water.

POLLARD: I cannot describe it in detail because it involves a lot of plane diffusion constants. The procedure was essentially that of Mazia and Blumenthal. Two separate monolayers, one of catalase and one of bovine serum albumin, were deposited on a chromium-plated glass slide which was placed in a clean water solution, with precautions for no oxygen, and then X-irradiated. Loss of function was measured by the ellipsometer technique in one case. In the case of the catalase, it was described in terms of the amount of hydrogen peroxide converted. In the case of the bovine serum albumin, it was measured for the amount of specific antibodies that would hook on to the surface of the albumin. Both gave quite similar figures. But the significant thing about the experiment is that in both cases, exposures of the order of 200,000 r were needed to produce any effect. This is quite different from an enzyme that is distributed throughout the material; for example, for catalase in bulk dissolved in water. Catalase would undoubtedly be inactivated by a

fraction of this exposure, perhaps 5000 r or something of the sort.

The point is that because there is only one place where the catalase is, namely, on the slide, the time for diffusion is important, and if the time for diffusion is longer than the time for recombination, then the radical is not effective. By calculating for plane surfaces one would come out with a theoretical figure.

I don't really want to spend time on this part since I want to get to the details of direct action studies. What we have done has been to pick up where Lea, Smith, Holmes and Markham (7) left off. They investigated the effect of X-rays on dry myosin and dry ribonuclease and measured what is called their inactivation volume. This is the volume within which one ionization, randomly distributed, will cause inactivation or loss of function. It is to be thought of as a parameter and it is only by chance or, by what I hope to bring out, by some process that we would like to be able to describe, that this parameter agrees in any way with anything known about the molecule at all.

Lea and his associates found that in both of these molecules something like the molecular volume was involved in the figure for the inactivation volume, and that if, in particular, one allowed for the way in which ionization comes in clusters, the calculated molecular weights based on this method of inactivation agreed tolerably with the figures that were accepted at the time. Lea did not follow this up in the years before he died, and when we began our work on irradiation of viruses, it was suggested by Dr. Forro that we should study the effect of radiation on enzymes as well.

The experiments are threefold in character and quite elaborate. First of all, whatever you study has to be brought into a condition whereby it can be dried and handled stably. For most enzymes and antigens, this is easy. In fact, most of these things seem to have a higher stability in the dry than in the wet state. For example, catalase can be heated to 100°C when dry but is quite temperamental when wet. These are then irradiated with fast deutrons, slow deutrons, alpha particles, fast electrons of over 500,000 volts, and also with electrons of limited penetration, of energies below 4000 volts.

We have brought every piece of physical equipment that we could to bear on this major type of study. That is one advantage of being a physicist of some reputation. You can get hold of apparatus that otherwise is a little hard to get your hands on. We have not hesitated to go right after it and we have studied what is a surprisingly large array of things.

We have found, first of all, that you can consider a molecule as having an inactivation volume and an inactivation cross-section, depending upon whether you deal with ionization that is random in volume or with ionization that is confined to dense swaths and so can be considered as a sort of linear problem. The two usually go together, although not exactly. It is not a perfect fit unless you start to introduce other factors.

But the first thing to say is that in no case when we calculate the molecular weight do we come out with something wildly wrong. As a matter of fact, we have had some remarkable successes. For instance, we insisted that the molecular weight of urease would be somewhere in the neighborhood of 100,000 and we held to that in the face of opinion that it was 480,000. Well, later determinations are giving 100,000. We hit some things rather accurately. For pepsin we find a figure of 39,000 as against the accepted value of 36,000. For others we haven't done so well. We come out with a figure of 31,000 for trypsin,

whereas the present accepted figure is 17,000. We have a number of figures for radiation molecular weights which have not been checked in any other way. As they are gradually being checked, we find that our figures are quite often in the right league.

A very interesting one is that of DNA. If DNA is assayed as the pneumococcus transforming principle, then we come out with a molecular weight of 6,000,000 and we also require that the molecule be long and thin. The figure given is 45 Å wide and 3800 Å long. When we assay DNA, however, by something quite different -- the capacity to act as a substrate for its enzyme -- we come out with a figure of something like 2100 molecular weight and a cross-section of about 500 square Å. The conclusion is that perhaps 8 nucleotides are sufficient to be specific for digestion by DNA ase.

CARTER: What are the criteria for the enzymatic activity that you used?

POLLARD: This was done by Dr. Smith. I think he measured the amount of substrate converted in a fixed time, being certain that the amount of substrate was not the limiting factor.

CARTER: What was the endpoint?

POLLARD: No endpoint was measured.

CARTER: Was this loss of viscosity?

POLLARD: No.

CARTER: It would have to be done like everything we do in a Beckmann apparatus, and this would be measured by the amount of specific changes in absorption, probably at two wavelengths.

CHARGAFF: I am not sure that this is a very good criterion.

POLLARD: I am rather interested that you grabbed on to that as a method of measurement. That was not our point. Our whole point is that there is a completely different response to irradiation.

CARTER: The other point is that if you inactivated the desoxyribose nucleic acid by three different methods you might come out with three different answers.

POLLARD: We bombarded the nucleic acid to see whether it could still be used as a substrate. The enzyme was not bombarded in these experiments.

CARTER: That irradiation can do so many different things to the molecule is the point that we want to establish.

POLLARD: I would confidently expect that if one actually studied these separately there would be significant differences between them.

CARTER: There may be significant areas of agreement.

POLLARD: Yes, quite possibly.

CHARGAFF: The transforming molecular weight was about 6,000,000,

if I understand correctly.

POLLARD: That is correct.

CHARGAFF: That is not to say that if you break it in half it is no longer transforming or is that the minimum? I really don't know what you are measuring when you say 6,000,000. Is it the molecular weight?

POLLARD: What is done is what I call a mental transformation and one has to undergo this before he can understand it. You take a preparation of the transforming principle, dry it and then take part of the dried specimen out as a control. You irradiate it with fast electrons and fast and slow deutrons and you measure, after irradiation, the amount of activity which is left. In the case of the early experiments, this was a very difficult thing to determine. One had to determine the concentration of the irradiated material on which a fixed number of transformations would take place. It was rather nasty, and the observations were not very precise. In the modern experiments, it is much easier. You can simply measure the number of antibiotic-resistant forms that are transformed. These colonies can be measured as a definite number, and you can get some estimate of the activity of the transforming principle that is left at the end. This loss of activity follows approximately a logarithmic function.

I must say with regard to the transforming principle, in view of the crudeness of the assay, that we did take the logarithmic inactivation on faith. If you believe then that the logarithmic inactivation requires that there be a constant, which is volume in one case and area in another, the volume corresponds to the volume of the sensitive unit. This can be re-expressed as a molecular weight. Bombardments that measure the volume give a value of six million for the equivalent molecular weight. Bombardments measuring area do not agree with this unless the substance is very long and thin. Agreement between the volume and the area can be obtained by saying that it is 45 Å units across and 3800 Å units long. That is all we can say about this.

CHARGAFF: That is roughly a ratio of about 100?

POLLARD: Roughly, 100.

I have spent too long on this since I am not too sure that our work is at its best in these two cases because the assays are somewhat an open question. But the substances that we have studied show remarkable radiosensitivity in the dry state, sensitivity that is apparently confined to a region that is approximately that of the molecule.

I should like to summarize the facts as we know them. I think I have nine. Incidentally, I must stress that I have a very fine group that is doing all this work, and I am on top of a pinnacle that they support.

1. All the inactivation volumes are within a factor of 4 of the molecular volume, on the basis that a single ionization will inactivate the molecule. Just a single ionization, not primary, but any ionization.

2. The cross-section of a molecule measured with densely ionizing radiation, such as alpha particles or deuterons, is a varying function of what we call the ionization density. Dr. Zirkle and Dr. Tobias call it linear energy transfer. Either is all right. Generally, this shows a trend to a maximum value and that value corresponds ordinarily to the diameter and area of the molecule.

3. The curve for this cross-section versus ion density can usually be fitted by a theoretical relation, and the theoretical relation rests on the random production of a definite minimum number of ion pairs in a molecule of definite thickness. These measurements enable you to get an independent measure of thickness.

4. Both these quantities, cross-section and volume, vary with temperature during irradiation. If the material is cooled to dry ice or liquid air temperature, it is likely, although not guaranteed, that the sensitive volume will be smaller. If you want to get a most dramatic variation you can get it every time by irradiating just below the temperature where you would inactivate thermally. If you hold the material about 20°C below that for thermal inactivation and irradiate at the same time, the volume and cross-section will both be of the order of 3 to 5 times larger than normal. It is not a small effect. It is definite.

5. You can have partial damage due to ionizing radiation. This shows up in the case of hemoglobin. If you irradiate hemoglobin and then look for any change in it by any method you like, the first thing you need to do is to put it into solution. If this is attempted at an adverse pH, the irradiated material will not go into solution. However, hemoglobin is soluble at pH 4 or 5 and once in solution it will not appear to be damaged. Since there is a change in the solubility at high pH, partial damage of some kind has occurred (8).

6. Radiation action can migrate. It can migrate across an enzyme inhibitor or an enzyme substrate bond. We have measured the effect of trypsin and soybean trypsin inhibitor separately and combined and the effect of hyaluronic acid and hyaluronidase separately and combined. In both cases we conclude that energy can migrate. We are now studying this in the case of antigen antibodies.

7. On the other hand, radiation action does not readily migrate from one molecule to another in a dry solid.

   We have a rather simple experiment to show this, being done at the moment by Hutchinson. If you take electrons of finite range, e.g., 200-volt electrons, and you bombard a layer of invertase, you cannot burn off more than one monolayer no matter how long the radiation is applied. You only eliminate from this invertase preparation the top layer that corresponds to one molecule. This means that the transfer of radiation energy from the top layer to the second layer is very difficult.

   More recently, Hutchinson has shown that this is difficult even if the temperature of invertase is increased. So that the transfer from one molecule to another in dry solid is actually difficult in the case of invertase.

8. Previous treatment of a molecule, e.g., by heat, can condition its radiosensitivity.

9. Loss of solubility is an important response to radiation. It is not necessarily the most sensitive index, although on occasion, this is the case. For example, the main effect of irradiation of bovine serum albumin in bulk is the loss of solubility. If it is put on a

monolayer and irradiated, then its antigenic property is lost or its ability to combine with antibodies is lost, but it is lost after considerably more radiation than will remove its solubility.

Now, to start the discussion, I should like to suggest that we have an explanation for these events. This is largely aimed at Dr. Platzman. If we can get him started we have succeeded.

We feel that two things occur. I rather like the method of approach that is used by Augenstine in the remarkable little book on "Information Theory In Biology", that Quastler edited (University of Illinois Press, 1954). Augenstine analyzed protein denaturization in the following stages:

1. The breaking of a bond such as an S-S bond, which is a definite strong bond. This is associated with no entropy change and involves an energy change of about 20,000 calories per mole.

2. The breaking of a number of hydrogen bonds which opens the structure. They have entropy associated with them, and each has a much smaller amount of actual energy, in the neighborhood of 6000 calories per mole.

3. Another bond is joined, and, in Augenstine's approach, this is a new S-S bond, not the right one for the original configuration.

We should like to take almost exactly the same viewpoint for radiation action. Being a physicist, I know no chemistry and, therefore, I shall just draw the whole structure.

A physicist's idea of a protein backbone, with cross-linkages here and there is shown in Figure 1.

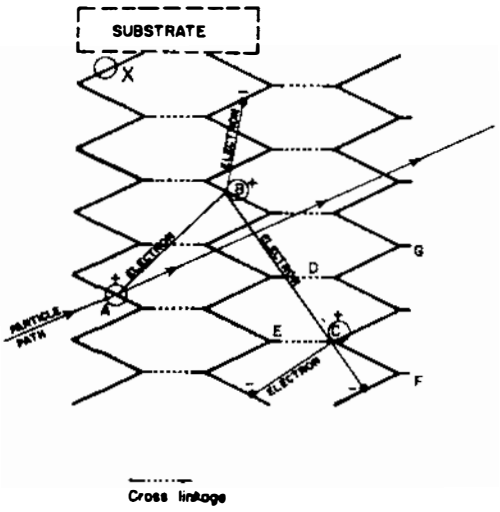

Figure 1. Schematic representation of events associated with the passage of a fast-charged particle through a protein molecule.

What is said about protein obviously can apply to nucleic acid also. Let us imagine that the particle traverses the molecule as shown in the diagram. This is the path of the fast-charged particle that does the ionizing. We will say that all it does is produce a primary ionization at A. As a result of the primary ionization, first of all, a plus is formed at A and then an electron is also released. We will say that the path of the electron is as indicated and that it ionizes again at B and then moves away. There are now two electrons produced, one of which comes to rest while the other ionizes at C before coming to rest. There are now three pluses and three minuses where electrons have been captured. Now all this must happen in the order of 10^{-13} seconds, perhaps even less because very little time is required.

What follows this and how it is related to the loss of biological function

of the molecule? Well, I feel that the things one has to think about are these: In the first place we have atoms that have lost an electron. The positive attribute, it seems to me, cannot possibly stay there, or at least there is no reason why it should. It would be quite natural for the neighboring atom to feed an electron into it, in which case, the plus is now in the next atom, even though the positive charge itself does not move physically. But from the place where it started it can go all the way along these chains and probably does so very rapidly. So we have a concept of migration up and down from one end to the other.

We have a specific functional region in the molecule, and let's say that this is attached in some way to the substrate or is hooked on to something else. It won't matter. Let us say that a bond is broken. Suppose I indicate a broken bond at X. The breaking of the bond is my conception of the removal of a valence electron by the migrating positive charge. This broken bond will mean that the structure will essentially break here, and the fragment can move off with the material of the substrate, or whatever you like, that is bound to it. In which case, the molecule no longer has its specific configuration and its biological activity is lost. This is inactivation by a single event and corresponds to the fact that the single event occurs in a place where just that one event is sufficient to cause inactivation. This might, for instance, be a prosthetic group that dropped off. The concept I want to state is that of the high energy single event. Let's call that category 1. This, in Augenstine's picture, would be the equivalent of the breaking of an S-S bond.

Now let's look at something else that can occur. If these positive positions wander around, they can move, for instance, into a place like E and that can mean that for a moment a bond will be broken. Now suppose that for some other reason, e.g., thermal agitation or another ionization, the bond at D is also broken temporarily. Then there can be a motion of the whole end of the chain outward.

Bear in mind that Figure 1 is not drawn to scale because I have drawn it linearly and, in actual fact, the ends are closer together. It is possible that this outward motion will then cause a cross-linkage between, for example, F and G, and this cross-linkage will make permanent the sort of damage that has occurred. This second method, too, is clearly dependent on the strength of the hydrogen bonding. This is something that may be dependent on temperature.

I feel that there is a lot of significance to the fact that proteins have a high coefficient of thermal expansion, and this may mean that they contain bonds that are actually capable of being weakened just by the fact that they are a little further apart when the high expansion is taking place. When this type of inactivation involving two bonds takes place, we observe a temperature effect.

In any event I should like to point out that the migration of the energy up and down these chains may take place by means of migration of the plus charge; this seems to me to be the significant thing.

I have concentrated on the plus charge, but what I have said also applies equally well to the minus, which will be stopped in the vicinity of an atom. Of course, in time these opposite charges will come close enough together so that a recombination can occur, and in a period of time of about 10^{-8} seconds recombination will be completed. It must be as small as that or we would not observe time-dose rate reciprocity in radiation action.

CURTIS: If I can get one thing clear, both of these events really occur at the outside of this molecule. That is, you have a volume here, and if I have

understood you correctly, the charge is passed along the bonds until you sort of get to the outside, to the periphery, and in that condition these two things that you mentioned can occur.

POLLARD: Actually what I want to do is to make Augenstine's third point, and that is that some place a new bond must be capable of formation. This molecule isn't inactivated until it has gone wrong. If the molecular pattern is undisturbed, it will recover within 10^{-8} seconds. But if something has been broken that can form a wrong configuration, that is the thing that inactivates the molecule.

As I have indicated here, the serious events take place at the periphery, but that might not necessarily be the case if you have a helical structure that is bonded in a certain way. The bonding might go inside and instead of the helix holding it in place, you would have a momentary deformation of the helix that stays there. So that the site where wrong bonding occurs is the place where radiation action is finally manifest. The other concept I have is the free travel of this type of energy -- you can think of it either as a broken bond, as surplus charge, or lack of charge -- free travel up and down.

BARRON: The theory of the electron traveling through the protein structure was formulated by Schmidt in an article published 3 years ago, and since then Franck and Livingston (9) have said that no such thing exists.

PLATZMAN: The discussion by Franck and Livingston has nothing to do with what Dr. Pollard is proposing. It was specifically restricted to the consequences of electronic excitation -- e.g., by light absorption, and was not concerned with consequences of ionization by ionizing radiation.

POLLARD: I am not speaking of an electron traveling and I am not speaking of a proton of a nucleus traveling. I am speaking of the location where there is positive electricity.

BARRON: Do you mean then, that it is the amino group, because you have a protein that is in essence, a polymerization of amino acids?

POLLARD: I think I have something much more fundamental than that, Dr. Barron. I will point it out this way: Suppose I have three nuclei as those diagramed in Figure 2 a, b. They have electrons around them and they have a valence electron that is also shown. This is the P state kind of valence electron.

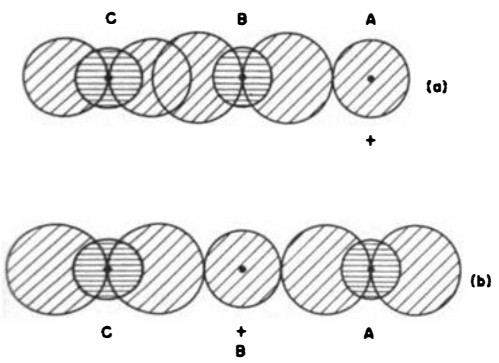

Figure 2. Transfer of electrons between neighboring atoms.

If I take away the electron from atom A, it becomes positively charged, but why will it not be quite possible for a P state electron to move from atom B to atom A in which case B becomes positively charged, and then later moves to C, in which case C becomes positively charged? Why does the positive have to remain at the atom that has lost the electron?

There is a strong interaction between all these atoms, and there is no reason why an atom should not capture a P state electron from its neighbor. When it does so, the neighbor becomes plus, and so on down the line. I feel that

one thing that may be very significant in radiation action is any type of bonding in which this is unlikely to happen. Another thing I feel may be very significant, is the ring structure in which this, so to speak, is held up by just going around and around the ring for a while and, therefore, not traveling.

PLATZMAN: What is important about postulating that?

POLLARD: The important thing is for this type of broken bond, as I see it, to be able to migrate.

PLATZMAN: Why is that important?

POLLARD: So as to have an event anywhere in the molecule apparently produce an inactivation.

There are two points of view on this. One is that a protein molecule is so sensitive that anywhere you hit it, it dies. I look on that as being a little superstitious. I just don't think any biological system is quite that critical. I used to feel that way, but I no longer do. If that is not so, then you have to say the protein will cease to function biologically under a condition in which a bond is broken and the wrong bond is formed. The place where a bond can be broken and a wrong bond can form isn't just anywhere in the molecule. Apparently it does not matter where we put the radiation energy in; we are able to find the place where the bond is broken and the wrong bond is formed. So I feel there has to be some means for the migration of this effect.

PLATZMAN: But perhaps not with 100 percent efficiency.

POLLARD: No, not with 100 percent efficiency; in fact, almost surely this is not 100 percent because we now find molecules which require at least 3 ionizations to inactivate them. Not 1 but 3, and that alone means that there is an efficiency factor.

It certainly did not seem obvious to me that because you "ionize" an atom in a solid molecular configuration like this, that that particular atom had to stay ionized. It would seem to me that it would be just the other way around and there would be every reason for the place of ionization to have a statistical chance of moving all around.

BARRON: You are acquainted with our work in which we irradiated enzymes containing the sulfhydryl groups as the active group and where the only thing that happened was the oxidation of the SH group without destruction of the protein molecule. That is completely reversible because you can reduce the SH group and enzyme activity is restored. There, you see, you cannot use your criteria for explaining inactivation of the enzyme.

POLLARD: No, that is a chemical reaction.

BARRON: That is correct. I am very glad that Dr. Pollard is talking about the direct theory, but we have to make sure that what he is talking about has nothing to do with the indirect action.

POLLARD: That is right.

PLATZMAN: Is someone going to tell us just how good a distinction one can make between direct and indirect action?

POLLARD: I should like to know that, too.

PLATZMAN: Perhaps that should come before we talk about them as though the difference had been clearly delineated.

BARRON: I think I will have something to say this afternoon.

KAMEN: What was that business of no migration in the dry state? How does that fit into this picture?

POLLARD: No migration?

KAMEN: In your fact No. 7 you said that there was no migration of radiation energy in the dry state.

PLATZMAN: It was not "no migration." It was limited migration.

POLLARD: I said it does not readily migrate. I would like to go on with this and to have some discussion. Particularly, I should like Dr. Platzman to comment on this because one of the things that I am highly interested in is the return of the electron to the positive ion. Last year, he gave quite an interesting discussion of electrons below the energy of the first excited state and how they behave. I am inclined to think that is also very crucial in this context, because, if what I have said is right, the events are devastating to any molecule, but something terminates the holding of excitation energy, and it may terminate so fast that possibly this is still not a very important process. In other words, maybe the recombination occurs before these things have time to migrate at all. This should be calculable.

The feeling I had from your discussion on water was that recombination was not likely to occur very fast, and if recombination does not occur in this case either, then I am quite sure that some sort of mechanism like this of energy migration will be of great importance.

PLATZMAN: But if I may quote Pollard, from the last conference, the medium here is not water. One should take care in extrapolating the results from one medium to another.

POLLARD: I believe that I have been going a little fast on one point, and so I would like to illustrate the kind of thing that we can do by showing you a slide of an apple blackening.

This shows our basic method of working, in a very raw way. It is not something that we have published and I don't want to have it pinned on me that this is how I measure my molecular weights. But I do want to illustrate how you could go about measuring molecular weight with only these data. These data were taken by Mr. Bellamy at General Electric.

The picture represents pieces of apple that have been exposed to the air for fixed lengths of time. They have been bombarded by ionizing radiation -- mainly, I think, fast electrons. The control sample has become brown as an apple does. The samples that have been irradiated get less and less brown, and finally the one that has had 1,000,000 r is preserved and is as white as the original apple before its exposure to air.

I asked Bennett, just before everybody gathered, to look at this and to estimate the percentage of color remaining in the various samples. I have set

up a rough logarithmic plot here on the board of Bennett's estimates. In any event, you can look optimistically at this straight line. With the data we have for pepsin, trypsin, invertase, and so on, this straight line is unquestioned, because we have a true estimate and not just subjective estimates of color. Nevertheless, even these points lie on the line and they show a reasonable biologic relationship.

We claim that they have a very definite relation. The enzyme is causing the oxidation of the surface of the apple, thus giving the brown color. The per cent survival of the enzyme then follows the relation:

$$\ln \frac{n}{n_o} = VI$$

"I" is the number of clusters of ionizations per cubic centimeter volume and "V" is the quantity I have been talking about, the inactivation volume. $\frac{n}{n_o}$ is the fraction of activity surviving the irradiation, n being the amount left, and n_o the amount at the start.

This is really answering Dr. Chargaff's question. When I say it is a mental transformation this illustrates the process. Now I have merely written down the number of roentgens that correspond to this figure of $\frac{n}{n_o} = .37$. I multiply that by the number of clusters per cubic centimeter in protein, which you can work out from the Bethe formula, and then I come out with a value for "I".

The inverse of that is then the volume, V. To find the molecular weight, I multiply the volume by the density of the protein, 1.3, and I multiply that by Avogadro's number. Then we conclude that this enzyme has a molecular weight of 760,000.

It is assumed that inactivation of the enzyme on that apple surface is due to an effect caused by radiation deposited inside the molecule. I have no proof that this is the case. Actually, a wet surface-migrant energy is perfectly possible. So that I am not claiming that the molecular weight of tyrosinase is 760,000.

What I should like to debate this afternoon, or rather, should like to be informed about, is the extent to which this type of reasoning might be true. Is it possible, in point of fact, that an inactivation process of the sort described is close to the truth, or is there something completely different that greatly dominates this whole process and that actually renders this whole derivation invalid?

It would be a nice thing (and I am surprised that more people haven't done it) to study the loss of activity of enzymes in systems such as this and also the same enzymes under equivalent conditions in vitro. We have done a little of this with extremely dry preparations. We have studied the enzymes amylase, invertase, cytochrome oxidase, and succinic dehydrogenase in essentially in vitro systems and in living cells. For example, the amylase was in barley; invertase in yeast cells; and the cytochrome oxidase and succinic dehydrogenase in B. subtilis cells. In all of these studies the effect of irradiation of commercial samples in the dry state and of the organic systems was the same.

Now I should like to consider the following question. Suppose an ionizing radiation passes near a biological molecule, e.g., a respiratory enzyme unit, a mitochondrion, and produces a primary effect. We know that a part of the action -- Tobias and Zirkle have made this essentially a complete theory (10) -- is that

there can be wanderings of agents produced at the primary ionization point and that these will get into the molecule and cause an inactivation. However, only a certain class of agents may wander.

The kind of primary event that occurs at one point cannot wander through the cytoplasm and arrive at another spot. Something that has a characteristic more related to chemistry than to the direct physical event that occurs must wander. This must be something with quite a long half-life, one that lasts long enough to produce an effect. In addition, it must be something that is free to diffuse. That is to say, the entity itself must move and not merely the property possessed by the entity. So this will be a different class of process from the one of which I have been speaking.

That is well-illustrated by the fact that, roughly speaking, for an enzyme molecule, with the exception of the very sensitive sulfhydryl enzymes that Dr. Barron has worked with, it takes of the order of 10 ion pairs to produce an effect by indirect means. In the other process that may occur, which is not the passage at a distance through a liquid medium, the ionization event seems to somehow distribute its effect right through the molecule. It then ultimately produces an effect at some critical place, or alternatively causes the molecule to split open, so that it no longer acts as a molecule but becomes an opened-up system of some kind that is no longer specific.

The part that is my primary concern this afternoon concerns this direct effect. This is the only subject of which we have really made a study, and the extent to which one unit of this type of radiation action can cause an inactivation of a molecule is remarkable. Looking at this thing in general, one is forced to accord the process some respect in radiobiological materials because it does seem to be as potent as it could possibly be.

I should like to reiterate what I was discussing when I talked about the migration of the positive charge. If we take a polypeptide chain and, let us say, we ionize a nitrogen atom, it loses an electron, and so we actually have at the moment in this chain, a "carbon" atom. It has lost its electron and it has a positive charge.

Why is it necessary that it retain its inherent new carbonlike properties? As a matter of fact, it won't have the right valence. Nothing will be really fitting right. Is it not possible for this erroneous valence to migrate instead of staying in place? That is to say, why won't this ersatz carbon nucleus that is, after all, a nitrogen nucleus with a right to crystallize 7 electrons around it, take 1 of these 7 electrons from the next atom, so that it is now restored as a nitrogen atom and we no longer have a carbon atom as the next neighbor, but we have effectively a boron atom which will now be plus?

That may not last. The electron may come back or it may wander on, and you have, therefore, a random walk migration of this plus outward from the center. It is a random walk that is not in an area, but along lines and so it will, in time, move anywhere on the chain. The motion will be very rapid, because the exchange of an electron between 2 atoms like this takes place at the velocity of electronic motion and over the distance of 1 $\mathring{A}$.

The decision to transfer takes something of the order of 10^{-16} seconds. The number of decisions to transfer that can occur in 10^{-8} seconds is 10^{8}. This would mean to my mind that the migration would have had a chance to cover the whole long chain and possibly even to branch out through the residues on the side in some sort of way. This is the exchange that I was talking about before.

You will notice that I am not speaking about anything actually moving as such. No one electron travels. It is just the transfer of the electron from one atom to another that takes place. Just as we might say this is true for the rather easy case of the positive part of ionization, it would be equally true if an electron had decided, for example, to stop in a certain atom. Then we would now have a carbon nucleus with a nitrogen structure, and this in turn could then begin to move.

CURTIS: May I ask a question at this point. This event happens in 10^{-16} seconds?

POLLARD: That is the transfer of positive charge from one atom to the other.

CURTIS: And this time presumably is not time enough for anything very radical to happen as far as the bond there is concerned. The charge can travel back and forth until, as you mentioned this morning, it gets to the surface, in which case something may happen. Have you considered the possibility of two such traveling charges arriving at a bond simultaneously?

POLLARD: Yes. We have been kicking an alternative theory around. For instance, if you have a second ionization and both travel around, some bond may be broken when the two happen, by chance, to come together. It is a very attractive idea and might be all right.

PLATZMAN: What are the entities that are supposed to come together?

POLLARD: Suppose, for instance, that one atom broke off. It might weaken the bond of the nearby residue for a moment just as it went by, and if it weakened it at the right place at the same time the first was weak, you might actually get these two just simply breaking off together.

PLATZMAN: The two positive regions would repel each other and the tendency would be for them to keep apart and not to come together.

POLLARD: Well, we are thinking of the two bonds being broken so that there is a momentary chance for a new chemical configuration to form.

PLATZMAN: Yes, but the possibility of forming a new configuration would not help to induce the two initial episodes. They would have to be independent and coincident.

POLLARD: The coincidence might aid it.

BENNETT: What you want your event to do is to occur at a special place, and if these two things are going along together, it seems to me rather unlikely that that would hit at a special place, which is one of the earlier requirements that you set up.

ZIRKLE: It would require a double ionization, wouldn't it? Don't your data indicate that 1 does the trick?

POLLARD: In half the cases, 1 does the trick. In some, however, 3 are necessary. In one case, 4.

ZIRKLE: I am not entirely clear, from your earlier discussion, as to

just what can bring an end to this random walk. That is, what sort of setup could there be in some part of the molecule where this process could finally result in something irreversible. The thing surely does not keep on bouncing back and going the other way.

POLLARD: That is why I tried to relate this to Augenstine's idea of denaturation. I must admit that his idea of the sulfur bond breaking and a new sulfur bridge forming may be satisfactory, but there are molecules in which this is unlikely, e.g., molecules that contain no sulfur. So I felt that there must be a variety of ways of reforming bonds. My feeling is that the same atoms in every protein molecule are capable of being bound in different ways; that they don't have to be uniquely bound as proteins. My idea is that this migrating, weak bond, if you like, merely gives an opportunity for some of these other things to form, and if they do, then you have the loss of biological function. If they don't then you have recovery. In cases, for instance, like bovine serum albumin where it takes three ionizations to produce the inactivation, it is obvious that one ionization will do nothing. It has the chance of doing something, but on the whole, 1 ionization is not sufficient. But with 3, apparently this multiple probability of something happening may cause the inactivation.

Hutchinson (11), who discovered this effect of bovine serum albumin, thinks that a great part of solubility loss is due to multiple ionization. The proof is not complete, but, in this session, we have to talk about hunches.

MAZIA: It seems to me that what you need are experiments where you can assess the effects of radiation on two measurable activities of the same molecules that you know to be located in different parts of the molecule. One case that comes to mind where I think that the measurement would be possible is myosin. Szent-Györgyi has shown that myosin is an association of two entities which he calls meromyosins, and which are linked together by peptide bonds. One of these sub-units has an enzyme activity -- splitting ATP -- and the activity of the other can be measured as contraction. It would be predicted here that the radiation effects on the two activities would be parallel, would it not?

POLLARD: Yes, that should be the case. That is a good experiment.

BARRON: You can decrease the activity of myosin by irradiation and you can bring it back. In other words, the only thing you do is to oxidize the sulfhydryl groups without destroying the architecture of the molecule.

POLLARD: That again, however, would apply to indirect processes. That is one difficulty.

BARRON: Unfortunately, in reality you have to remember that the biological system contains 80 percent water saturated with oxygen; therefore, if you are interested in biology you have to think with this in mind.

MAZIA: But for the purpose of testing this theoretical formulations, it seems likely that you could irradiate in the dry condition.

BARRON: You cannot dry myosin and have it contracted.

MAZIA: Well, you could soak it in glycerol and serve the same purpose.

BARRON: Then you oxidize the glycerol.

POLLARD: I think you could take care of that. You could, for instance, have glutathione present during irradiation. In other words, if the recovery system is present any radiation effects will be superimposed on that.

KAMEN: I am surprised that none of the experts have hopped on this point. You would expect that if you got a positive charge on the carbon, this would stop the "walk". I don't know what the times are.

POLLARD: I have not had a chance to say what I now realize is the key to everything here. All of these times are such that no heavy thing can move. This is a key all the way through. It is a key to the very nature of ionization itself. The proton or the nucleus in the ionization process never moves, and it is interesting that in neutron studies with solids, the damage can be related to the number of recoils that actually do cause a motion of a heavy part, the part that is usually unable to move and really is insignificant here. I feel that the times involved are such that there isn't any chance for a free radical to form. If a free radical did occur I would be confident that you would no longer have a specific protein. So, possibly, one of the things to look for is, as Dr. Curtis has said, effects at the end. It might well be that all that is necessary is that something break way from the end and, having broken away, the molecule is then inactivated.

MAGEE: You have, in addition to this freedom of motion of the positive charge, a competition with the motion of the nuclei, i.e., vibrations. So when the charge gets into a certain region, you freeze the charge and it no longer moves freely. You freeze the charge into the region because of the excitation of motion of heavy nuclei; some of the energy is transformed into vibrations and the electronic energy is reduced. Then, the charge stays in one vicinity and chemical reaction occurs. I think, in general terms, that this is the explanation for the specificity of the direct action of ionizing radiation. In radiation chemistry, the fact that there is specificity of effects in certain functional groups of a molecule is known. This has been investigated, I think, rather extensively for decarboxylation of aliphatic acids. I believe that a relatively high fraction of total absorbed energy goes into decarboxylation (12).

POLLARD: You mean that it comes to a place where you can transfer from electron excitation to vibrational. It then freezes in position. That suits me fine.

ALLEN: This may shed some light on why this energy apparently does not migrate from one molecule to another. We know, of course, from the liquid scintillation counters that certain kinds of energy will do this. You can get fairly good scintillation out of dilute solutions of some aromatic compounds in normal hexane. This means that the energy absorbed by the normal hexane molecule travels from one hexane to another until it reaches the aromatic molecule. This aromatic molecule then fluoresces and produces the light that you see.

One might ask, why does this not happen with these protein molecules? Why does the energy not migrate from one to another as it probably does with normal hexane? I think it is because the protein molecule contains groups that have an affinity for hanging onto the positive charges, thereby producing decomposition, and this process is in competition with the transfer of charge from one molecule to the neighboring molecule. The fact that the protein molecules apparently do, in general, possess these reactive groups is the reason the energy stays in the same molecule.

PLATZMAN: May I make a few remarks about the charge migration?

I am certain that Dr. Pollard does not mean to give the impression that this process is a new conception. In case anyone is confused, my opinion is that it is neither new nor questionable. As a matter of fact, instead of saying that the charge moves, one could rephrase the argument and state that there is no justification for saying that the charge is localized in any one atom of the molecule -- until, of course, the charge is found in a region where part of the energy of the system may be converted into vibrational energy, i.e., heat. As Mazia has just said, once dissipation starts it cannot be reversed.

Less is known about the mode of migration of energy inside a molecule than about the related (but by no means identical) phenomena involving migration of excitation energy. The latter have been studied extensively in a variety of systems. For example, Weissman (13) has investigated the migration from a carbonyl group at one end of a molecule to a rare earth atom at the other, under a variety of conditions. Bucher and Kaspers (14) have shown that, in the carbon monoxide-myoglobin complex, light absorbed in the protein component can dissociate the CO from a prosthetic group. Franck and Livingston (9) have analyzed these and other cases and have concluded that the mechanism of energy migration is most likely of the "sensitized fluorescence" type, in all cases.

One wishes that some information were available on the extent to which Pollard's "ionization migration" can occur. A point to bear in mind is that the distance through which sensitized fluorescence can occur is determined essentially by the wavelength of light, which is much greater, of course, than the wavelength of the migrating "electron." The most stable position, if it could be reached, is at a site that one might crudely identify with the atom of lowest ionization potential. This statement must be interpreted loosely, but the fact that it is not strictly correct does not mean that it is completely wrong or may not be of great help in qualitative reasoning.

CURTIS: You say the lowest ionization potential?

PLATZMAN: Yes, for that is where the greatest amount of energy would be available for heat.

CURTIS: To put this into more visual terms, this charge sort of goes back and forth and up the side chains?

PLATZMAN: To some extent.

CURTIS: Hunting around until it kind of samples all the different atoms, and then finally picks out the one that has the lowest ionization potential and nestles there.

PLATZMAN: Except that it goes so fast you cannot say it is at any particular place at any particular time.

POLLARD: It has a higher probability of nestling there.

PLATZMAN: In radiation chemistry this argument is not uncommon. For instance, Kamen's objection that ionization ought to cause dissociation at once is met by the observations that in certain molecules this does not occur, at least with a high yield. For example, in benzene the probability of dissociation is comparatively small.

KAMEN: When don't you get it?

MAGEE: Aromatics don't give it.

KAMEN: What happens?

PLATZMAN: The excess energy is converted to heat.

MAGEE: This is a basis for protection theory. It sometimes happens that in a sensitive region, a molecule can dissipate energy that is trapped without dissociation, and then you have a protection from radiation. All we need is a recipe for favorable atomic configurations.

PLATZMAN: I should like to raise the question of the mechanism whereby a single ionization brings about the suggested effect in a protein. What is your opinion of the theory for this that Franck and I advanced (15)?

POLLARD: You tell me about it, then I will tell you.

PLATZMAN: It involved the simultaneous breakage and reorganization of many hydrogen bonds as a result of rotation of water dipoles about the freshly formed charge.

POLLARD: In other words, this occurs the moment water hits it. Being dry, how does this work?

PLATZMAN: Dry protein still shows strong dielectric absorption: therefore, it contains groups that reorient under the influence of electric fields. This reorientation is, without question, associated with the hydrogen bonds, and the sudden production of an electric charge within the protein must cause the breakage of many hydrogen bonds over a great region. Subsequent reforming of the bonds would then be irregular and might not give the original configuration.

POLLARD: I think Hutchinson does not like this. Now I am a little out of my department, at the moment, but Hutchinson's low voltage electron experiments are really very informative about this (11). What you find is that you don't get a really large effect on a molecule like bovine serum albumin until you get up to about 15 electron volts, indicating that you do have to get ionization first. You can put a great many electrons into your bovine serum albumin, so that it is certainly getting considerable charge; this does not seem to me, however, to be reorienting hydrogen bonds and producing an effect.

PLATZMAN: It would have to be pretty carefully proven that electrons get in.

POLLARD: That is right. I don't want to be dogmatic about this, and it would be better if he were sure that the electrons are in there. He is not, of course, but he says this is an indication. A figure of 15 is a sort of plausible broad ionization figure. I think these low voltage electron experiments are very informative. Obviously, they should be done on a larger scale so that we get data more quickly.

Could I go on for another couple of minutes, putting in the seamy side of this? I want to say why I think this is important first. In considering a cell that has a nucleus, chromosomes, mitochondria and so forth, distributed through it, one fact that must be borne in mind is that radiation that occurs within the molecular region will produce a certain effect. We like to say that you can make a fairly good estimate of the proportion of radiation damage that will occur as a result of this direct process by simply taking the total volume of every one of

these items, which are responsible for biological action, and calling it the sensitive volume as far as radiation action is concerned.

If I think about this from the point of view of the cell, suppose a mitochondrium is damaged. If an effect is produced at one point in it, I am sure that this will not inactivate the whole mitochondrium. It will probably inactivate a little cytochrome enzyme, 1 of a total of 10. So the effect of this on the total operation of the cell cannot, I believe, be very great. On the other hand, the inactivation of a nucleic acid molecule will also occur, and if you put one ion pair inside the molecule, and if that has transforming properties (it may or may not), biological consequences may follow, and you can estimate them in terms of this very simple idea. That I think is a contribution to radiobiology because, as I say, it enables you to pigeonhole one class of biological action in one corner.

HOLLAENDER: Do you call that a direct effect or an indirect effect?

POLLARD: This I call a direct effect. I am speaking of the case where energy is produced and is released inside the molecule. That is my differentiation between direct and indirect effects. Where does the primary action take place? If it takes place inside the molecule I class it as direct. I am saying that, for a first order, if this occurs inside this molecule, you can then say that this same molecule and not its neighbors will cease to have biological function.

HOLLAENDER: Could you modify this by some secondary treatment; possibly prevent the direct effect.

POLLARD: I would expect that the direct effect could be modified. That is one thing I want to talk about in a minute, because there are more features to it than I have been mentioning. I am quite sure that there are ways in which this could be modified on the basis of the picture that I have drawn of the radiation migrating, and the suggestions that Dr. Kamen and Dr. Platzman came up with that you could have groups which, so to speak, absorb the radiation where it does no damage. It could be done deliberately and on occasion something like it does occur.

However, speaking in the first order only, I should like my pigeonhole to include the statement that the whole molecule, and not its neighbors, in inactivated when energy is released inside. By energy, I mean ionization. Clearly, as Dr. Barron correctly says, 80 percent of the matter in this space is water, and what effect results from water action I don't particularly want to debate. I wanted to contribute a part that is not related to water and which I believe to have a part in radiobiological response; certainly not a dominant part. My estimate is that it can be between 25 percent and 75 percent. This is only an estimate.

Now I should like to give the more seamy and, may I say, the more ordinary radiobiological side of this. I should like to describe two experiments that show that you cannot quite accept my overwhelmingly simple concept.

The first concerns the loss of ability of a virus to combine with antiserum. We take a virus, T-1, irradiate it and observe whether it will still combine with the specific antiserum (16). We let unirradiated and irradiated viruses compete for antibodies and we see whether the competition is interfered with in any way by irradiation.

If we use a sample of T-1 from a very clean solution, we find that loss of ability to combine with the antibody follows exactly the pattern I have described. You get a nice one-hit curve. Everything is straight-forward. In fact, we can work out the molecular weight of the antigenic surfaces, and it fits very nicely. It is a 22,000 molecular weight for the unit represented.

If, on the other hand, we use T-1 from a solution containing a lot of broth (not pure T-1), then we are apparently unable to inactivate the surface at all. Jane Setlow, who is working with me on this, found that when the bombardment was hard enough, she could detect the stage where the activity had been lost by looking at the changes in color occurring in the samples; if the color did not change, there was no loss of ability to combine with antibodies. We found that loss of activity was very slight until heavy bombardment was applied, after which it increased rapidly. I do not know precisely what phenomenon occurs here. But it is quite clear to me that it is possible for a virus to combine with some of the protein and other molecules in broth in such a way that the surface is now radiation-stable. Why and in what manner this happens I do not know.

This is one experiment that I won't call disquieting, but it shows that we have to think a little more than we have already.

I might say that when we deal with commercial preparations and other enzyme systems, we don't find such curious anomalies. Most of our work is pretty straight-forward. But when we observe the hemagglutinins of the Newcastle virus, which constitute a number of units on the surface of the virus, and if the virus has been dried in gelatin and then irradiated, we always get a single hit type of inactivation for the process of losing ability to agglutinate red cells. It is a single hit inactivation but its behavior is such that apparently 3 to 4 ionizations are necessary (17).

Some of you may wonder how we arrive at this conclusion. It is very easy. Dr. Tobias and Dr. Zirkle will understand. In deference to their terminology, we apply the linear energy transferred below and plot the process observed in the reaction and we arrive at the sigmoid type of curve with points something like those indicated in Fig. 3. We also measure the initial slope of electron bombardment like this. There isn't any question that such a sigmoid curve cannot be explained by 1 ionization event, but actually 3 or 4 ionization events will give you this sort of relation.

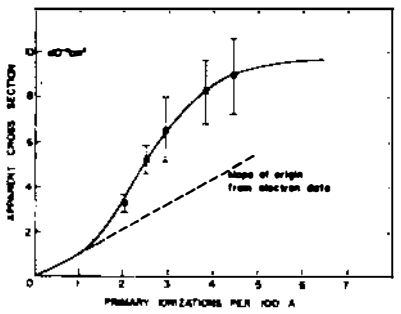

Figure 3. The effect of bombarding NDV with different energy deutrons as measured by its hemagglutinating ability. The cross-section changes follow an S-shpaed curve. The slope at the origin may be deduced from electron bombardment. The line drawn is a theoretical line based on an effective thickness of 50 A. and a sensitivity requirement of four ionizations.

If the virus is dried-out-of-phosphate buffer, which can be done, then the kind of curve that is obtained is of a more usual type. It still gives nearly the same maximum figure. We may not be quite accurate enough to be able to tell that for sure. In other words, in a dried-out-of-phosphate buffer, 1 ionization is adequate.

So in the case of these two admittedly complicated systems, which are, nevertheless, more in keeping with what radiobiological systems are like (after all, biological material is not made up

of commercial enzymes put in solution), there is present an aggregated unit with a definite biological function. The part that we measure is the part that is concerned with hemagglutination and it seems to differ in its radiobiological sensitivity according to whether it is dried-out-of-gelatin (whatever that does to it) or whether it is dried-out-of-phosphate buffer, (whatever that does to it). We get a tendency to greater sensitivity in the phosphate buffer case than in the gelatin.

These two phenomena are disquieting, in a sense, but not to me, because they tell me that there is more color and more definite information to be gained from radiation action. Since one of my primary aims is to use radiation action to study structure, the more "color" we can develop, the better I like it. Now I should like to throw this open to discussion.

KAMEN: What percentage of your dry material is still virus?

POLLARD: In the case of T-1, between 90 and 100 percent. In the case of influenza, the hemagglutination is intact. The drying does not touch it, but the infectivity is, of course, largely gone.

KAMEN: There is not enough impurity to talk about the effect any more even in the dry film ?

POLLARD: No. I would say that in the dry state there could hardly be any indirect effect.

CHARGAFF: Is there any such thing as a dry protein or a dry nucleic acid? We must have prepared hundreds of samples of nucleic acid. When we recover the material after pumping off the water at 10^{-2} mm. Hg., we invariably end up with 12 percent water in our "dry" nucleic acid which I have always considered structurally bound.

Furthermore, as to the evidence from X-ray diffraction, Wilkins and his associates have shown that they get these pretty pictures only at relatively high humidity. I don't doubt that you can pump out all the water if you let it go long enough, but I would hesitate to call that a protein or nucleic acid until I have been shown that it has not been changed.

You see, the transforming principle is really damaged even by dialysis against an electrolyte-free medium. I think that no one has been able to restore the viscosity of nucleic acid after drying, and that goes even for drying to 12 percent moisture. When you go down to 0 I doubt very much that you can really reconstitute the solution so that it has the properties of the original nucleic acid.

POLLARD: I get this very often, Dr. Chargaff. Let me ask a question. What does that tell you?

CHARGAFF: It does not tell me what has happened but it tells me that something has happened. In biology, it is always very easy to recognize degradation. It is not easy to recognize the native state. We don't know what the native nucleic acid looks like; but we can recognize the degraded state.

POLLARD: I quite agree about the degraded state. However, we are considering whether a separate effect due to diffusion of activated molecules is present. Diffusion involves motion through a medium. I claim that in these experiments I have removed that medium. That is all. I do not claim that I have

removed all the molecular H_2O.

CHARGAFF: It may be that you have irradiated this 12 percent moisture.

POLLARD: It is just like having air inside a belljar. I can never pump all the air out of the belljar, but, nevertheless, I cannot hear the sound in it.

BARRON: Then you have not removed the indirect effect.

POLLARD: I may not have removed the indirect effect because I think there is a certain amount of religion connected with the indirect effect. I have removed the medium through which diffusion can occur. There is no hydrodynamicist in the room who will disagree with me that the indirect effect is something that can diffuse as through a liquid. I am afraid I could not stand up as an objective scientist, if I did not recognize that fact. There is no medium through which diffusion effects can occur.

PLATZMAN: Easily.

POLLARD: Well, could occur easily, if you like. That is right. Of course, diffusion of lead into gold can be observed. It must not be taken as an arbitrary statement. But I am not trying in these experiments to study nucleic acid for its own sake. I very deeply regret the unfortunate fact that this transforming principle is undoubtedly degraded. I rejoice over the fact that it still works when I put it back in solution. I find that when they have their other coats on, some of the people who are the most critical of my experiments do much worse things to nucleic acid than I even think of doing, yet continue to study the effects there. I have tried to isolate only one side of action radiation. I want to say, furthermore, that I do not consider this to be the only side.

I liken myself as a physicist to a person studying electrical discharge. I am looking for one aspect. I am not saying I am explaining all the phenomena of a neon sign. If I can only find out, for example, what the simple phenomenon of ionization by collision is like, I will be content. I have a finite life.

CHARGAFF: My definition of a biochemist is a chemistry major who did not get into medical school, and he usually is quite sensitive about many other things. For instance, when I read Schroedinger's book, "What Is Life", I noticed with amazement that he had left out water. Since that time, I have been sensitive to H_2O, and that is the only reason for my question.

PLATZMAN: I have lost the threads of the debate now. Do you disagree with what Pollard said?

CHARGAFF: I don't know. I have no license to disagree. But I doubt very much that you can still call it a completely anhydrous nucleic acid or protein molecule.

PLATZMAN: We don't care what the names are. Are any of his conclusions wrong?

CHARGAFF: I would at least conclude that there is probably still plenty of water left in these molecules.

PLATZMAN: But if the water is left after the treatment, which he suggested, then it is different from the water that he has removed. As he points out,

it does not affect the argument.

CHARGAFF: You may remove 10 percent of the water from all molecules or you may remove 100 percent from a part of the molecule and keep the other part hydrated.

PLATZMAN: But if that hydrated water is different from the semi-liquid water that he removed, it does not matter.

COHN: If you have this water which is not diffusable and is part of the crystalline structure of the dried nucleic acid or protein, might it not contribute to the sensitive volume? Might it not be connected so intimately with the structure that it obeys all of the things that you are talking about so that you would not be able to distinguish an indirect effect from a direct effect because it would contribute to this molecular volume?

POLLARD: That is right. I agree. The only thing is, you see, there would still have been ionization taking place within the molecular structure. The water would have been part of the molecular structure.

PLATZMAN: If one wished to take the water out of ethyl alcohol, he could convert it to pure absolute alcohol. A harsh critic, however, might point out that the formula is still C_2H_6O, and insist that the true anhydrous form is ethylene. It seems to me that a little of this kind of thinking might be read into Chargaff's objection.

CHARGAFF: I don't think you can apply the conception of absolute alcohol to extend it to something like an absolute protein. I think there is an essential difference.

PLATZMAN: There is also an essential difference between isolated water molecules bound into a foreign structure, and a liquid drop of water.

POLLARD: Let me direct a question to either Dr. Zirkle or Dr. Tobias. You have a diffusion theory of radiobiological action, which really is an extention of what I have mentioned here. Don't you feel that you have to have a medium through which the diffusion can take place?

TOBIAS: When you expose the proteins and virus particles to radiation, you take elaborate pains to assure that these materials should be dry, that they should contain as little water as possible. Yet, when you test for the effect after irradiation, you actually place your dry molecules in aqueous medium again. Do you have any evidence at all that the effects, denaturation, inactivation, or change in structure occur immediately after exposure and still in the dry state, or do they occur when you resuspend the molecules in water?

POLLARD: I am sure the effects occur when we put the preparations into water, for the most part. We have looked for spectroscopic changes. The last case at which we looked was hemoglobin. Appleyard did this work on hemoglobin (8) and expected to find spectroscopic changes in the dry state. He used a quartz slide where everything could be observed. The largest effect does seem to occur when the material is put in water. The only thing I can say is that my whole concept has been that although there is a rejoined bond of some kind, this rejoined bond actually won't produce any change in the over-all ultraviolet absorption spectrum because ninety-nine percent of the usual bonds are still there. However, this 100th bond will affect the biological action. That you cannot see until the material is in water. So water does play a part in the effect.

But many other things can be said. It makes no difference how long you wait before you put it into water. You can heat it after it has been irradiated. You can heat it for a considerable time and then put it in the water. We have tried all of these things and they have no effect unless the heating is excessive.

TOBIAS: A charge from an ion pair does not migrate very far. So it seems to me that the major effect occurs mostly when you put the irradiated molecule in water. Then we should look for a mechanism that can preserve ionization or the excitation for a long time.

POLLARD: That is why I supposed the wrong bond formation preserves it.

TOBIAS: I would like you to discuss further the assertion that an ion pair causes the effect in large dry molecules. Most of your evidence appears to stem chiefly from the fact that if one assumes a plausible value for the energy necessary to produce an ion pair, this leads to a volume per ion pair, which is close to the correct molecular volume. Can you completely rule out excitation as the cause of the biological effect in dry molecules?

POLLARD: Well, Hutchinson's experiments with low voltage electrons speak against excitation. Also, the action of ultraviolet light itself is not very great. The quantum yield is low, of the order of 1 in 100.

TOBIAS: But you could have a wavelength in the far ultraviolet region where the quantum yield is presumably high.

POLLARD: We have just been looking in the far-off field.

PLATZMAN: How far off?

POLLARD: We have gone out to about 1500 now. We have begun to look for absorption, and it is very interesting.

TOBIAS: I am under the impression that large molecules in the dry state might have considerable charge accumulated on their surface. Do you have any observations available on the net molecular charge?

POLLARD: I haven't any figures on that.

KAMEN: Do you know about the recent work of Alexander and Charlesby (18)? They have studied methacrylate polymers and find a linear relation between radiation dosage and cross-linking. On the other hand, Little (19) thinks that all their data can be explained as straight-forward breakdown of the linear chains. I wanted to ask you whether you knew about this work.

POLLARD: I don't know much about it. It is in Nature (18), and I have read it as you have.

ALLEN: I should like to ask one question on this allusion you made to indirect action in the case of the monolayer of catalase, I believe it was. Can you give a figure as to the deduction with regard to the lifetime of the species in water? What was that lifetime?

POLLARD: The closest I think is 3 microseconds.

ALLEN: To what distance of diffusion does this correspond?

POLLARD: I cannot remember that. It is in the paper that Smith (3) worked out.

ALLEN: This seems awfully short for the lifetime of a radical in pure water. Could it be possible that the catalase, when it is in the monolayer stage, is less radiosensitive than it is when dispersed molecularly in water?

POLLARD: I think if you assume that the catalase has an unequal sensitivity, the lifetime does not become any shorter.

ALLEN: Is there some basis for an estimate of the probability of collision of the radical with a monolayer and of its doing anything?

POLLARD: Well, that was what worried Smith. He measured the ionic yield for the catalase and got 20 ion pairs to inactivate 1 catalase molecule. I think he assumed that this corresponded to a high sensitivity region on the surface, which was 1/20th of the whole surface, and he used that 1/20th of the surface as the region on which the radical would have to go. Otherwise, he would come out with a figure like Lea's, which he didn't believe. He has a check against this in the bovine serum albumin where again you get a figure of the same order. I do know that he does not treat the whole molecule as the sensitive region. I know that it is a fraction and it is a fraction determined by the measured ionic yield. Of course, if a multiple number of ions is needed to arrive even to a sensitive place, then it would be different.

ALLEN: Is it not still possible that the whole structure of the catalase, and particularly its hydration structure, may be changed when it goes into this monolayer?

POLLARD: It seems to work on hydrogen peroxide in nearly the same way.

MAZIA: But the solubility certainly has changed.

POLLARD: It is just the enzymatic activity that apparently hasn't changed appreciably.

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THE IN VITRO EFFECTS OF RADIATIONS ON MOLECULES OF BIOLOGICAL IMPORTANCE

E.S.G. Barron

We have heard Dr. Pollard's very thorough discussion about the action of ionizing radiations on "dry" matter. I am now going to speak on the action of ionizing radiations on aqueous solutions and the role of oxygen. As Dr. Pollard has already stated, the living cell contains about 80 percent water. Moreover, in most cases, it is oxygen-saturated water. The biologist is therefore mainly interested in the action of ionizing radiations upon oxygen-saturated aqueous solutions.

I have been asked to talk about the action of ionizing radiations upon substances of biological importance and to attempt to draw from these studies conclusions that are of interest to the cell physiologist. I will start with the oxidation-reduction reactions that are brought about by ionizing radiations. In oxygenated aqueous solutions, we have the formation of three powerful oxidizing agents: the radicals OH, O_2H, and H_2O_2. Atomic hydrogen seems to recombine quickly to form the unreactive molecular hydrogen. The only reduction reactions, reported to be caused by irradiation, have been reductions of inorganic compounds, such as ceric sulfate in acid solutions, and permanganate, bromate, chromate, iodate, systems with an Eo above $+0.9$ v, which are of no biological interest. Substances of biological importance, such as the respiratory pigments, ascorbic acid, glutathione, lactic acid, ethanol, dihydrodiphosphopyridine nucleotide (DPNH), coenzyme A, and formic acid are all oxidized, whereas the oxidized states are not reduced at all (1).

The hydrogen atoms, which are presumably formed by irradiation of water, show little activity. Probably the rate of recombination to molecular hydrogen is too fast. For example, ferricytochrome c, which is easily reduced by black platinum and hydrogen, is not reduced by X irradiation up to 100,000 r, the maximum exposure that can be used without producing protein denaturation (2). The same thing occurs with glutathione. Whereas reduced glutathione is oxidized by irradiation, oxidized glutathione is not reduced (3).

KAMEN: Did you see any hydrogen produced?

BARRON: We did not measure hydrogen production. What we say is that it has been impossible for us to produce reduction reactions by irradiating aqueous solutions of substances of biological importance.

The point has been raised concerning the extent of ionization and the ionic yield. This is of special importance because we have to remember that when we pass an ionizing track through an aqueous solution containing a number of reactants, we have not only the action of the free radicals produced on ionization of water but also the action of free radicals produced during the oxidation of the reactants. If we accept Michaelis' theory of compulsory univalent oxidation, there must be formation of intermediate free radicals whenever we oxidize a bivalent compound. These free radicals will then produce oxidation-reductions, perhaps of different systems than those reacting with the OH and O_2H radicals. The high yield of oxidation of glutathione may be explained by a chain of reactions produced by the free radical RS, besides the free radicals OH and O_2H:

$$RSH^+ \quad OH \longrightarrow \dot{RS} + H_2O \qquad (1)$$

$$RSH + O_2H \longrightarrow \dot{RS} + H_2O_2 \qquad (2)$$

$$\dot{RS} + \dot{RS} \longrightarrow RSSR \qquad (3)$$

$$RSH + H_2O_2 \longrightarrow \dot{RS} + H_2O + OH \qquad (4)$$

$$RSH + OH \longrightarrow \dot{RS} + H_2O \qquad (5)$$

$$RS + \dot{RS} \longrightarrow RSSR \qquad (6)$$

There are thus 4 molecules of RSH capable of being oxidized by the 2 radicals, which would give 12 molecules per 100 ev. if we assume that 32.5 ev. produce the 2 oxidizing radicals:

$$H_2O \longrightarrow H + OH$$

$$O_2 + H \longrightarrow O_2H$$

PLATZMAN: How did you compute the ionic yield?

BARRON: The ionic yield was computed by measuring the oxidation of ferrous sulfate in acid solutions.

The great sensitivity of the sulfhydryl groups to ionizing radiations is clearly shown on irradiation of phosphoglyceraldehyde dehydrogenase. With 100 r, there was 21 percent inactivation. That this enzyme inhibition was produced entirely by oxidation of -SH groups in the protein molecule was shown by the complete reactivation of the enzyme on addition of glutathione. When the exposure was increased to 500 r, there was 94 percent inhibition of enzyme activity and only 10 percent reactivation on addition of glutathione. (Table I) We may assume that the irreversible inhibition was due to action on other groups of the protein, such as the OH groups of tyrosine or serine, the NH_2 groups, or to rupture of hydrogen bonds. How much of this second action -- irreversible inhibition -- is due to the direct collision between the ionizing track and the protein molecule cannot be calculated from these experiments.

POLLARD: I would answer that none of this is due to the direct effect of the track going through the enzyme.

PLATZMAN: Are these solutions?

TABLE I

INHIBITION OF PHOSPHOGLYCERALDEHYDE DEHYDROGENASE AND ADENOSINE-TRIPHOSPHATASE BY X RAYS
REACTIVATION WITH GLUTATHIONE (added after X irradiation)

Enzyme	X ray Exposure	Inhibition	Reactivation
	r	Percent	Percent
Phosphoglyceraldehyde Dehydrogenase	100	21	Complete
	200	50	62
	300	80	-
	500	94	10
Adenosine-triphosphatase	100	27	97
	500	41	56
	1000	73	22

BARRON: Yes.

PLATZMAN: None was a concentrated solution?

BARRON: The experiments reported in this table were performed with solutions containing 14 Mg. per ml., i.e., 1.4×10^{-7} M.

PLATZMAN: Did you use different concentrations in other experiments?

BARRON: Yes. The effect was independent of concentration. That the effect of radiation varies with the different proteins is shown in Table II, where the data on X-ray-induced inhibition of enzyme activity have been assembled: The highest ionic yield was obtained with the -SH enzymes, alcohol dehydrogenase and phosphoglyceraldehyde dehydrogenase, and the lowest with catalase. Not all -SH enzymes, however, have the same ionic yield, because of the different spatial

TABLE II

IONIC YIELDS OF ENZYMES INACTIVATED BY X IRRADIATION

Enzyme	Ionic Yield
Yeast alcohol dehydrogenase	1.1
Phosphoglyceraldehyde dehydrogenase	0.93
Carboxypeptidase	0.18
D-Amino acid oxidase	0.1
Hexokinase	0.07
Ribonuclease	0.03
Trypsin	0.025
Lysozyme	0.01
Catalase	0.003

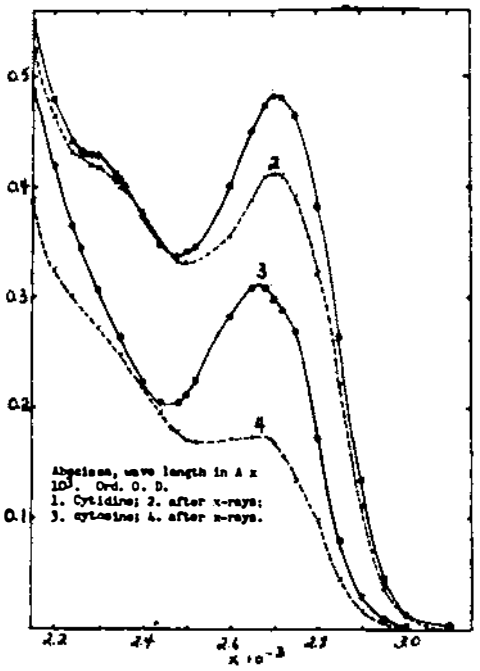


Figure 1. Effect of X-irradiation on the absorption spectrum of cytidine and cytosine X-ray dose, 20,000 r.

distribution of the -SH groups will hinder oxidation. In Table II, we have the -SH enzymes, D-amino acid oxidase and hexokinase, which are more resistant to X irradiation.

MAGEE: How did you calculate the ionic yield?

BARRON: In these experiments ionic yields were calculated by assuming that 1.61×10^{12} ion pairs are formed per g. of water per r unit.

PLATZMAN: Thirty-five volts per ion pair?

BARRON: 32.5 volts.

I want to discuss now the problem of induced oxidation-reduction reactions by the free-radicals produced during oxidation with OH and O_2H radicals. I would like to call this process enhancement of irradiation. These radicals, having a longer half-life than the OH and O_2H radicals, may diffuse longer distances. This, in my opinion, is of tremendous importance to the biologist because it may explain effects produced by small doses of ionizing radiations, and effects observed at long distances from the ionizing tracks. While reduced pyridine nucleotide (DPNH) is oxidized by X irradiation, the oxidized form (DPN^+) is not reduced (4). In the same manner, ethanol is oxidized to acetaldehyde, and lactate to pyruvate, while the reverse process does not occur. Swallow (5) found that when aqueous solutions -- nitrogen saturated -- of DPN^+ and ethanol were X irradiated, there was formation of DPNH as shown by spectrophotometric measurements. Swallow did not measure enzymatic activity of the irradiation product. We have confirmed Swallow's experiments. Moreover, the same reduction, although to a lesser degree, was found after irradiation of lactate plus DPN^+ (Figure 1), and also after irradiation of isoproply alcohol plus DPN^+. We have, in this case, the following reactions taking place with ethanol and DPN^+:

$$CH_3CH_2\dot{O}H + OH \longrightarrow CH_3\dot{C}HOH + H_2O \quad (1)$$

$$CH_3\dot{C}HOH + DPN \longrightarrow DP\dot{N}H + CH_3COH \quad (2)$$

$$CH_3CH_2OH + OH \longrightarrow CH_3\dot{C}HOH + H_2O \quad (3)$$

$$CH_3\dot{C}HOH + DPNH \longrightarrow CH_3COH + DPNH + H^+ \quad (4)$$

The OH radical oxidizes ethanol to the half-oxidized radical, $CH_3\dot{C}HOH$, which in turn reduces DPN to the half-reduced radical $DP\dot{N}H$. A second molecule of alcohol radical completes the reduction of DPN. The reduction is thus pro-

duced by the oxidation product of irradiation. In the presence of lactate, the free-radical formed is $CH_3COHCOO$, and in the presence of isopropyl alcohol, it is $CH_3\dot{C}OHCH_3$.

The enzymatic activity of this reduced compound was measured with alcohol dehydrogenase from yeast and was found to be 50 percent active.

KAMEN: Don't you think that 50 percent yield is accounted for by the fact that the enzymatically active DPNH has stereospecificity, whereas, in your experiments, the position of hydrogen in the nicotine-amide portion of the molecule is randomized?

BARRON: I thoroughly agree with you. These experiments suggest that in the enzymatic oxidation of ethanol there is intermediate formation of a free radical. They are also a confirmation of the beautiful experiments of Westheimer and Vennesland (6).

KAMEN: Can you reoxidize this enzymatically reduced 50 percent?

BARRON: Yes.

I want to speak now about the effect of ionizing radiations on proteins. Here my point of view is rather different from that of Dr. Pollard. Proteins are attacked selectively by ionizing radiations and at different points. If we take, for example, proteins with a $\frac{\text{tyrosine}}{\text{tryptophan}}$ ratio greater than 1, such as serum albumin, irradiation produces an increase in the absorption spectrum at 2800 Å, which is proportional to the X-ray exposure (7). This increase is due to oxidation of the tyrosine residue and it is also found after irradiation of tyrosine solutions and during the first minutes after addition of tyrosinase to tyrosine. X irradiation of a tryptophan solution, on the other hand, produces a decrease in the absorption band at 2800Å, which is proportional to the dose. If changes in the absorption spectrum around this wave length are due to a tryptophan attack in the protein molecule, then one would expect a decrease in the absorption spectrum after irradiation of proteins having a $\frac{\text{tyrosine}}{\text{tryptophan}}$ ratio lower than 1. Chymotrypsin and lysozine were taken as examples of such proteins, since both are rich in tryptophan. X irradiation of these proteins produced the expected decrease in the absorption spectrum at 2800 Å. X irradiation of proteins with small doses leads first to oxidation of the -SH groups and nothing else. Then comes oxidation of the OH groups of serine, and deamination of the free amino groups. When the exposure is increased to 75,000 r, aqueous solutions of serum albumin are precipitated. This phenomenon, which is temperature dependent, seems to be due to rupture of the hydrogen bonds. X irradiated solutions of serum albumin can be kept for hours at 3°C without precipitation. As soon as the temperature is raised, precipitation occurs. Polymerization may also take place. When -SH- containing proteins are irradiated, there may be formation of a dimer, a disulfide protein:

$$2\ \text{SH-protein} \longrightarrow \text{protein - S-S-protein} + 2H^+$$

This phenomenon takes place with serum albumin that has one -SH group per molecule. Sedimentation studies of normal and X irradiated serum albumin indicated that the S_{20w} value of the second peak of the irradiated protein agrees with the values calculated for a dimer.

CHARGAFF: Does the amount of the second peak depend upon the dose

or can you produce more at will if you irradiate longer? Does it reach an equilibrium value?

BARRON: That is a difficult question to answer because, when the exposure is increased to produce more dimer, protein damage is also increased and precipitation takes place. Furthermore, the presence of other solutes also has great influence. A dilute solution of serum albumin that precipitates with 75,000 r remains optically clear if irradiated in the presence of salts, NaCL (0.1M) or phosphate buffer (0.01M).

WORF: Would small concentrations of amino acids have the same protective effect?

BARRON: Yes. When aqueous solutions of albumin were irradiated in the presence of cysteine, there was no dimer formation, presumably because of reaction of the free radicals with cysteine, the "protecting action" of Dale.

MAZIA: Do you ever get gel formation in concentrated solutions?

BARRON: We have never irradiated concentrated solutions.

MAZIA: I ask this in connection with Dr. Pollard's suggestion concerning the breakage of disulfide bonds. If these bonds were broken and then reformed in new positions, it would be probable that a certain number of intermolecular S-S bonds would be formed, and such a polymerization might lead to gel formation.

BARRON: This relation between -SH groups and gel formation reminds me of the experiments of Huggins (8) who found that in the thermal coagulation of serum albumin, the nature of the coagulum was influenced by minute amounts of -SH reagent. At pH values from 6.9 to 7.4., thermal coagulation produced a soft, opaque gel. Previous addition of -SH reagents produced clear, elastic gels. The clot produced in the presence of -SH reagents could hold 3 to 4 times as much water as the control opaque gel.

MAZIA: Pollard proposed the opening of the S-S bonds and reformation in other places. You would, under these conditions, expect gelation.

BARRON: Few proteins have -S-S- bridges.

CURTIS: You actually do get this. Nims, in our laboratory found an increased tendency of fibrinogen solutions to clot following the massive exposure to radiation. Although the average size of the molecule was greatly reduced according to the sedimentation constants, the clotting capacity of the solution had increased.

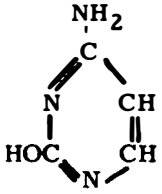
BARRON: Polymerization resulting from irradiation has been demonstrated, and it is conceivable that substances containing a number of -SH groups in their side chains may, on oxidation of these groups, polymerize to form gels and macromolecules.

I will speak now about some experiments we have done with nucleic acid, pyrimidines, and purines. Taking advantage of the intense absorption of light in the ultraviolet, we tested the action of X radiation on adenosine triphosphate. There was a decrease in light absorption proportional to the X-ray exposure. The same phenomenon occurs with all these substances. Purines and pyrimidines can add 1

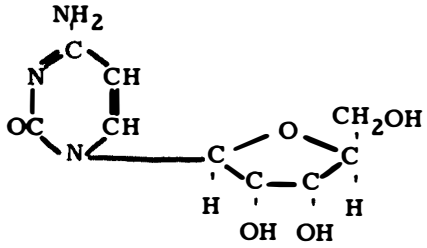
pentose molecule to give a nucleoside. They may add phosphoric acid residues, thus increasing the complexity of the molecule. Finally, they may combine with each other to produce the nucleic acids. We have found that as the complexity of the molecule increases, the sensitivity to the action of X-rays decreases. For example, irradiation of cytosine with 50,000 r produced a large decrease in the absorption spectrum. Addition of pentose to cytosine to form cytidine decreased considerably the action of X-rays (Figure 1).

ALLEN: Could you tell us what these molecules are?

BARRON: Cytosine is 2 hydroxy-6-aminopyrimidine



Introduction of a pentose residue to the N in position 3 gives cytidine, 3 β-ribofuranosido-cytosine



Addition of ribose protects the cytosine molecule against the effects of X irradiation.

ALLEN: What is the ion pair yield for this loss of ultraviolet absorption?

BARRON: I will come to it later.

ALLEN: May I ask what group is responsible for the ultraviolet absorption?

COHN: It is the pyrimidine ring and primarily the double bonds in this ring. There are 3 double bonds in the cytosine ring.

ALLEN: Are the double bonds reduced?

BARRON: According to Cavalieri (9), the absorption is due mainly to the -C = C - C = N or -C = C - C = O chromophore of the ring. Reduction of the double bonds is rather difficult, although it may be produced by hydrogen in the presence of colloidal palladium.

COHN: Destruction of the double bonds does not necessarily mean reduction. It could be by oxidation.

ALLEN: If it were by oxidation, you would form alcohol.

KAMEN: Did Sinsheimer do this?

COHN: He destroyed the ultraviolet absorption of uracil and brought it back by chemical treatment, an indication that there was no cleavage.

CARTER: This reaction is undoubtedly different from that produced by X-rays. Is the decrease produced by irradiation reversible?

BARRON: No. A good compound for study of the relationship between the complexity of a molecule and its resistance to X radiation is adenine. (Table III). When an aqueous solution of adenine

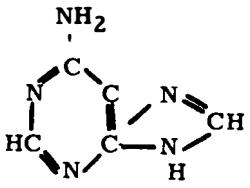


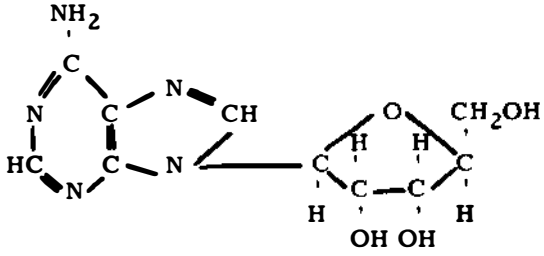
TABLE III

COMPARATIVE EFFECT OF X IRRADIATION ON THE ABSORPTION SPECTRUM OF ADENINE COMPOUNDS.

Concentration, 4×10^{-5} M dissolved in water. X-ray exposure, 20,000 r. The figures given are the decrease in optical density ($\log \frac{Io}{I}$) after irradiation and measured at 2600 Å.

Substance	Δ, 2600 Å
Adenine	-0.090
Adenosine	-0.045
Adenylic Acid	-0.035
Adenosine diphosphate	-0.023
Adenosine triphosphate	

is irradiated, the optical density at 2600 Å decreases by 0.09. Addition of ribose to form adenosine (9-β-D-ribofuranosidoadenine),



decreases by half the sensitivity of the compound. Further protection against the effect of X irradiation is observed on addition of phosphoric acid residues. The ionic yields of several of these compounds is given in Table IV. It can be seen that while the ionic yields of thymine, uracil, cytosine, adenine, and guanine are around 1 per 100 ev., they are considerably less for ribonucleic and desoxyribonucleic acids.

TABLE IV

RADIOCHEMICAL YIELD ON X IRRADIATION OF PURINES AND PYRIMIDINES IRRADIATED IN DILUTED AQUEOUS SOLUTIONS

Substance	G (100 ev.)
Sodium Desoxyribonucleate	0.000385
Sodium Ribonucleate	0.00724
Thymine	1.245
Uracil	0.64
Guanine	0.261
Cytidine	0.616
Cytosine	1.845
Adenine	0.676
Adenosine	0.196
Adenylic Acid	0.161
Adenosine diphosphoric acid	0.138
Adenosine triphosphoric acid	0.109
Uric Acid	0.37
DPNH (Oxidation)	1.51
DPN	0.02

CARTER: The latter are more stable as measured by one criterion?

BARRON: That is correct; stable around the chromophore groups responsible for light absorption in the ultraviolet.

CHARGAFF: How do you explain the difference between thymine and guanine?

BARRON: Perhaps the addition of the iminazole ring to the pyrimidine in guanine made the molecule more stable than thymine, which has only a pyrimidine ring.

ALLEN: Are these ionic yields all for air-saturated water?

BARRON: Yes. Irradiation in nitrogen-saturated water diminished the yield.

I am going now to present to you some experiments we have performed on respiratory pigments with iron-porphyrin as the prosthetic group. X irradiation of ferricytochrome produced an increase in absorption spectrum in the region corresponding to the protein moiety, around 2800 Å, and a decrease of the Soret band corresponding to the porphyrin nucleus. The Soret band, 4050 Å, is more easily destroyed by X radiation when cytochrome is dissolved in 0.005 M HCL than when it is dissolved in neutral or alkaline solutions. This effect is due to the oxidizing action of the OH and O_2H radicals; it is decreased by half when irradiation is performed in the presence of nitrogen. The decrease of light absorption in the Soret band can also be demonstrated on irradiation of protoporphyrin. Here, as the Soret band is decreased, there is also a decrease in the red fluorescence characteristic of porphyrins.

SHERMAN: Did you observe this effect in the absence of water?

BARRON: I was informed that experiments had been performed with nucleic acids dissolved in carbon tetrachloride and that they were presented as an indication that the effect produced by irradiation was not due to the OH and O_2H radicals. Similar experiments were made with protoporphyrin. On X irradiation, the porphyrin became green and the Soret band was greatly reduced as well as the fluorescence. The same effect was obtained when porphyrin was dissolved in X irradiated carbon tetrachloride. Porphyrin was converted into a biliverdin.

Pure, dry carbon tetrachloride, free from impurities, is relatively stable. When it is X irradiated, a free radical and atomic chlorine are formed which on recombination give hexachloroethane and Cl_2:

$$CCl_4 \xrightarrow{\text{irradiation}} \dot{C}Cl_3 + Cl \quad ;$$

$$\dot{C}Cl_3 + \dot{C}Cl_3 \longrightarrow C_2Cl_6$$

$$Cl + Cl \longrightarrow Cl_2 \quad .$$

The oxidizing action of chlorine is responsible for the attack on the porphyrin molecule. It consists of the opening of the methane bridge and the oxidation of this group. In the presence of water, chlorine gives ClOH, which also is a powerful reagent.

KAMEN: Was carbon tetrachloride irradiated in air?

BARRON: It was irradiated soon after redistillation and in a closed vessel.

ALLEN: If you add chlorine to your system, does it have the same effect as irradiated carbon tetrachloride?

BARRON: Chlorine and hypochlorous acid produce the same effect as

irradiated carbon tetrachloride.

We will continue with the respiratory pigments and consider hemoglobin. Irradiation of a dilute aqueous solution of hemoglobin with 20,000 r causes the following reactions to take place as observed spectrophotometrically: Oxidation of the tryptophan groups of globin, as shown by a decrease in the absorption spectrum at 2800 $\mathring{A}$; an attack on the porphyrin, as shown by a decrease of the Soret band; and oxidation of the Fe^{++} porphyrin to Fe^{+++}, as shown by an increase in the absorption spectrum at 6300 $\mathring{A}$. In fact, the oxidation of oxyhemoglobin to methemoglobin proceeds in linear relation to the X-ray exposure.

We may conclude from all of these experiments that ionizing radiations acting on aqueous oxygenated solutions show definite specificity in agreement with the assumption that the effects are essentially those produced by the free radicals, OH and O_2H. It is essential to remember that observations from studies using large doses of X-ray cannot be extrapolated to those with doses used to irradiate living cells, which do not produce immediate death. Large amounts of radiation produce effects qualitatively different from those obtained from exposures in the thousands. Whether the effects of thousands of roentgens can be extrapolated to effects produced by hundreds or tenths roentgens is not known.

The second conclusion I would like to make concerns the biological importance of the induced oxidations, what I have called enhancement of radiation action. These are reactions produced by the free radicals originating from the primary oxidations due to OH and O_2H radicals. The former are more stable than water radicals, and, as a consequence, can diffuse more efficiently. The cell has continuously a large number of oxidizable substances that, on x irradiation, will form free radicals. These will act by themselves either as reducing or oxidizing agents.

Regarding the effect of radiations on nucleic acids, I would venture the opinion that they are rather resistant because they are well-protected by other groups in the vicinity. This opinion is in agreement with the observations of cytologists. What is inhibited by small doses of X-rays is the synthesis of nucleoproteins, as was found by Mitchell and by Hevesy.

The work done with pure enzyme solutions cannot be extrapolated to the cell. Irradiation of enzyme solutions was performed in a system where the enzyme was the only reactant. In the cell, there are hundreds of enzymes, proteins, carbohydrates, fats, and electrolytes, all capable of reacting with the free radicals. However, as a general rule, one may say that a system that is stable when irradiated in aqueous solutions will be stable when so-treated in the cell.

I am, of course, disappointed to see that the -SH groups in the cell seem to be more resistant than when they are irradiated in solution. However, I still believe that the enzymes for nucleic acid synthesis and for protein synthesis are -SH enzymes with freely-reacting -SH groups that are extremely sensitive to oxidizing agents. In this respect, I want to remind you of the experiments of Van Heijningen (10), who found inhibition of -SH enzymes in the lens of X irradiated rabbits, whereas enzymes possessing no essential -SH groups for activity were not decreased.

PATT: But this is somewhat removed in time from the initial event of irradiation. Sulfhydryl inhibition, in this case, occurs days after irradiation and therefore, probably does not represent a direct effect of oxidizing agents formed

during the exposure.

BARRON: Yes.

PATT: That, I think, is quite important.

BARRON: You are absolutely correct. We have to demonstrate inhibition of -SH enzymes in the cell soon after irradiation. I am still hopeful of being able to make such a demonstration when we start our work on synthesis of nucleic acid components. I still believe they are -SH enzymes and that they are very sensitive to oxidizing agents.

CARTER: I don't want to take the part of defender of nucleic acids, but I would like to get back to this business of what you have to measure when you are talking about the relative sensitivity of nucleic acids to irradiation. I think the criteria Dr. Barron discussed may be the least sensitive. In the case of biological activity of the transforming principle, it is known that minor changes in the organization of the molecule brought about by heat, pH change, and ultraviolet radiation are associated with loss of activity.

MAZIA: Carter's statement raises the question, whether anyone has irradiated solutions of transforming principle. This is the only means now available where one could measure an effect on the biological activity of nucleic acid.

CARTER: Zamenhof has done some work in this regard. I have not seen his published figures.

CHARGAFF: I do not think he has worked with X-rays. He has studied ultraviolet and similar things and some pH changes. I have the feeling that I have seen a paper on it.

CARTER: The ultraviolet has been done. Heat also causes inactivation.

CHARGAFF: If 99 percent of the molecules have nothing to do with the transforming principle, if the transforming principle is more sensitive to all kinds of agents than the DNA present in the preparation, there could be inactivation because you have really hit the most sensitive part of the mixture. That is a possibility. As long as we really don't know what makes the transforming principle, it is hard to say. You see, the old finding of McCarty about inactivation of the transforming principle by ascorbic acid has stuck in my mind as something very funny. I don't know whether that has been repeated. It has never been shown in any event how ascorbic acid inactivates the transforming principle. The analytical methods are too crude. You can analyze within plus or minus 2 percent. You can account for 98 percent of your bases, but trace-constituents can still be present and escape detection for the moment.

CARTER: For the most part, you know from the chemical work that those trace constituents are not protein. So what it comes back to is what is the molecular identity of this material?

CHARGAFF: It is undoubtedly true that it is the nucleic acid that has the transformation activity, but whether this property is due only to a particular sequence of the constituents or whether there is something else in its structure, which we cannot describe yet, is unknown.

MAZIA: I think that one could challenge Dr. Barron's last point; namely, that the irradiation effect is not on the nucleoprotein but on the enzyme system synthesizing nucleoprotein by drawing upon a fundamental experimental design in radiation genetics. One irradiates mature sperm in the male and observes a genetic effect of the radiation when the male is crossed with an unirradiated female. The sperm cell is not synthesizing nucleoprotein; this had been synthesized some time earlier. The sperm nucleus carries a complete genetic code that is waiting to be transported into an egg where it will go to work. The fact that irradiation of the sperm nucleus does produce genetic effects - in fact, irradiation of sperm is a favorite tool of the geneticist - tells us, I think, that the nucleoprotein is, in fact, radiosensitive. In a way, the chromosomal nucleoprotein appears to be the most radiosensitive of all systems. This may be explained by the fact that the techniques of genetics and cytogenetics permit us to observe effects at dose levels where you could not hope to do so on enzymes under physiological conditions.

BARRON: I agree with Dr. Carter that we have just one parameter, but you can do the same thing with amino acids. You irradiate amino acids and you determine the diminution of the amino acids. You make a peptide. Immediately the deamination becomes more difficult. From the peptide you make a protein. In other words, the more complicated the molecule becomes, the greater its stability against the action of the ionizing radiation, because you have the effect of steric hindrances and of electronegative groups in the protein molecule, which protect the sensitive spot where the product of ionization is going to act.

MAZIA: The larger molecules may be more resistant to radiation effects that can be detected by chemical methods and yet be more sensitive in terms of biological detection. They may have specificity and may be denatured completely as a result of very minor structural modifications. In many cases, such as the genetic system, there may be present only one or a few of a given species of molecule, so that modification of one molecule will produce a large effect biologically. Perhaps we should distinguish between the intrinsic stability of molecules, which you have been discussing, and their stability in terms of the probability that a radiochemical event will have measurable biological consequences.

CARTER: Of course, there are some aspects of organized systems, such as Mazia has worked with, that deserve some comments. As I understand them, the combination between nucleic acid and protein is an exquisitely sensitive system in terms of certain parameters.

MAZIA: Yes. Dr. Maurice Bernstein, working with Kauffman at Cold Spring Harbor, has found that desoxyribonucleoprotein of nuclear origin shows a sensitivity to X-rays beyond what would be predicted from information about effects on pure nucleic acids or pure proteins.

HOLLAENDER: Carefully isolated nucleoprotein that has a very high viscosity will respond by change in viscosity to less than 100 r. The ultraviolet absorption spectrum will not change under such conditions. The sensitivity of this nucleoprotein resembles the sensitivity of biological material to X radiation. At least the energy values are of the same order of magnitude. (11).

BARRON: I want to emphasize that when you irradiate two substances you have not only the action of the ionizing radiation but you have also, the action of the free radical that is produced when two systems are irradiated. I want to extend this to the cell and to say that when you irradiate the cell, you produce,

besides the free radicals from water, free radicals from the ionization of the innumerable substances that are in the cell, and they are going to contribute to the overall effect.

CARTER: I would certainly agree with Dr. Barron in his interpretation in this regard. I believe, on the basis of prejudice more than anything else, that the primary events probably are concerned with the smaller molecular weight components and those that are exquisitely sensitive to ionizing radiation. These may transmit their effects to the higher molecular weight components. The point that I was arguing (and I believe Dr. Mazia was) was that these high molecular weight structures are quite sensitive; that they do have parameters of structure that probably we don't quite know how to explore at this time and how to correlate with biological activity, but insofar as we can make estimations at this stage of the game, they do seem to be sensitive.

BARRON: I want to recall for you the experiment with DPN. I have irradiated DPN with 100,000 r and it has been impossible to produce reduction to DPNH. But when I irradiated with 35,000 r in the presence of lactic acid, there was a reduction of DPN produced by the oxidation product of lactic acid. This is the sort of thing that we have not considered in radiobiology.

KAMEN: I should like to bring up a point in connection with this DPN experiment. I believe that when you talk about an enzyme substrate like that, to talk about what happens to it away from the enzyme may be misleading. Have there been any experiments to show that there is reduction of DPN with a DPN-dependent enzyme and its substrate present? For instance, in photosynthesis, despite the fact that we produce in extracts carrying out the Hill reaction, an oxidizing system with a potential near that of the oxygen electrode and simultaneously a reducing system with a potential near that of the hydrogen electrode, we cannot reduce DPN or TPN directly. But if we throw in enzymes, like Ochoa's "malic" enzyme together with pyruvate and CO_2, then, even though we cannot show any accumulation of TPNH or DPNH with H-acceptor systems, we can get reduction of the pyruvate and CO_2 to malate. But if you throw in enzymes like alcohol dehydrogenase, then even though you cannot detect any DPN reduction, you do get reduction of the fumarate to succinate. It may be then, that in the cell where the DPN is bound to some characteristic enzyme, such as a dehydrogenase you could be getting reduction of the DPN because there is something to pull it. As long as there is nothing for it to do, it probably does not get reduced. So that is the kind of thing that I think ought to get looked at more carefully.

BARRON: I presented this experiment to demonstrate that there are effects produced by free radicals present in the cell. So it is not only DPN. There may be other systems, too, that contribute.

KAMEN: Most of the DPN in the cell is bound DPN. There is very little free DPN in the cell.

BARRON: I agree with you. But what I am saying is that this is an experiment to demonstrate the enhancement of action.

KAMEN: I am just saying that your model experiment may not be a model experiment.

POLLARD: I have one experiment that worries me very much, and I should like to ask whether anyone here has anything analogous to it. We measured the ionic yield for invertase in aqueous solution and came out with a very

reasonable figure of about .016, or something like that, for the ionic yield. Then we just took some yeast cells and put them in distilled water and irradiated them, extracting the invertase afterward, and we measured how much we had lost. We didn't lose any. Even when yeast cells in distilled water were irradiated with 200,000 r there was no observed effect on the invertase, whereas 1000 r given to the commercial preparation in distilled water led to a definite effect.

KAMEN: Isn't that the same as Forssberg's work on catalase where he found that irradiating the liver gave no inactivation of catalase whereas in vitro the free stuff was inactivated readily?

POLLARD: I find myself, therefore, a little bewildered at taking over experiments that are designed to be true in aqueous solution to actual biological systems because, after all, normal yeast is in its right environment.

COHN: Did you ever set up a row of tubes with 10 units of invertase, 20 units, 40 units, and on up to 100 units of invertase and then give all of them a fixed amount of radiation?

POLLARD: Oh yes.

COHN: What happens to them?

POLLARD: If you do this in aqueous solution, when enough invertase is present, you get the same amount inactivated per roentgen. If you have too little invertase, the radical is gone before it can be effective.

COHN: Suppose you have just enough radiation to give 100 percent inhibition in the tube with 50 units of invertase and then you give that amount of radiation to all of them?

POLLARD: You won't get just 50 units inactivated all the way along. It won't work quite that way.

COHN: But you might get 10 percent inhibition in the tube that had 100 units.

POLLARD: Yes. The number of units of invertase we measured in the yeast cells, I think, was well within the range of our in vitro experiments.

COHN: Of course, yeast has a lot of other things besides invertase.

POLLARD: That is right. That, I think, is perhaps the thing I thought of. I believe that the interaction of the other things present is paramount in interpretation.

JONES: I should like to ask Dr. Barron whether the idea originally put forward by Dale of simple protection by ionization product capture still holds.

BARRON: Yes, that is very well shown by irradiation of protein. An exposure of 75,000 r will precipitate protein irradiated in water solution. If you irradiate the same protein with sodium chloride present, there is no precipitation. Of course, one might say that the protein has combined with the chloride to form a more stable protein. So this possibility must also be con-

sidered. It is very difficult to determine the system that is going to protect and the one that is going to act as a source of free radicals to enhance radiation action. What I should like to do is to extend Dale's concept and to say that you not only have protection but you may also have enhancement of X-ray action by the constituents present.

PATT: Do you mean to imply that the sodium chloride protection is due, in this instance, to the sharing of free radicals?

BARRON: I think it is a combination of the chloride with the protein.

PATT: Dale's original protective effect was, I believe, somewhat different and was more a matter of competition or sharing than of combination. He showed subsequently that there were different types of protective agents and that the relationship between the concentration of the protective substance and the degree of protection was not entirely simple. While most of the effects could be thought of in terms of the sharing of radicals, the protection by certain agents fell off with increasing concentration.

JONES: I think Dr. Barron's point as to the enhancement effect is a very important one. I, for one, have found it very difficult to see how you can get any irradiation effects comparable to a simple water system at the high concentration of original substance existing in the cell. Everything would protect everything else.

PATT: Yes, but then you have to go back to the thought that you are not dealing with a homogeneous system. As suggested by Dale, there may be dilute areas alternating with more concentrated areas and surface effects of various sorts. In other words, you simply cannot compare the cell with a concentrated solution.

JONES: It seems that the balance of this dual system, on one hand enhancing radiation effect, on the other minimizing the damaging reaction of ionization, would be quite sensitive and dependent upon the density of ionization and upon the type of protective substances used.

POLLARD: There isn't a consistent story on density of ionization. Chromosome breakage is greater with more dense ionization. I see no simple explanation that is possible.

PATT: Yet in solution there is generally a greater effect with the low than with the high ion density radiations.

POLLARD: And for other things too, but not for chromosome breakage, let's say.

PATT: For most types of biological effect, the effectiveness generally increases with increasing ionization density; yet we find the reverse situation when we work with simple aqueous systems.

MAGEE: We often think about the high energy of ionizing radiation as supplying the energy to make reactions go and perhaps intuitively we think that radiation-induced reactions have high free energies, but we can also have reactions in the cell that are exothermal and have a negative free energy change. In such cases, we can have terrific enhancement. You just sort of trigger these off and they go on, so that the enhancement in such cases, can be very high. In pure chemical systems there are reactions which have G values of many, many

thousands, like the photochemical chain reactions.

CARTER: Isn't it conceivable that in some systems, an enzyme would split a substrate more rapidly because of the radiation energy that has been absorbed into the system?

POLLARD: Very little energy is put in there measured in terms of the metabolism of the cell.

CARTER: I think, in one of the cases you mentioned this morning, the enzyme protein was acting upon the substrate. Can any of the energy that is absorbed into the protein be transmitted into the enzyme substrate complex?

POLLARD: It certainly can be, but that would affect only the one substrate molecule that happens to be on the protein at the same time, and the enzyme is good for several thousand.

CARTER: And the molecular transformations are taking place much slower.

POLLARD: I feel that we are ignoring the innate character of radiation action which is that for the amount of energy it puts forth, its effect is enormous -- larger than any other character of energy. That is the basic quality that radiation has. So I don't think we ought to look upon it as though it were just a form of heat or something of that sort.

CARTER: But it propagates over long periods of time.

POLLARD: It is an autocatalytic action of some kind.

CURTIS: In terms of enzymes, enough radiation to kill a cell will inactivate only one in every 10^8 molecules of the enzyme or something of that order of magnitude. On this basis, you don't have very much of an increase in the reaction.

CARTER: Unless the substrate were on there at that time, and then it is an event of very short duration. Is that the point?

POLLARD: That is my point.

I think that Dr. Barron's point about enhancements is a new concept in this type of work, and if we agree that enhancement can occur as well as inhibition, we might make a lot of headway. Incidentally, the enhancement must be a particular kind of enhancement and obviously, systems other than his must show enhancement. So if you can find one or two that do and concentrate on them, that might be very revealing for radiation action.

ALLEN: Dr. Barron raised a point early in his talk to the effect that you do not get reduction very readily in solutions in pure water. It has always seemed to me that this is to be expected if the water decomposes, at least roughly, into equal numbers of oxidizing radicals and H atoms. They ought more or less to neutralize each other, so that you should get little reduction or oxidation.

BARRON: However, you do get oxidation.

ALLEN: In some systems, even in the absence of oxygen, you do get

a net oxidation, and I think this is due to the fact that the water under irradiation decomposes not only to give radicals but also to give a certain, but smaller, yield of peroxide molecules and hydrogen molecules. As the peroxide accumulates in the solution, it can either act directly on the substrates or it can react to some extent with H and OH to form oxidizing radicals that can produce this net oxidation. The hydrogen molecules are, relatively speaking, inert. Is that point clear?

BARRON: Yes, that is what I thought; the reason there was no reduction was because the hydrogen atoms combined.

ALLEN: No, I don't think this is so because the yield of molecular hydrogen that one sees in many systems is much smaller than the yield of radicals that one has to assume is present. But I think that what usually happens is that the effects of the H and the OH in oxidation and reduction cancel out and any net oxidation that you get (this is all in the absence of oxygen) is due to the presence of molecular peroxide that is formed directly from the water, to some degree simultaneously with these radicals.

BARRON: But there is no hydrogen peroxide formed by X radiation in the absence of oxygen.

ALLEN: That is true only if the water is very pure. In that case, the radicals can act on the molecular hydrogen peroxide and the whole thing goes back to water. If you add anything to the water, it will generally protect the molecular hydrogen, which is less active than the peroxide, so that you do get a net formation of hydrogen peroxide, and the peroxide can go ahead and subsequently produce oxidation.

BARRON: In all our experiments, we have saturated the water with nitrogen, purified by passage over copper wire heated at 800°C. We have never found hydrogen peroxide in water saturated with pure nitrogen.

ALLEN: If the water is purified, there is a radiation-induced reaction between any molecular hydrogen and hydrogen peroxide that may be accumulating. The steady-state concentration of peroxide may be too low to detect.

BARRON: However, we have one experiment with steroid hormones where we can demonstrate reduction that is very similar to the reduction with atomic hydrogen. The steroid has an absorption peak at 240 mμ. This is due to the carbonil groups. Irradiation in nitrogen gives a greater diminution of the absorption spectrum than irradiation in the presence of oxygen. I thought this was a reduction. So what we did was to pass hydrogen over colloidal palladium and we found exactly the same diminution.

ALLEN: Dr. L.H. Gray told me about some experiments that have been done on irradiation of suspensions of T-1 phage. Inactivation of this phage proceeded with a much higher yield if the solution was saturated with nitrogen, and a still higher amount of inactivation was found if both oxygen and hydrogen were present. It was concluded from this that the main agent for inactivation of the phage was the hydrogen atom.

PATT: Along the same lines, Bachofer reported recently that oxygen apparently protected phage against X radiation in contrast to the usual protective effect of oxygen removal. It appears, however, that this effect may be due, or at least related, to the presence of certain salts in the medium. I think the work to which you refer was done by Alper; it has been reported in the British

Journal of Radiology.

POLLARD: I find that kind of work a little trickly unless you actually determine what it is that the virus has lost. For instance, has the virus simply lost the ability to attach to the host or has it actually lost the ability to multiply or what has happened? Dealing with a virus as an indicator of radiation action has its troubles.

MAGEE: Bachofer's only test was multiplication. I have often wondered, whether there is any simple rule-of-thumb way of knowing, in thinking about the various parameters you can use, which are the most sensitive and which are the least so?

POLLARD: No, there is not. It depends again on the class of inactivation. It is almost certain that the indirect effect is on the surface and probably does involve such things as attachment. Possibly, it may even pull the envelope out so that it releases nucleic acid. That sort of thing may happen but isn't established. The modern feeling on viruses is that they are not molecules, but rather are systems and should be thought of as such.

TOBIAS: Dr. Barron in his discussion commented chiefly on the role of the -SH group. As I understand, there is a protective agent for the -SH group, β mercaptoethylamine. Would you care to comment on the mode of action of this substance and on the sensitivity of other groups besides the -SH group?

BARRON: Yes, I know the work of Bacq. The protective action there is probably due to the reducing power of the mercaptoethylamine. I think it has much more reducing power than glutathione and therefore it is a more reactive agent for the free radicals formed from the irradiation of water. I think all this protective action comes by combination with the free radicals, the competition theory that Dale was talking about. I have tried to do the Swallow experiments with glutathione and DPN, to see whether the radicals of oxidized glutathione do produce the reduction of DPN. Unfortunately, the experiments were negative. I was unable to reduce DPN with glutathione on irradiation. It demonstrates that the potential of the system is too positive to cause the reduction of DPN. All these things depend entirely on the potential of the system.

PATT: A few things could be added to what Dr. Barron has said, although I think we are in general agreement on the interpretation.

DUBOIS: Dr. Barron, did you mix any of the materials such as oxyhemoglobin with lactic acid-DPN to study the distribution of the effects between two sensitive systems?

BARRON: No, the experiments that I have reported were done 10 days ago, after we confirmed Swallow's work. The experiment on the reduction of DPN with propyl alcohol, for instance, was done only yesterday. We are studying these experiments, and I know that we are going to try quite a number of enhancing agents. We intend to try to reduce the cytochrome by this kind of coupling action. I became interested in it because I tried to demonstrate that there is formation of free radicals in the oxidation of alcohol.

TOBIAS: It seemed that the concentration of these agents, for example, the alcohol, was quite high, probably higher than the occurrence of the same substance *in vivo*.

BARRON: Yes, and this was so in Swallow's work. The concentration of the alcohol was continuously diminished in our experimental procedure because of the nitrogen bubbling. We do not know how much alcohol we had in the solution. I think that when we repeat the experiment we will have to determine the actual amount of alcohol present. However, we could not have lost more than half. So we must have had 0.2 molar. I think that the concentration of alcohol has to be high enough to produce a large number of radicals. If we diminish the concentration of alcohol, I am afraid we will be unable to find the reduction of DPN. But those are points that we have not yet worked out.

Also, the concentration of DPN was high. We did that purposely in order to demonstrate quite conclusively that there was a large formation of reduced DPN.

CHARGAFF: What about those experiments of Joseph Weiss with high dosage irradiation of nucleic acid? He used very high dosages and got fearful effects.

BARRON: The same thing happens with sulfur mustard and nitrogen mustard. When large amounts are used, all kinds of effects appear.

CHARGAFF: Is it possible that the same effects but, of course, in a much lower concentration, do take place in physiological doses but cannot be detected because the particular methods are not good enough?

BARRON: To that question, I have no answer. We have tried very hard, with the most accurate methods for the determination of phosphorus, and we have never found any inorganic phosphorus. You see, Weiss irradiated with 1.5 million r. With amounts within 150,000 r we were unable to find any diminution.

CHARGAFF: If nucleic acid has a molecular weight of six million, that would mean 20,000 nucleotides. If 1 or 2 out of these 20,000, say, were dephosphorylated, you would not see it analytically.

BARRON: We have used adenosine triphosphate, that has a much smaller molecular weight, and have observed a decrease in the absorption band at 260 mμ. It was strictly proportional to the amount of radiation. But no inorganic phosphorus was formed.

CARTER: Scholes and Weiss have an explanation for this in terms of the increase in acid lability of the phosphate, not necessarily a splitting out of inorganic phosphate from the molecule. They explain the after-effect, the long period of drop in viscosity that takes place subsequent to irradiation, in terms of slow hydrolysis of the labile phosphate compounds.

Also they have recently reported conversion of monoethyl phosphate to acetylphosphate, which, at least, is the model for this type of reaction.

CARTER: They say that the 4' hydroxyl group of the desoxypentose moiety is extremely susceptible to attack by the perhydroxyl radical leading to the production of an acid labile phosphate ester.

BARRON: Coming back to the sulfhydryl groups, you may recall that coenzyme A was oxidized in vitro with an ionic yield of 3. It is extremely sensitive.

BENNETT: All of your values are for compounds in solution. In the solid form, some compounds, e.g., choline, appear to be particularly susceptible to irradiation. G values of the order of 500 are obtained from self-irradiation of choline with carbon-14. Dr. Lemmon has presented the data in several papers (12-14).

He has also investigated the effect of structural changes in the choline molecule on the G value. For instance, with choline, if an analogue is made that contains the 3-hydroxy propyl group instead of the 2-hydroxy ethyl group, the G value drops by a factor of 10.

BARRON: Is the choline oxidized?

BENNETT: It goes to acetaldehyde and trimethylamine. As far as can be determined, these are the only main products. The acetaldehyde has not been determined quantitatively, but when experiments are done with methyl labeled choline, then trimethylamine is the only radioactive compound that is obtained. The G value depends upon the type of irradiation. In other words, if the irradiation is done with cobalt-60 as the source, the G value is around 180, and if the irradiation is done with high energy electrons, the G value is around 20. Experiments are in progress to determine the sensitivity of different compounds and the effect of structural changes. These preparations are dry and they are irradiated in the absence of oxygen, in other words, evacuated systems.

POLLARD: It is obviously a reaction that goes from molecule to molecule. It is a lot of fun to start putting things between the molecules.

BENNETT: For instance, this is choline chloride. If you change the system to choline iodide, which is in a sense putting in something different, the G value goes down considerably -- by a factor of about 10. The G value for choline chloride in the 2-to-4 Mev. bombardment is around 20, and for the iodide appears to be of the order of 2. There are some experimental difficulties in accurately determining the G value. If one goes, for example, to a choline analogue where there is chlorine in place of the -OH, the G value is also quite low, so that there are profound effects with changes of structure.

POLLARD: Did you try to irradiate this at liquid air temperatures?

BENNETT: No. Choline was chosen for these studies because of a number of compounds that we had prepared with carbon-14 and stored, it appeared to be the most sensitive. For instance, adenine was relatively insensitive, as were most of the amino acids. Many compounds can be tried, and undoubtedly we will try more of them.

PATT: I believe that your group has studied radiation effects on coenzyme A in tissue.

BENNETT: I would say that the effect appears to be small. In an earlier report it was stated that there was considerable effect, but there now appears to be little effect of X irradiation on coenzyme A or DPN (15).

DUBOIS: I would agree with that conclusion on the basis of experiments that we have done. In our experiments, sulfanilamide acetylation was studied in irradiated rats. We gave a dose of 100 mg. per kg. per day of sulfanilamide. This normally results in excretion of about 50 percent of the injected dose in the acetylated form and the rest in the free form. By determining daily urinary excretion of acetylsulfanilamide throughout the survival time after

lethal doses of X-rays, we obtained a comparison of acetylation by the livers of normal and irradiated rats. With this procedure there was no effect of X radiation on acetylation. Fifty percent of the administered dose of sulfanilamide was excreted as the acetylated derivative in both the normal and irradiated rats. Therefore, it appears that the coenzyme A level is not reduced, and Bacq's suggestion that mercaptoethylamine may act by preserving coenzyme A is not supported by our experiments.

PATT: A similar conclusion has been reached by Thomson in our laboratory.

COHN: Coenzyme A might be poor some place else.

DUBOIS: Yes, the acetylation reaction that we studied occurs in the liver.

PATT: But I think his remarks concerned Bacq's suggestion.

DUBOIS: Yes, the suggestion that mercaptoethylamine exerts part of its protective effect in the liver was the point under consideration.

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CELLULAR BIOCHEMISTRY

Frederick G. Sherman

The discussion yesterday was encouraging because it is becoming evident that it may be possible to construct some models of the interaction of radiation with complex molecules that might be applicable to living systems. It is encouraging, too, that radiobiologists are beginning more and more to utilize technics that can uncover some of the physiological effects that take place relatively soon after irradiation. Evidence for short-term effects of irradiation has been obtained with a wide variety of organisms. The period I have in mind as being covered by "short-term" ranges from a few minutes to not more than a few hours.

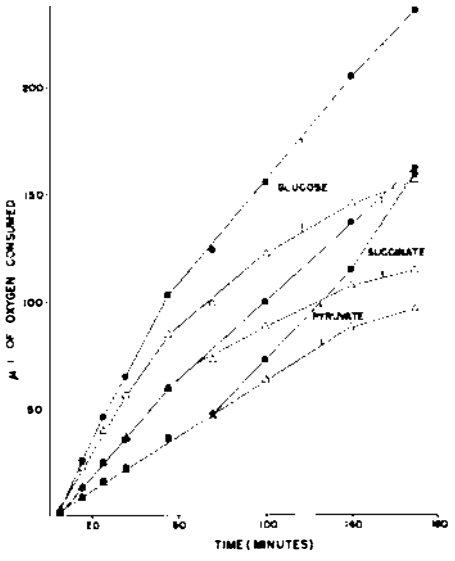

Figure 1. The respiratory activity of X-irradiated Escherichia coli, Strain B/r, on several substrates. The control cups contained 20×10^8 and the experimentals, 20×10^4 colony-forming organisms. Filled circles represent the controls; triangles, the irradiated cells.

A good place to start the discussion would be to consider some of the experiments done by Billen et al. (1) They have observed an inhibitory effect on respiration in E. coli with exposures as low as 5,000 r. This effect varies with the carbon source in the medium. (Figure 1).

They observed a period of normal respiration in the B/r strain in every instance. This was longer with succinate or pyruvate than with glucose as the substrate. However, in the Texas strain, the difference was in the opposite direction. When the substrate was pyruvate, respiration was inhibited immediately. The length of the period of normal respiration was a function of temperature. When the cells were incubated at 26°C, respiration stayed at the control level for 1 to 2 hours, whereas at 37°C, the period of normal respiration was reduced to 20 to 40 minutes. (Figure 2)

The explanation advanced was that enzyme synthesis is interfered with by radiation. Under the conditions of

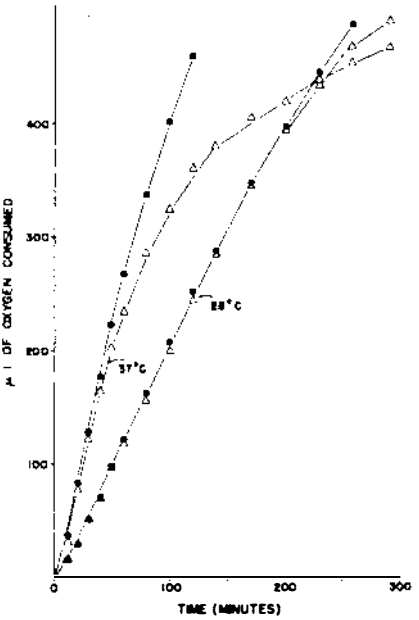

Figure 2. The influence of temperature on X-ray damage to bacterial respiration. The control cups contained 54×10^8 and the experimentals, 56×10^4 colony-forming organisms. Filled circles represent the controls; triangles, the irradiated cells.

their experiments, there is a continual breakdown and repair, which results in the maintenance of the enzyme level in the unirradiated cell at some equilibrium value. In the irradiated cell this process of repair does not take place or it is interfered with in one way or other.

BARRON: Were those cells suspended in a medium containing nitrogen?

SHERMAN: The cells were suspended in nitrogen-free medium. But, even so, Billen's hypothesis was that there is still some normal repair in these cells and that this is inhibited. The experiments of Billen and Lichstein (2) on the adaptive formation of hydrogenase, which is interfered with markedly by irradiation, were used as evidence for this. However, this enzyme may be a special case because it requires certain amino acids, glutamate, among others, for its formation.

That protein synthesis can go on after irradiation is well established. Holweck and Lacassagne (4) long ago observed that yeast cells would form giant cells, and Brace (5) showed that the formation of giant cells was not due to the uptake of water, but that protein synthesis was taking place. Also we have some data that show that the nitrogen in the medium is utilized as rapidly by irradiated as by nonirradiated cells.

Another kind of evidence that protein synthesis can take place in X irradiated cells is furnished by the experiments of Spiegelman, Baron and Quastler (6). They reported that galactozymase formation was not impaired by exposures that resulted in inability of more than 99 percent of the cells to form new colonies.

These examples suggest that enzyme synthesis can and does go on in irradiated cells. What may be happening in the experiments by Billen et al., is that cellular repair and protein synthesis continue, but that the repair is aberrant. This results in the gradual establishment of a set of abnormal enzymes. Their data (Figure 1) is suggestive of a progressive deterioration.

Another explanation proposed by Billen was that there may be a certain proportion of enzymes in excess of the enzyme requirement, which comes into play to replace those that have been damaged. However, after exposure of 5,000, 15,000 and 60,000 r, the period of normal respiration appears to be nearly the same. Cells exposed to 5,000 r were inhibited least; at 15,000 r, an intermediate inhibition of respiration was observed, and at 60,000 r the rate of O_2 consumption fell quite rapidly to low values.

The curve we have been discussing was from an experiment conducted at 37°C (Figure 1). Cells treated in the same way but incubated

at 26°C instead of 37°C, had a longer period of normal respiration (Figure 2). The total amount of oxygen consumed by irradiated cells at 26°C was larger than that at 37°C. The processes resulting in deterioration of enzymes associated with respiration appear to have a higher temperature coefficient than respiration.

A point I would like to emphasize is that microorganisms that have received large doses of X-rays may keep a large part of their metabolic machinery intact and functional even though, for example, they may not be able to divide. Yeast cells can utilize glucose and phosphate for periods of at least 24 hours after irradiation. They are not dead cells. It is only when they are asked to do something they can no longer do that they seem to be dead.

PATT: I should like to ask whether oxygen consumption levels off at the same place at both temperatures or at a somewhat higher level with the lower temperature.

SHERMAN: It looks as if it might level off at a higher value.

PATT: Perhaps then it is not entirely a matter of slowing up the deterioration but also of promoting some recovery at the lower temperature.

SHERMAN: That may be the case, but the experiment wasn't continued long enough to establish whether the respiration of irradiated cells incubated at 26°C would eventually fall off to the same level as the irradiated cells incubated at 37°C.

BARRON: In our early work, we observed also that the ability of cells to divide and form colonies might be inhibited even though respiration was perfectly normal. At that time we pointed out that we have to differentiate between the death of the cell and the loss of ability to divide.

ZIRKLE: Don't be too hard on us biologists. We are just lazy like everybody else, and sometimes use terms like "death" and "lethal action" loosely. In precise discourse, it is, of course, necessary to state just what we mean by "death" in that particular context. In one context, e.g., a person may use the term to mean inhibition of cell division, in another to mean cessation of motility.

SHERMAN: In our experiments with yeast, there was an initial period during which the rate of fermentation of glucose was normal. There was little inhibition for a period of about 2 hours, but after 4 hours, the inhibition of fermentation reached a maximum and this maximum did not change for over 24 hours (7).

It would be interesting to compare the life span of irradiated and non-irradiated cells from the standpoint of maintenance of their capacity to ferment glucose.

COHN: I am not clear about these curves. If enzyme replacement has been inhibited, does not this delay period simply constitute a measure of the survival time of the enzymes that are there to start with, and, couldn't this survival be longer at lower temperatures?

SHERMAN: I would go along with this interpretation of Billen's experiments. A couple of years ago, we published a paper on the effect of X radiation on the fermentation of glucose by "low nitrogen" and normal yeast (8). The period of "normal" fermentation after irradiation appeared to be similar in both low-

nitrogen and normal yeast. However, fermentation was inhibited by much lower doses of X radiation in low-nitrogen than in normal yeast cells. We have no data on the effect of temperature; however, if the period of normal respiration, or fermentation, represents the survival time of the enzymes directly concerned, there might be a considerable difference between normal and low-nitrogen yeast.

COHN: What is the relation between this curve and the curve you get from nonirradiated cells? Is there any evidence that you have impaired enzymatic activity from the initial portion of the curve?

SHERMAN: No. These curves (Figures 1 and 2) indicate that the activity of the enzymes associated with respiration are not impaired immediately after irradiation. However, there is evidence that other cellular activities are delayed (9).

COHN: You don't really know that you have done anything to the enzymes except impaired their replacement.

ZIRKLE: Isn't there evidence that the actual enzyme content is not impaired to any appreciable extent by doses that you are talking about. If you break the cells open and analyze for enzyme content, wouldn't you find practically 100 percent of the control value? In that case, wouldn't the difference in these curves be due to something else?

SHERMAN: Yes, in fact, experiments recently reported by Bair and Stannard (10), indicate that alteration in the composition of the medium may change radiation effects on fermentation from inhibition to enhancement. Their data indicate that some enzymes are able to function at 100 percent or more of the control value.

POTTER: I was about to ask if you intend to get into a discussion of coenzymes, and if you are, then it might be premature to comment.

SHERMAN: As a matter of fact, this would be a good time to consider the role of cofactors.

POTTER: Well, it seems to me that before one can speculate on the formation of wrong enzymes or the failure of synthesis of right enzymes, one has to break the cells open and find out whether there actually is less of the enzyme that you are measuring. Of course, this must be done in the presence of appropriate coenzymes and substrate. If the enzyme is present, then one would have to reject the idea that wrong enzymes have been formed and that the right ones have not. My own explanation is that this is more likely to be an interference with continuing synthesis of the necessary coenzymes.

SHERMAN: I think this interpretation is as reasonable as the one suggested by Billen and the modification of it that I have suggested.

HOLLAENDER: I believe that you will find that not only one enzyme is affected by the radiation but probably a large number of enzymes and coenzymes. We have tried to give irradiated cells an opportunity to repair some of the damage. The experiments which Dr. Sherman discussed really grew out of these studies. If a cell is given a chance to repair some of the damage before cell division is initiated, some recovery may be possible. We have studied this in E. coli, B/r. This organism has its optimum growth at 37°C. If our strain of coli is kept in the presence of certain nutrients at 18°C instead of 37°C, 100 times as many colony-forming organisms will be obtained from cells irradiated

with 60,000 r. The respiration experiments that Dr. Sherman has discussed were designed for the purpose of finding the energy source for this repair, and, as he has shown, the total oxygen consumption is considerably higher at suboptimal temperatures than at 37°C. The nutrient necessary for this recovery process can be obtained from yeast, beef or spleen extracts. A synthetic medium consisting of inorganic salts, glucose, glutamate, uracil, and guanine is also effective.

Although it is possible to omit some of the constituents of our synthetic medium and still produce a few pinpoint colonies. This is not a reliable method for studying the recovery phenomenon.

The basis for these experiments is the feeling that cell division places a great demand on the available enzymes and nutrients. If the cell is not able to synthesize these quickly enough, it will die. The process of cell division is extremely slow at 18°C. After the repair process has taken place at 18°C, the cell can be warmed to 37°C and many of the cells that have been damaged by radiation will recover and grow quite normally.

POTTER: Is your synthetic medium rich in certain nucleotide precursors?

HOLLAENDER: Yes. Actually the synthetic medium was better than yeast extract in restoring ability to form new colonies.

POTTER: Have you ever examined the fluid that these cells are in, from the standpoint of UV substances? That sort of thing has been done many times, but is there a correlation in this case?

HOLLAENDER: Very little ultraviolet absorbing material will diffuse from X irradiated cells kept in salt solution. However, if the cells are given some glucose, the ATP as well as other substances absorbing at 2600 Å will diffuse out quite readily. (12)

POTTER: I think that is highly relevant.

SHERMAN: I wonder if acid soluble nucleotides are decreased in irradiated cells because of leakage.

HOLLAENDER: We do not have quantitative data yet in regard to the leakage problem.

POTTER: If isolated rat liver mitochondria are suspended in isotonic sucrose, there is no leakage of nucleotides from the mitochondria at 0° in the presence or absence of substrate. When the temperature is raised to 30°C, there is a progressive leakage of nucleotides from the mitochondria in the absence of substrate. However, this is not the case when substrate for mitochondrial respiration is present. I think that this would provide an excellent and reproducible test system for study of effects of irradiation.

SHERMAN: Can you get enough mitochondria to do some sort of fractionation?

POTTER: Yes, readily, and you can get the complete profile of the nucleotides. They contain 25 or 30 different nucleotides.

CARTER: In a supplement of the British Journal of Radiology, there

is a paper (13) which perhaps bears on this point, showing inhibition of oxidative phosphorylation in mitochondria from irradiated animals. But certainly the kind of correlation that Dr. Potter is talking about is not made.

BENNETT: I am not clear about this protein turnover. These are non-dividing E. coli cells, are they not?

SHERMAN: That is right.

BENNETT: What were the experiments of Monod? Were they not with non-dividing E. coli that shows no protein turnover?

MAZIA: Monod could find no evidence of protein turnover in growing E. coli.

BENNETT: If the proteins are not being degraded and re-formed in the cells, it would rule out an explanation such as you have offered.

SHERMAN: If this is so, then some other repair or some other factor is interfered with.

BENNETT: Not the protein parameter or the enzyme parameter?

MAZIA: As I understand it, Monod claims that enzyme is extremely stable in E. coli. If the cells are under non-growing conditions, the enzyme stays put and if they are under growing conditions, the enzyme is diluted out. On the time scale within which he is working, the enzyme molecule would seem to be immortal.

SHERMAN: I would like now to discuss some experiments on yeast by Bair and Stannard (10). Yeast cells irradiated in potassium phosphate with 90,000 r of 250-KV X-rays had a significantly larger $Q^{N_2}_{CO_2}$ than nonirradiated controls. Those receiving the same exposure but suspended in triethylamine-succinate-tartrate buffer at pH 4.5 showed marked inhibition of anaerobic CO_2 production.

The role of electrolytes in the metabolism of irradiated yeast was investigated by treating the cells with a cation exchange resin (Dowex-50). The influence of potassium on fermentation (14) and the early work of Nadson and Zolkevic (15) led Bair and Stannard to suspect that depletion of cellular potassium might expose radiation damage to catabolic processes.

Yeast cells were suspended in Dowex-50 (50-100 mesh) that had been converted to the triethylamine form. The resin exchanged triethylamine for the cations of the yeast suspension. This treatment did not reduce the number of cells that were able to decolorize methylene blue. Treatment of yeast with Dowex-50 after irradiation resulted in a larger inhibition of respiration and fermentation than treatment before irradiation. Glucose uptake by irradiated cells did not differ markedly from that of nonirradiated cells. The inhibition of respiration and fermentation was due to the inability of the yeast to utilize the substrate.

Dowex-50 can remove the potassium or sodium or other cations that happen to be in the medium. Spectrographic analysis of Dowex-50 treated yeast indicated that the level of intracellular potassium and sodium was unaltered (10). However, treatment of irradiated cells with Dowex-50 resulted in a reduction of

the intracellular concentration of potassium and sodium to 25 percent below that of nonirradiated cells. Potassium was also shown to leak out of irradiated cells that were not treated with Dowex-50, whereas there is no appreciable leakage of potassium from normal yeast cells.

BENNETT: Were controls run to check the pH under these conditions?

SHERMAN: Yes. The cells were suspended in triethylamine-succinate-tartrate buffer during and after treatment with Dowex-50. The pH of the buffer was adjusted to 4.5 or 6.5.

TOBIAS: Was the fermentative ability of Dowex-50 treated normal cells decreased?

SHERMAN: Yes.

TOBIAS: But the irradiated cells decreased more?

SHERMAN: As much as 90 percent more.

Bair's experiments (11) suggest that irradiation results in the loss of ability of yeast cells to retain cations. When Dowex-50 is present in the medium after irradiation, the potassium concentration outside the cell is reduced to very low levels. The fact that post-irradiation treatment with Dowex-50 was more effective in revealing damage than treatment before irradiation supports this interpretation. The inability of irradiated yeast cells to retain intracellular cations may be the result of interference with the active transport system or conceivably it could result from changes in physical properties of the membrane.

BARRON: Muntz demonstrated that fermentation will not take place in yeast extracts in the absence of potassium.

SHERMAN: In E. Coli it has been quite clearly demonstrated that potassium is necessary for growth, synthesis, the incorporation of sulfur into proteins, and the utilization of phosphate (16), so that there seems to be some possibility of relating the radiation effect in potassium-deficient yeast cells to some difficulty in synthesizing protein or at least in getting energy for the synthesis of protein.

COHN: You said that the cells were treated with Dowex-50?

SHERMAN: Both cells and medium were shaken with the resin. Potassium deficiency in E. Coli does not interfere appreciably with glucose uptake. These cells apparently have other pathways for glucose utilization that do not involve potassium. But these pathways result in the production of energy that is not available for synthesis so that the synthetic activity is interfered with. Perhaps this is why these cells are so sensitive to irradiation. It is not that the irradiation has done anything different in potassium-deficient than in normal cells, but in normal cells energy is available for repair. In potassium-deficient cells it is blocked.

We did some experiments a couple of years ago in which we made yeast cells nitrogen deficient by growing them in low concentrations of ammonium sulfate (8). These cells had a reduced total nitrogen content and also a reduced nucleic acid content. Their anaerobic CO_2 production could be inhibited measurably with exposures of the order of 5000 r to 10,000 r. There was no appreciable inhibition of fermentation by these exposures in cells grown in the presence

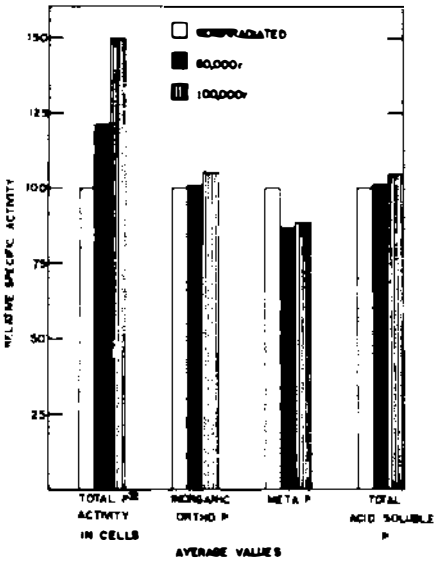

Figure 3. Comparison of the specific activity of various fractions from X-irradiated and nonirradiated yeast (T. utilis). Inorganic orthophosphate, cts./min./μg. P relative to control; metaphosphate and total acid soluble phosphate, cts./min./μg M-P or TAS-P divided by cts./min./μg. inorganic P relative to control.

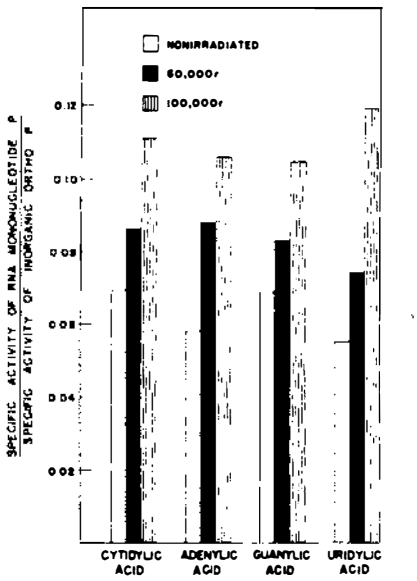

Figure 4. The relative specific activity of RNA mononucleotides from irradiated and nonirradiated yeast. (cts./min./μg. RNA-tide P/cts./min./μg. inorg. O-P).

of concentrations of ammonium sulfate that were nonlimiting for growth. Our interpretation was in terms of the relative amount of irreparable damage to the fermentation enzymes.

More recently, we have been looking at the effect of irradiation on the phosphorus metabolism (17). This has been done by incubating the cells after irradiation with inorganic P^{32} for periods of about an hour. In these experiments, the cells are put into a medium that will allow them to divide. However, an appreciable number of new cells does not form in an hour at this incubation temperature. Nonirradiated cells begin to form buds, but bud formation is not apparent in the irradiated cells. In Figure 3, the activity of various fractions is compared in irradiated and nonirradiated cells. There is a marked increase in the total uptake of phosphorus by the irradiated cells. However, there is little difference in the specific activity of inorganic orthophosphate or of total acid soluble phosphate. The metaphosphate picture isn't as clear. In some of the 100,000 r experiments, the metaphosphate values for irradiated cells are fairly near those for nonirradiated cells; in other experiments, they are considerably depressed.

BENNETT: What fraction accounts for the increase then?

SHERMAN: That isn't shown in this figure. The increase in activity is apparently in the RNA fraction.

BENNETT: This could represent just a difference in the phosphorus percentage in the cells.

SHERMAN: Perhaps, but the amount of P^{31} in each of these fractions is the same. The ribonucleic fraction from irradiated cells has a much higher activity than RNA from control cells. This is shown for the mononucleotides of RNA in Figure 4.

CHARGAFF: Does this include all the mononucleotides?

SHERMAN: This does not in-

clude the mononucleotides that are soluble in acid. The cells are first extracted with TCA and fat solvents. RNA and DNA are extracted from the insoluble fraction with hot sodium chloride. The RNA is hydrolyzed to its constituent mononucleotides, and these are separated on paper by ionophoresis. We have also separated RNA-mononucleotides on a Dowex-1 formate column with essentially the same results.

POTTER: Is there something special about these cells?

SHERMAN: No, these were normal yeast cells grown in a synthetic medium containing as many of the cofactors and vitamins as we know. Perhaps growth is not quite as good as it is on a non-synthetic medium but it approaches it at least.

The cells are irradiated in potassium phosphate buffer, then put into a nonirradiated growth medium containing 2 mc. of P^{32} per ml., and incubated for 1 hour at 30°C.

CARTER: This represents a 1 hour period of incubation then? Do you have time sequences that show the distribution earlier?

SHERMAN: No, we are planning to do that.

BENNETT: Do you have quantitative data for the amount of nucleic acid present? Is it increased or decreased?

SHERMAN: There is an apparent increase in the amount of RNA in all but the first experiment. DNA does not appear to change significantly. The data are shown in Table 1. Both RNA and DNA were extracted by the method of Ogur (18). The DNA extraction, I think, is not nearly as clean as the RNA extraction. I am not as convinced by the DNA values as by the RNA figures. I think the probable error in the RNA determination is smaller than it is in the DNA determination.

TABLE 1

RIBOSE AND DESOXYRIBOSE NUCLEIC ACID IN IRRADIATED AND NONIRRADIATED YEAST

(mg/mg dry wt. of yeast) 100

Experiment	RNA			DNA		
	Control	60,000 r	100,000 r	Control	60,000 r	100,000 r
1	5.81	5.38	5.25	0.42	0.42	0.41
2	5.38	5.50	6.78	----	----	----
3	5.56	6.00	6.28	0.55	0.61	0.59
4	5.28	5.50	----	0.40	0.41	----
5	5.74	6.15	6.05	0.59	0.62	0.61
6	5.50	5.95	6.38	0.52	0.50	0.52
7	5.66	6.10	6.52	0.57	0.55	0.56

Under these conditions there is not much of an increase in the number

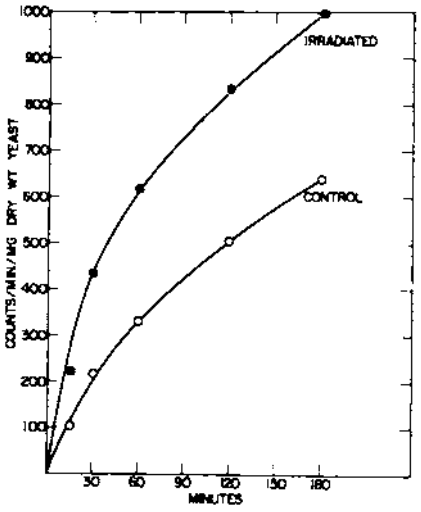

Figure 5. The uptake of inorganic P^{32} by nondividing yeast cells (S. cerevisiae) in a phosphate-glucose buffer. Irradiated cells exposed to 60,000 r of 200-KV X-rays. Time, minutes after irradiation.

of cells. I think there is an increase, however, in the amount of cellular protoplasm in this time.

CHARGAFF: An increase?

SHERMAN: Yes, because the irradiated cells can utilize nitrogen from the medium.

POTTER: Do you have the specific activity of DNA?

SHERMAN: Not for yeast because there is not very much DNA in that organism. So far, we haven't been able to purify the DNA that we do have. We have some data for liver cells but not on yeast cells.

KAPLAN: There is evidence for an increase in RNA per cell in other studies that might be construed as supporting this. We found some time ago that RNA phosphorus per cell in irradiated thymic tissue goes up by a matter of 200 or 300 percent within 4 days after irradiation. We have not studied it earlier. It remains up for a long time. Recently, Gardella and Lichtler (19), reported that after irradiation ascites tumor cells showed an increase in relative cytoplasmic volume within something like 18 or 24 hours. That is a decrease in the nucleocytoplasmic ratio, and as these cells increased in cytoplasmic volume, there was an increase in RNA and in nitrogen content of about the same magnitude. There was no change in DNA content per cell, which is in agreement with other findings.

PATT: Klein and Forssberg (20) observed the same thing for ascites cells a few hours after X irradiation.

CURTIS: What is the time here, Dr. Sherman?

SHERMAN: These are 1-hour experiments.

CURTIS: On the basis of this, if you had waited 12 or 24 hours, you might have seen more of a change, or is this not possible from the experimental point of view?

SHERMAN: It is quite possible from the experimental point of view, but what we were hoping to do was to avoid second order effects. I couldn't predict whether total nucleic acid content might still be up or even higher than it is after 1 hour.

CURTIS: If some of your cells die as others grow, it must introduce serious complications.

SHERMAN: We haven't extended it in either direction in time. We are now trying to get shorter times because we have just recently completed some experiments on phosphorus uptake by nondividing cells, in which we found an increased rate of uptake very soon after irradiation. (Figure 5).

COHN: Is this P^{32} uptake or total phosphorus?

SHERMAN: This is counts per minute per milligram dry weight. The earliest point we have is at 15 minutes, and we have carried these out to about three hours.

COHN: This is not specific activity?

It could be specific activity only if the phosphorus content had a linear relationship to milligrams dry weight.

SHERMAN: That is right. If it does not, then you might guess that the cells are taking up P^{32} preferentially.

COHN: No, I would think that possibly you could have here an enormous net increase in the nucleic acid, a doubling or tripling of the actual milligram content of the culture, let us say. If you have an increase in total phosphorus you will naturally have an increase in P^{32} if you select the medium that way. These are irradiated in a phosphate buffer though.

SHERMAN: These are irradiated in a phosphate buffer and these cells are not starved cells. There was nothing wrong with them before irradiation as far as we knew.

COHN: You see it is important. The only point that I am trying to make is that it is important to consider specific activities not only in phosphorus but in terms of dry weight and in terms of the cell itself and in the degree of polyploidy if you are talking about DNA. Even using DNA as the criterion for the number of cells, you can run into trouble unless you know the degree of polyploidy also at the time of measurement. The mg. dry weight, I think, is not a very good measurement. One can duplicate this type of thing by the loss of some nonspecific weight constituent of the irradiated cells.

SHERMAN: Yes, I agree with you, but I wonder if you would have this kind of change in 15 minutes.

COHN: My point may be theoretical, but there is a tendency to bring up experiments that we never heard about, and I do not think that all of them have paid too much attention to the denominator.

POTTER: I would hazard a guess that if you put it in the terms that Dr. Cohn wants, the effect might be even more striking; that these irradiated cells are in effect phosphorus-starved cells and that, in the course of irradiation they have lost ground which they are now gaining back, whereas the normal cells are not turning over as fast.

COHN: If what you say were correct this would bring the two curves together essentially, because what you are saying is that the irradiated cell has to gain phosphorus back to make it up. If it does, it will also gain P^{32} and you really won't have an effect.

CHARGAFF: You don't know whether the cell lost phosphorus during irradiation?

SHERMAN: I don't know that, but I do know that there was no loss of P^{32} at the end of the 3-hour period when the cells were washed and suspended in P^{32}-free buffer.

CHARGAFF: It would be important to have a comparison analysis right

after irradiation, before they were put back into the nutrient medium and after they had been in it for an hour.

MAZIA: It may be a little misleading to focus our attention on the phosphate, since in yeast, phosphate is apparently carried into the cell in association with sugar, or serves as a carrier for the sugar, depending on the way we look at it. There is no evidence of straightforward diffusion of phosphate ions into the yeast cell.

CHARGAFF: I realize that, but still it is possible during irradiation of the cell.

SHERMAN: If this happens, the transport mechanism has been restored during the 3-hour incubation period because afterward, the specific activity of these cells remained constant for as long as 20 to 24 hours.

CHARGAFF: The experiment I have in mind would be to take yeast cells grown in P^{32} to begin with; to irradiate them in the buffer, and to determine right after irradiation what they have lost or what the analysis is and then to put them back in as you have done.

JONES: Do the control cells have the same exposure to phosphate buffer?

SHERMAN: Yes, they do not lose phosphorus either and this has also been reported by Goodman and Rothstein (21).

DUBOIS: What was the length of the irradiation period?

SHERMAN: These were fairly large doses. It was about 38 minutes or something like that.

CARTER: There are two things that I should like to bring up. These are good data. Obviously, we all want to talk about it as much as you do. But essentially we are doing a kinetic experiment from one point, which is probably a fine place to start but not an adequate place to make an evaluation.

There are several explanations for the difference in specific activity. One is that your normal decay curve has simply come down below the place of the decay curve in the irradiated cell. But I think that the most important problem we should get at is what is the immediate precursor of ribonucleic acid phosphorus, because if we are told to interpret this as a new synthesis, then we have to know about the comparative rates of assimilation of the immediate precursor to the nucleic acid. That is why I think it is important that you do rate studies at 4 or 5 different times, and these rate studies should include the specific activity of the acid soluble nucleotide phosphorus; then the nucleic acid specific activity should be related to these figures. If you do that, I think that you are on very sound ground. At this stage of the game I think it is merely conjectural.

SHERMAN: We plan to do exactly that kind of thing.

BENNETT: I would gather that you are inferring that the rate of synthesis of RNA is, say, double normal from these experiments.

SHERMAN: Approximately.

BENNETT: I might point out that this is somewhat in contrast to what one gets in mice in adenine experiments of a similar type. Here, the main effect to be noted is on the DNA, and small but varying effects on RNA are observed, depending upon the tissue and the time after irradiation.

SHERMAN: I don't know that I would go along with that exactly because the few experiments we have made with mouse liver go along quite like this.

BENNETT: I have done numerous experiments using adenine in mice, but perhaps tomorrow is a more appropriate time to discuss these results further.

SHERMAN: I think Dr. Jones also showed that there was an increase in the incorporation of P^{32} in cytoplasmic RNA.

JONES: Yes, that is a post-irradiation change we have noted.

BENNETT: But the effect of X irradiation on the incorporation of P^{32}-phosphate or adenine into RNA is small compared with the immediate effect observed on the incorporation into DNA. It is a factor of 2 at the most on RNA whereas the incorporation of P^{32}-phosphate or adenine-C^{14} into the DNA of the liver or bone marrow is decreased to 5 to 10 percent of that in a normal animal. These results are similar whether they are carried out with adenine-4, 6-C^{14} or P^{32}.

SHERMAN: In our experience, at least, the DNA story isn't as good as the RNA story from the point of view of getting out DNA that is not contaminated with other phosphorus fractions.

KAMEN: From the standpoint of continuity, I remember that we made much last year of the experiments that Kellner did on ultraviolet irradiation of E. coli followed by reactivation with visible light. Kellner's thesis was that maybe the reactivation phenomenon was a better, more sensitive criterion for singling out real irradiation effects from those that occur in the more drastic case of X-ray, and Kellner's conclusion was that there was an inhibition of DNA synthesis within a minute after cessation of UV irradiation.

Now with the X-ray, the general burden appears to be that it is the RNA that is activated and accelerated and that the DNA stands still. I am a little worried about how this fits together now. I have not seen any more from Kellner's laboratory about this work. I am wondering if anybody knows how that has developed since a year ago.

SHERMAN: It is my impression from the experiments that we have done, that the pattern of P^{32} activity in the various RNA nucleotides is a fairly variable one. In many instances with 100,000 r, uridylic acid seems to have the largest part of the activity, and there are experiments in which this is exaggerated. (Figure 4).

In the 60,000 r experiment and in others, cytidylic, adenylic, and guanylic acids have about the same activity, while the uridylic acid appears to be short-changed. In all cases, however, the pattern is different from the pattern in the nonirradiated cells. This suggests that the mononucleotides may be put together differently in irradiated cells than in nonirradiated cells.

CARTER: It means that they are entering at a different rate, not that they are put together differently.

BENNETT: It depends on your pool size. You don't have data, I suppose, on the amount of soluble nucleotides of these derivatives in yeast. This sort of data would be most important if they are intermediates in the formation of nucleic acids. You can get variable amounts.

POTTER: The specific activity of all the nucleotide precursors in the acid soluble pool is different, because all of those nucleotides are in equilibrium with all of the coenzymes and they are mixed in different ways, so you can have very remote effects.

SHERMAN: But this seems to be a variable thing from one experiment to another.

POTTER: But it affects your interpretation very much. You must avoid the interpretation that the nucleic acid is being put together differently.

COHN: One wonders what these would look like at a different time, at 30 minutes or at 3 hours.

SHERMAN: Forssberg and I (22) did a series of experiments in which the mice were killed 5 minutes after injection of P^{32}. We compared the specific activities of phosphorus in inorganic o-phosphate, acid labile phosphate, the 7-minute hydrolyzable fraction, and the total acid soluble phosphate. A marked increase was seen in the inorganic-phosphate fraction and in the acid labile phosphate immediately after exposure to 800 r. The activity of these fractions tended to return to the control values 60 minutes after irradiation but they were still significantly higher 24-hours later. The 7-minute hydrolyzable fraction increased in activity over the 24-hour period.

BENNETT: What organ?

SHERMAN: Liver.

BENNETT: I think we should distinguish between organs because our experience is that every organ is entirely different.

SHERMAN: The ommission was an oversight. The acid labile fraction is possibly inorganic pyrophosphate. It was separated from inorganic o-phosphate by the method of Ernster, Zetterstrohn and Lindberg (23).

The analysis scheme does not give a clean separation of acid labile phosphate because under the conditions of hydrolysis of acid labile phosphate, about 10 percent of the ATP in the extract is also hydrolyzed. Therefore, the acid labile fraction is diluted to a considerable extent by acid soluble nucleotide P. If 10 percent of the ATP in the extract is hydrolyzed, this can contribute as much as 50 percent of the phosphate found in the acid labile fraction. In spite of that, there is a marked difference in the activities of these 2 fractions in the irradiated animals.

POTTER: Was the P^{32} given intravenously?

SHERMAN: It was given intrapleurally since there is probably a faster uptake of P^{32} from the lung than from the peritoneal cavity.

In muscle, the story is quite different. In order to get enough labeled phosphorus into the muscle to determine conveniently the activity in the various fractions, we had to wait 30 minutes after injection before sacrificing the ani-

mals. Under these circumstances the specific activity of inorganic ortho P, acid labile P, 7-minute hydrolyzable P, and total acid soluble P was reduced by irradiation. Except in the acid labile fraction, there was no significant change in the specific activities of these fractions from muscle taken from animals injected 60 minutes after irradiation instead of immediately afterwards. The specific activity of acid labile phosphate appeared to be approaching the control value in the 60-minute experiment.

In in vitro experiments with irradiated liver, small increases of the order of 5 to 10 percent were observed in inorganic o-phosphate, acid labile phosphate, and 7-minute hydrolyzable phosphate. I doubt if these differences are significant. These data suggested to us that the effect of irradiation on the liver was being influenced by the effect of total-body irradiation.

POTTER: Before I would accept that conclusion, I should like to know how well the inorganic P^{32} in the medium equilibrated with the acid soluble pool of these slices in the controls.

SHERMAN: These were incubated for an hour. I cannot answer your question except to say that we had a high specific activity of inorganic phosphorus from our tissue slices. There were about 2 μc. of P^{32}/ml. in the medium and this was enough to give high rates in all the fractions, so that a lot of P^{32} was taken up. I don't know whether or not this was equilibrated because these studies were done only at 60 minutes.

POTTER: Even with liver slices of 0.5 mm. thickness, glycogen was formed only in the few cells on the outside of the slice according to the studies by Buchanan and Hastings. Inorganic P^{32} may equilibrate with some ATP in the outer cells of the slice, and then, of course, when you go ahead and do the remainder of the experiment, you have some odd ATP.

SHERMAN: These samples were washed repeatedly and I don't think there was very much inorganic phosphorus hanging on.

POTTER: No, I don't mean that.

BENNETT: You would have to express this as a ratio of the activity of what you had in the external medium and inside and in ATP to enable you to evaluate it properly.

SHERMAN: This still would not satisfy Dr. Potter's objection.

DUBOIS: The animal studies indicated only turnovers in the total quantity of acid labile phosphorus in the control and in the irradiated tissue.

SHERMAN: There is not much difference in the total quantity of acid labile phosphorus between the control and irradiated animals.

BENNETT: I don't think this varies appreciably.

DUBOIS: It has been our impression from analysis of total concentration that there isn't any appreciable change in the total inorganic phosphorus in the irradiated liver, and I wonder whether you agree with this.

SHERMAN: I think that there is no appreciable change in the total quantity of inorganic P. I am not sure that I would agree that there is not a change in the total acid soluble P.

DUBOIS: In 1 hour?

SHERMAN: Yes.

BENNETT: I would say that such a change is less than 25 percent.

KAPLAN: If you irradiate as much as half the liver in vitro how do you make sure that the internal liver cells have access to oxygen? I think Vincent Hall showed some time ago that on irradiation of tumor fragments in vitro, the radiosensitivity of the tumor was very highly dependent on the size of the fragment used and even quite small fragments showed radioresistance of the cells in the interior of the fragment simply because they didn't get the same oxygen concentration.

SHERMAN: That is an important point. We tried to minimize the "oxygen" effect by cooling the liver immediately after removal and by irradiating it in the cold within 5 minutes. The irradiation period was 1 minute, and the tissues were kept at 0 to 1°C, until they were sliced and put into the incubation medium.

TOBIAS: Mr. Chairman, I am not a biochemist and probably my question will seem naive, but I came here with the hope that the biochemists would answer some very simple questions. For example, as you all know, about 1/10 of the dose necessary to kill will cause a very great delay in the division process in a microorganism, and at the same time the cell itself will continue to grow, perhaps to 20 times its normal volume and presumably it will continue synthesizing proteins. I wonder if there are any clues as to what enzyme system would be affected by this small dose of radiation.

SHERMAN: Is this a simple question?

TOBIAS: I assumed that it would be a simple question for a biochemist. To answer it is beyond me, of course.

CHARGAFF: A physicist can ask more questions than a hundred biochemists can answer, I am afraid.

I don't think that what produces cell division is so simple. I think it is the most complex question in biochemistry. If you interfere with cell division you probably interfere not with just one reaction but with many.

COHN: I would suggest that the day we can equate growth or reproduction to a number of enzyme systems we will all take a long vacation.

CARTER: I think you can paraphrase that by saying that the answer to such a simple question would be given by a very simple biochemist.

POTTER: I would say that that comment discourages further discussion. I think Dr. Mazia has something worthwhile to say.

MAZIA: Since it has come up several times, just for the sake of kicking it around, let us suppose that radiation affects some DNA synthesizing mechanism; that in order for a cell that has been produced by a division to make the next division, it has to double its DNA; that low-dose irradiation knocks out the DNA forming mechanism; and the delay in division represents the time required for its reformation. Let us suppose that this is the only important event when you irradiate with the dose you have in mind. Would not the protein-synthetic

processes associated with growth just go on until the DNA finally doubled and the cell was ready to divide? In such a case, you would have a larger than normal cell by the time division was possible.

I just put this out as a basis for not abandoning Tobias.

BARRON: The lack of inhibition of protein synthesis and inhibition of synthesis of nucleic acid was shown by Abrams in irradiated rats.

MAZIA: The first diagram (Figure 6) shows the results of a series of weighings of pairs of daughter amebae; that is, the two daughters resulting from

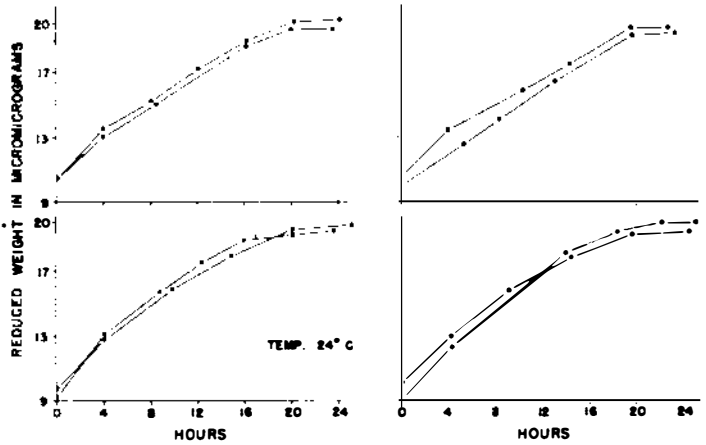

Figure 6. Growth curves of Amoeba proteus cells. Each pair of curves represents the growth of sister cells from the time of separation by division of the mother cell until the time of their division. Reduced weight is measured by the Cartesian Diver Balance, and is essentially the weight under water.

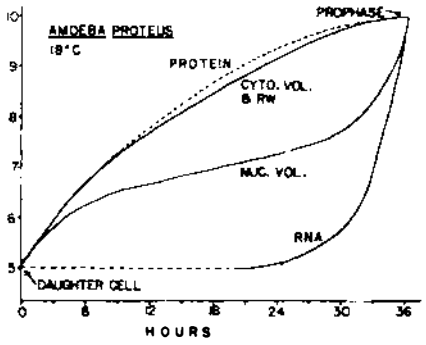

Figure 7. Interphase growth of amoeba, showing course of changes in reduced weight (proportional to dry weight), volume, protein content, nuclear volume, and RNA content.

a division were weighed immediately at "birth" and their weights were followed until they divided. The second diagram (Figure 7) is a summary of various data on the events taking place during growth between divisions. The time scale is slightly different because these data were obtained on amebae grown under conditions where the time between divisions was longer than 24 hours. These experiments are made possible first of all, by techniques that permit measurements on single cells whose history is known and secondly by a new technique of growing cells in such a way that they divide synchronously, thus permitting observations on groups of cells of known history. Data on the increase in "dry weight" during the life of the cell are obtained by

individual cells as they grow. These measurements were made by my student, David Prescott, who used a sensitive Cartesian Diver technique.

Let us consider the growth in weight. The initial point is that at which the ameba comes out of a division - is "born". Twenty-four hours later, this ameba divides. The initial rate of increase in mass is the highest; growth progresses at a steady but decreasing rate and levels off some hours before the next division. It levels off at a weight just double the birth weight.

The ameba knows what its mature weight is going to be but doesn't determine it by simple arithmetic. It does not necessarily double. If we have a case where 2 sister amebae are of unequal size, one abnormally small, the other correspondingly large; the smaller one starts growing more rapidly than the larger one, and both end up at the same weight - the weight of their mother cell.

Water content keeps pace with dry mass, that is, the curve for growth in volume runs parallel with that for growth in dry weight.

We don't have data on growth with respect to DNA, but just to round out the story, we may refer to the findings of Pelc and Howard and others, on other kinds of cells. They find that the doubling of DNA goes on between divisions and is completed some time before the next division.*

The story of RNA - and I would not for a moment represent it as general - is rather unexpected. This study was made by Dr. Thomas W. James. The RNA per cell does not increase at all during the period of maximum protein synthesis, but undergoes a doubling during the period between the leveling off of protein synthesis and the onset of division.

In a discussion of radiation effects, we have to consider the relation between cell growth and cell division. The cell is not going to divide until it has reached what I might call "maturity." The growth in mass, the growth in volume, and the doubling of DNA are completed some time before the cell is reading to divide. It is waiting for something to happen before it can divide.

I should like to raise, in a general way, the question, whether it is legitimate to think about a trigger to cell division. Does the absolute quantity of something in the cell have to reach a certain level? Does some new reaction have to take place before division can go forward? There are some cute experiments on the ameba that make me think that it is legitimate to invoke a trigger mechanism to cell division.

Some 30 years ago, it was shown that an ameba will not divide unless it has achieved a full complement of some X that it must contain. The cell divides once every 24 hours and it is growing during this period, as I have described. All you do is this. Each day you amputate a big chunk of cytoplasm from the cell, undoing the growth it has accomplished during that day. Its growth cannot catch up with the amputations. It does not divide but remains alive indefinitely. This experiment tells me that the ameba must pile up a certain amount of X before division is triggered, and because of our frustrating operations, the level of X neve gets to the triggering amount.

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* - Since the Highland Park meeting, Dr. Walter Plaut in our laboratory, has obtained evidence that the synthesis of DNA in ameba is completed during the first 18 hours of the 24 hour interphase period.

POLLARD: You take out a chunk of cytoplasm?

MAZIA: A chunk of cytoplasm, that is right. Cells treated in this way have been kept alive for as long as 6 months without dividing. No limit has been found as yet.

SPIEGELMAN: Do they keep on making nuclei?

MAZIA: No, no.

SPIEGELMAN: But if you amputate just before it divides, then it keeps a double nucleus.

MAZIA: We don't have any measurements on that. It does not matter. Suppose that it is twice the normal --

SPIEGELMAN: It does not matter to me; it might to the ameba.

MAZIA: The nuclear contents would either regress to the diploid value or remain somewhere between diploid and tetrapoloid.

SPIEGELMAN: The suggestion then, is that this is controlling not only division of the cell but also division of the nucleus because otherwise the nucleus would keep on doubling.

MAZIA: Yes, the processes I am speaking of would be controlling everything that went into division. That is why I am using the term trigger and implying that the trigger sets off the whole chain of events in cell division, including the nuclear changes. James did a simple experiment. He waited until the cell had begun the mitotic process. The nucleus was still present, but he knew the ameba and its history well enough to know that it was about to go into prophase. At this time he chopped off a big chunk of cytoplasm. He could no longer stop division. The cell simply went through with the division and produced two small daughter cells, each with a nucleus. Obviously, the trigger had already been pulled.

BARRON: The RNA starts increasing only after 10 hours?

MAZIA: Yes. It begins to increase during the second half of the period between divisions.

BARRON: Wouldn't you say that the increase in RNA is the trigger mechanism?

MAZIA: It follows the predicted pattern for the trigger mechanism. The farthest I would go would be to say that it may be a tracer for the trigger mechanism. It could, for instance, be an index of the multiplication of some cytoplasmic particles.

CARTER: These are total amounts of RNA?

MAZIA: Yes.

CARTER: So that actually RNA could be the precursor, or could be providing parts of the precursor, for synthesis of DNA molecules, and it is only when DNA has been synthesized and there is no further demand on the precursor that RNA accumulates.

MAZIA: The participation of RNA in protein synthesis is by no means ruled out. It is true that cell division takes place between the completion of RNA synthesis

and the onset of protein synthesis, but if we could overlook this interlude, the data would tell us that the cells are building up a maximum concentration of RNA before undertaking protein synthesis at a maximum rate. From the ameba's standpoint, the fact that RNA is laid down before division might mean that it can avoid a lag in protein synthesis after division. The data tell us that there is no net increase in RNA per cell during the period of rapid protein synthesis. They tell us nothing about turnover.

SPIEGELMAN: Let's imagine that RNA is actually destroyed while making protein. Then the rate in that steady state might acutally be faster than during the rise.

MAZIA: That could be. These data deal only with absolute amounts of RNA, and only tell us that the cell does not require a higher level of RNA during net protein synthesis than existed before division when net protein synthesis was not taking place.

POLLARD: Let me ask a question. Was it your preconceived idea that you don't want this to be destroyed?

MAZIA: I was coming to that Dr. Pollard. There is a lot of circumstantial evidence relating RNA to protein synthesis, but many of the people who work on it fail to distinguish between three entirely different things; a relation between RNA and protein synthesis, a relation between RNA synthesis and protein synthesis, and a relation between RNA turnover and protein synthesis. I have never head a discussion of this important problem where these three kinds of relations have been distinguished sharply.

CARTER: A gland stimulated to secrete a great deal of protein hormone must be synthesizing protein at a rapid rate and yet it shows no change in rate of nucleic acid turnover, measured with one or two precursors.

MAZIA: That does not dissociate the synthesis from the RNA that is there. That is my point.

CHARGAFF: I think there is very little evidence either for or against it.

SPIEGELMAN: Well, I will cite some evidence.

CHARGAFF: For RNA being responsible for protein synthesis?

SPIEGELMAN: The evidence available cannot be taken as establishing with certainty that RNA is responsible for protein synthesis, but I believe that the experiments are suggestive. The data I shall be concerned with deal primarily with the synthesis of certain enzymes.

A variety of enzymes has been studied, including β-galactosidase of E. coli and α-glucosidases of yeast. I might, perhaps, begin by noting that evidence concerning the nature of the precursor that is converted into active enzyme appears to be quite definitive. The work in Monod's laboratory on the synthesis of β-galactosidase in E. coli, and our own work on this system, as well as the α-glucosidases of yeast, indicate that the cell uses free amino acids in putting enzyme molecules together. There are no indications of peptide involvement. The data support rather the simultaneous utilization of the constituent amino acids. This view already has implications for the enzyme-forming mechanism. If one is to look for the machinery that puts the protein together, it obviously has to be as big and as complex as the thing that is being synthesized if this view of protein synthesis is correct. As long as one could imagine a step-wise mechanism, the

actual machinery could be simple, but if it is a 1-step process, than it must be complex. There are only 3 candidates that one can propose, which could serve this purpose. They are: Protein, DNA and RNA.

CHARGAFF: They are all that you have found ?

SPIEGELMAN: They are the only 3 candidates that I can name.

CARTER: What about polysaccharides?

SPIEGELMAN: They are large but they don't have the informational content necessary.

CARTER: Well, you have not postulated them yet.

SPIEGELMAN: There are no polysaccharides that I know that are sufficiently complex to serve such a purpose.

CARTER: The antigenic polypeptides are complex.

SPIEGELMAN: Antigenisis per se does not necessarily mean complexity. The addition of one simple group can convert an antigenically inactive substance to an antigenically active one.

CARTER: I don't want to deny that you probably are correct.

SPIEGELMAN: In any case, the candidates that we can perform experiments with are those that we can name, and those which we actually seek to test experimentally are those we think are the most likely.

In principle, one can perform elimination experiments to seek to determine whether the interference with the synthesis of a particular component has as a consequence the cessation of the ability to form enzyme. These are, in part, the kinds of experiments that we carried out. We have interfered selectively with the synthesis of DNA using a variety of procedures including the use of X-rays, low dosages of UV, sulphur mustard, and analogues of thymine. In none of the cases could we show any parallelism between the extent and the severity of the inhibition, and the effect on enzyme forming capacity. Indeed, in certain cases there was virtually no interference. Cohen has recently added another interesting example in the form of a thymineless mutant that will continue to synthesis enzyme after it has exhausted the thymine in the medium. We have repeated and confirmed these results with another enzyme system. It should be noted in this connection that this behavior is not observed, for example, with the uracil-like mutant or with an amino acid deficient mutant. In these cases, the elimination of the required metabolite from the medium leads to the complete cessation of the ability to form new enzyme molecules.

These experiments taken together certainly do not encourage one to postulate the personal involvement of DNA in the act of fabricating new enzyme molecules.

The situation is quite different if one turns to RNA synthesis. In the first place, relatively slight impairment (50 percent) of RNA synthesis by UV leads to complete abolishment of the ability of yeast cells to synthesize α-glucosidase. Further, unlike thymine analogues, analogues of uracil and adenine can cause immediate cessation of β-galactosidase formation in E. coli. I have already noted that uracilless mutants of E. coli are unable to synthesis enzyme in

the absence of the metabolite they require, and the same is true for adenineless mutants. Further, growth of uracilless and adenineless mutants under limiting conditions of the required metabolite, leads to the temporary loss of enzyme-synthesizing ability even in the presence of the metabolite they need.

CARTER: Could that mean that the uracil polyphosphate or adenine coenzymes could be implicated as well as ribose nucleic acid?

SPIEGELMAN: I don't think so. Well, it could, yes.

CARTER: So, in other words, it may turn out to be a low molecular weight compound that takes us out of this holy trinity.

MAZIA: Except that this synthesis has to impart specificity, and therefore the substance has to be a nuclear product.

CARTER: He emphasized that it does not have to be a nuclear product.

SPIEGELMAN: What do you mean? There is no evidence in these experiments that you need nuclear products continually made by the nucleus.

MAZIA: No, but there was a nucleus there.

CARTER: It is a cell, in other words.

SPIEGELMAN: If it does not have the right gene, for example, it does not do any good.

CARTER: But it does not have a nuclear apparatus in this experiment that you are talking about.

MAZIA: But it has had one. It may still be operative via the specific products it has put into the cytoplasm.

SPIEGELMAN: The possibility that nucleotides and their phosphorylated derivatives are involved in some generalized and nonspecific fashion in the experiments that I described, is an important one to consider. Certainly, for example, the complete depletion in the cell of adenylic acid and its derivatives would completely abolish all activities, including enzyme synthesis. We have attempted to get around this difficulty by adjusting our treatments as well as our inhibitory agents, so as not to interfere with overall metabolic processes. Indeed, we have been quite lucky in being able to adjust the level of antagonist so that there was actually no interference with growth. Nevertheless, we were able to exhibit a specific interference with the synthesis of a particular enzyme. This specificity is due to the fact that the forming system involved was a poor competitor for the nucleotides. We have subsidiary evidence supporting the conclusion which I cannot detail now.

It might, perhaps, be of interest to note some other experiments with yeast which provide relevant information. We discovered the existence in yeast of a nucleotide pool quite analogous to the free amino acid pool with which we had been dealing in the past few years. It may be remembered that with the free amino acid pool we were able to show that suppression of the incorporation of any one of the free amino acids led to the cessation of enzyme synthesis. We were able to devise procedures whereby we could deplete the nucleotide pool in the cell and examine the consequence for enzyme formation. This was accom-

plished by means of a uracilless mutant which was forced to synthesize proteins rapidly. It was found that the free nucleotide pool was considerably depressed in a matter of 30 minutes. It was possible to replenish this pool rapidly by incubation in the presence of mixtures of purine and pyrimidine bases in the proper ratio. Restoration of enzyme-forming capacity in cells whose nucleotide pool had been depleted could be obtained very quickly by incubation with the proper ratio of purine and pyrimidine bases.

CHARGAFF: What is the right ratio?

SPIEGELMAN: The ratio we employed corresponded to the published analysis of the RNA of yeast. These data argue against nonspecific effects which might be attributable to the polyphosphate of the purines and pyrimidines in energy transfers and other reactions. If one takes a cell that is half induced, and, therefore, has the right systems to form the particular enzyme being studied, and depletes the nucleotide pool, any further capacity to form more enzyme molecules is abolished. The data thus far, would seem to indicate that the cell must be able to make new RNA if it is going to synthesize new enzyme molecules. It does not appear to be necessary for a cell to synthesize new DNA in the process of forming enzymes.

MAZIA: I think, that evidence for the nuclear origin of RNA, which has been fairly good, is growing stronger.

I should like to show you some simple experiments done on the amebae that bear on this. The amebae received no external nutrition during these experiments; we are dealing with endogenous processes.

Brachet had shown, a few years ago, that if you cut an ameba in half and follow the total RNA content of the half with the nucleus and of the half without a nucleus (in the absence of external food sources), you obtain the following result: the RNA content of the half with a nucleus declines very slowly over a period of days, while the RNA of the enucleated half declines steadily and rapidly. You could interpret these experiments as meaning either that the nucleus was stabilizing the RNA or that it was replacing RNA nearly as fast as it was broken down.

James, in our laboratory, was doing the same experiments at about the same time, but added a simple control: He followed the RNA content of intact amebae kept under the same conditions. Much to our surprise, the RNA content of these whole amebae dropped with time in a way that resembled the situation in enucleated halves more than that in nucleated halves!

At first glance, the experiment would suggest that Brachet was wrong in concluding that the nucleus was involved in maintaining the RNA level in the cell, for these whole amebae also had perfectly good nuclei. But a simple and interesting calculation by James showed that the new data actually proved the existence of a nuclear activity that is responsible for the maintenance of the RNA level of the cytoplasm as postulated by Brachet. Let us consider the simplest kind of replacement mechanism; namely, one in which the RNA in the cytoplasm is continuously breaking down, and can be replaced only by the activity of the nucleus. In the experiments of Brachet and of James, we have the simple condition that nothing is entering the cell from the outside; we are dealing with endogenous processes. Then we may use the rate of decrease of RNA in the enucleated half as a measure of the rate of breakdown of cytoplasmic RNA. The difference between the lower rate of loss of RNA by the nucleated half and that of the enucleated half gives us, by simple arithmetic, the rate of replacement of

RNA by the nucleus under the conditions of the experiment. If we assume that the cytoplasmic RNA is breaking down at the same rate in the whole ameba as in the half ameba, we can calculate the rate of loss of cytoplasmic RNA in the whole ameba. It will lose twice as much, in a given time, as the half ameba, because there is twice as much to start with. But if the nucleus can synthesize it only at the rate calculated from the data on the nucleated half-ameba, obviously it will not be able to keep up with the loss that is occurring in the whole ameba; hence, it will suffer a net loss, as observed. Using the actual quantitative values, we should be able to predict the curve for RNA loss from the whole ameba from the rates of loss and replacement calculated from the data on the halves. The predicted curve corresponds very well to the experimental curve, and therefore, I feel that these experiments strengthen the theory -- which has much other support -- that the RNA of the cytoplasm, which is most of the RNA of the cell, originates in the nucleus.

SPIEGELMAN: Have you followed the turnover of RNA in these enucleated ameba?

MAZIA: Yes. At least radioactive phosphorus data indicate that it is only 1/3 that of the nucleated part.

SPIEGELMAN: But Brachet claimed that there was considerable capacity for reformation of RNA even in the enucleated cells.

MAZIA: The figure is 1/3. The experiments of James show, however, that there is a net loss in the absence of the nucleus.

MAGEE: I don't understand the relation between the RNA in this cell and the one that you were talking about previously.

MAZIA: Earlier, I was speaking about a growing cell, one that was taking in food. The data of Brachet and of James are for starving cells. We used the starving cells in order to eliminate variation due to food intake, but, in fact, it is the simplification introduced by starvation that makes the evidence for the nuclear origin of cytoplasmic RNA so clean.

KAMEN: There are no DNA data on these cells?

MAZIA: Unfortunately, no.

TOBIAS: If you will apply these ideas to my question raised earlier, then one possible explanation for a delay in cell division after a small dose of radiation seems to be that the irradiation would inactivate a good deal of the RNA in the cell; it would take quite a while for the DNA to resynthesize enough RNA for division to get going. You may recall that there is some evidence for increased RNA in irradiated tissues.

MAZIA: There are some radiation data on ameba that Hirshfield and I published a few years ago that may be relevant, although we were dealing with UV irradiation. We measured the delay of division caused by UV and compared the effect of a given dose in delaying the division of a whole cell with the effect on a cell from which half the cytoplasm had been removed. We found that when the cytoplasmic volume was reduced by one half, the radiosensitivity (measured as delay of division) doubled; it took half the dose to produce the same effect. This would fit your proposal perfectly.

SPIEGELMAN: Isn't it true that you hit DNA synthesis first?

MAZIA: You hit DNA synthesis --

CARTER: If this is going to be our working formulation, could we get Tobias to state his idea again, because this is going to dominate radiobiology for a long time and we should have it clear?

MAZIA: Perhaps we should finish with Dr. Spiegelman's question. We think that you hit DNA to cause the delay in division, but that cytoplasmic mechanisms are responsible for the reversal of the radiation effect. We took photographs with the ultraviolet microscope at 2537 Å to see whether there was total absorption, in which case the difference between the whole cell and the half cell might be accounted for by shielding of the nucleus. We concluded that we didn't have total absorption because neither photograph was black. In fact, there wasn't much difference; the ameba cooperates by flattening out.

KAPLAN: I am not sure that I understand the suggested mechanism that activates mitosis in the nucleus. Is the idea that the piling up of RNA is the trigger mechanism?

CARTER: Could we get a clear statement of this again?

TOBIAS: I will try to state it again, but I hope you will take these ideas as mere suggestions and speculation without proof. Dr. Mazia made two assertions: (1) that a certain amount of RNA has to build up in a cell before a cell will divide, and (2) that DNA is responsible for the synthesis of RNA. Irradiation with a sublethal dose might affect the amount of RNA or the ability of RNA to have its normal biochemical function. DNA, or if I may go farther, the genes of the cell, are not materially affected; if they were then we would have a lethal effect instead of mere cell division delay.

Now the cell would like to divide, but part of its RNA is inactivated and it is necessary for the DNA to synthesize some new RNA, so cell division can be triggered. This will take time, however, and a cell division delay occurs.

There is other corollary information on the role of RNA in radiation damage. Already it was pointed out by Kaplan that RNA actually increases in the post-irradiation period.

At higher lethal doses this model would admit damage to the DNA molecules or to their ability to duplicate themselves.

Does the model sound plausible to you, Dr. Mazia?

MAZIA: Yes. But again we have to distinguish between two facts about DNA. DNA has to double before division in order to provide each daughter cell with a full diploid complement. But it does not have to double in order to do its normal job in the cell. Theoretically, you could have a situation in a radiation experiment in which you blocked division because you blocked the formation of new DNA, but did not necessarily affect RNA formation because you had not damaged the DNA that was already present.

SPIEGELMAN: Are you postulating a permanent involvement of DNA in the formation of every RNA molecule?

MAZIA: No.

POTTER: Spiegelman can probably think of other limitation. I am

just mentioning one and that is that we don't have the DNA curve to relate to the RNA and protein changes.

MAZIA: As I said, we are facing technical difficulties in measuring DNA formation in the ameba. We are trying to measure it photometrically, and the thing that is holding us up would not interest the group here; it is the fact that in Feulgen staining the DNA is so coarsely distributed as to make the situation very unfavorable for spectrophotometric operations.

In any case, answering your last question, I am proposing here a DNA unit that has something to do with the production of RNA in a cell that isn't dividing and that is undergoing duplication in a cell that is getting ready to divide.

To block division, one could conceivably block the duplication of this parent molecule without necessarily affecting its ability to produce RNA. Let's put it this way. We have a DNA unit of a certain kind and only one such unit is needed to produce RNA, but you have to have two of them before the cell can divide. So you can imagine situations where irradiation will block division without blocking RNA synthesis or others where irradiation will block RNA synthesis. It could even be a quantitative difference. It all goes back to elementary biological considerations that do not depend on any of our chemical assumptions. The genetic material of the cell (which may be DNA) clearly has two different functions. One is to serve the cell that it is living in. The other is to double itself so that both progeny after division will have as much of it as the mother cell.

KAMEN: The DNA goes up to a constant level just before division. You have doubled the thing before you start.

MAZIA: You double the DNA before division. If you observe a cell in a random population, it may have a single dose of DNA (actually the diploid amount) if the cell is young, a double (tetraploid) dose in a cell that has completed its growth and is going to divide, or intermediate values in a cell that is somewhere between divisions. This has been established as a fact by the cytochemists. Furthermore, the cytochemists find that cells that are unlikely ever to divide again (as in highly differentiated tissues such as kidney) almost always have the single dose. They make no DNA after their last division. On a statistical basis, the majority of cells in an actively dividing population will have the intermediate amount of DNA.

SHERMAN: Or more.

MAZIA: That's right. You will find a good many cells approaching division and therefore approaching the double dose of DNA.

BENNETT: In some tissues, I think, irradiation does not stop cell division after a certain time.

MAZIA: Dr. Hollaender knows more about this than I do. One can say that there is a time in the process of cell division when radiation can no longer stop it.

HOLLAENDER: Yes, that is true.

PATT: If the cell has begun to divide, it usually completes the division process.

ZIRKLE: That depends on the kind of cells and on conditions. With suitable doses, cells irradiated during division can be stopped at various stages.

PLATZMAN: There appears to be a substantial spread in the times for division. This may be a significant fact.

MAZIA: The spread is relatively small under the same conditions. A few hours between the first cell division and the last cell division.

PLATZMAN: Is it approximately a relative curve?

Given exactly the same experimental conditions, what differences are there in division times for a given kind of cell?

MAZIA: About 15 or 20 percent.

TOBIAS: Taking yeast cells, the fluctuation in time for cell division is about $\pm$ 8 percent at 30°C. At higher temperatures, the relative uncertainty in time increases.

PLATZMAN: It seems to me that this is a significant thing to think about with regard to the determining factors.

TOBIAS: Yes, after irradiation the fluctuation of cell division times increases as the time for cell division is prolonged.

Careful data have been taken by Victor Burns at Berkeley on the relationship of cell division delay to the fluctuation in time for cell division. Both are more or less proportionately increased by a small dose of radiation. We infer that the chemical order of reaction did not change much but that the time constants became slower. It is also interesting to note that cell division delay is longer for the second division following irradiation than for the first division. I do not know whether or not the fluctuation in cell division time is directly related to the steep rise of RNA content just before division.

We might finish our model for sublethal radiation damage. I would do that by having the RNA react back on the system initiating its production, that is, on the DNA; thus cell division would be triggered.

KAPLAN: You are suggesting that with the mere accumulation of RNA in the cell mass it divides.

MAZIA: It may not be that simple. RNA is certainly heterogeneous, and we might require a certain amount of a certain kind of RNA to realize the trigger reaction.

SHERMAN: In this model, is there an interference with DNA production in irradiated cells?

MAZIA: Yes.

SHERMAN: So that the DNA that is already present continues to produce RNA. This accumulates because a link in the feedback loop has been broken.

KAPLAN: Well, RNA might pile up in that way, but certainly the mere accumulation of RNA does not make the cell divide. That is what I am

driving at, at the moment. I have followed thymic weight in irradiated mice in studies of the development of lymphoid tumors. In normal animals, the thymus gradually decreases in weight over an age period which runs about 100 days. If we irradiate these animals the thymic weight will fall to a small fraction of the normal, perhaps to 10 or 20 mg., whereas the normal then is about 50 to 60 mg.

Let's take the case for fractionated periodic irradiation. The thymus will be reduced to this weight and then will stay at this low level with some minor wiggles for a matter of 50 to 70 days and will begin finally to grow, but at this point it already has a tumor in it. If we irradiate while shielding this animal's hind leg or inject it with bone marrow -- within 4 days after the last irradiation, thymic weight shoots upward and very shortly exceeds the normal, then settles back to the baseline, and no tumors form. We have followed both DNA and RNA per cell in unirradiated controls and in groups irradiated with and without thigh shielding at a series of intervals to 100 days. There is no change in DNA per cell at any of these intervals with the exception of a small increase beyond 100 days when the tumors appear. This has been measured either chemically on cell suspensions where we count the cells or histochemically using the Feulgen stain on imprints, so that we have both population and individual cell determinations.

For RNA, on the other hand, there is a pattern that is of interest. The RNA per cell stays reasonably constant for the unirradiated thymus. Within 4 days after irradiation there is a 300 or 400 percent increase in RNA per cell. It does not matter whether the cells are from shielded or unshielded animals. But in the shielded animals or in those receiving bone marrow, this falls promptly to normal and stays there. In the unshielded animals, the thymus cannot regenerate and yet, the RNA per cell stays up at these grossly abnormal levels clear as long as 100 days. Thus, the thymic cells of these animals have accumulated RNA but they are unable to divide.

SPIEGELMAN: We have some unpublished experiments with different materials that agree with these results. We tried to be very cute and to force the cell to make a lot of RNA, in the hope that we could then demonstrate that such cells could make enzymes more effectively. Indeed, we went further and tried to induce the synthesis of specific kinds of RNA. The attempt went along the following lines: If microorganisms are incubated in the presence of an amino acid analogue and a mixture of amino acids, they are unable to synthesize protein, but can form RNA. One can thus obtain cells with as much as 4 times the normal RNA content per cell. Such incubations were carried out in the presence of a specific inducer of the β-galactosidase system of E. coli. After the incubation was over, the amino acids antagonist was reversed by adding the corresponding homologue. One finds that the accumulation of RNA does little for enzyme synthesizing capacity, indeed, quite the contrary. The cells grow much more slowly than corresponding controls and they show little ability to form enzyme for quite a while. In fact, they continue this relatively poor physiological behavior until the RNA that they have accumulated is diluted out. This result may simply mean that the wrong kind of RNA has been synthesized, and this may be the situation described here today.

TOBIAS: Dr. Kaplan, presumably irradiation somehow inactivates the RNA that is there and more is needed.

JONES: Chick embryo RNA protein is a required factor for culture of the chick fibroblast.

KAPLAN: This goes back to Dr. Chargaff's idea that we should not be talking about RNA as if it were something discrete. RNA is a collection of differ-

ent kinds of molecules, in all probability, and we probably have to have about as many different kinds of RNA as there are biological functions for RNA to serve in the cell. The same goes for DNA. It may be convenient shorthand but it is very misleading shorthand to talk about one kind of DNA or RNA.

BENNETT: Isn't it also true in irradiation effects, that the number of reactions one can expect to take place are considerably less than the total number of molecules by many factors of 10. This effect cannot be occurring in every DNA molecule; it would have to be on a very small number of the DNA or RNA molecules.

POLLARD: If you assume that irradiation acts only on RNA in yeast and that it is a definite factor of 1, i.e., that you get 1 molecule inactivated in order to take 10 mg. of RNA per cc. of yeast cells down to 9 mg., then the RNA will have to have a molecular weight of about 5×10^7.

CHARGAFF: Would that also hold if the RNA were together with a hunk of protein?

POLLARD: This means no recombination of the radical. No effect on the protein at all.

TOBIAS: What is the radiation dose?

POLLARD: 100 r.

TOBIAS: That is a pretty low dose for yeast. We would give maybe 600 r.

POLLARD: On the other hand, yeast is rather dry.

TOBIAS: No, yeast contains a reasonably high percentage of water. My calculations are done in a somewhat different way. We know that there is about 10 times as much RNA in a yeast cell as DNA. Further, we may take the molecular weight of each RNA and DNA as 10^7, and endow RNA particles with approximately the same radiosensitivity as DNA particles. From elementary probability considerations it follows that the number of cells in which at least 1 RNA molecule out of 10 would be inactivated is about 10 times greater than the number of cells in which a DNA molecule is inactivated.

POLLARD: I wonder if you should not look for some sort of propagation. Wouldn't it be more reasonable to say that there is a sort of biological multiplication that increases the number of inactivated molecules? Every time I have made this calculation I have stopped; it seems to be unreasonable. It seems to me more reasonable to suppose that you are hitting one molecule which divides many times and it is that process that has to come to a stop.

SHERMAN: With just one wrong molecule do you not have to postulate that this is being propagated selectively? It is in competition with a lot of other fairly normal molecules.

POLLARD: I suppose that competition ultimately wins so that the cell goes on its way and survives, but in the case where it does not survive, what happens? There must be some selective mechanism.

SHERMAN: I am not trying to say that I know what happens. I just say that this is a rather disturbing number, although it does not seem to bother Dr.

Chargaff very much.

CHARGAFF: I don't bother easily. I really don't know. I seem to gather that the theory now is that DNA makes RNA and RNA makes protein. This may be so in special cases. I think there is some evidence that DNA makes DNA and RNA makes RNA. In fact, there is little chemical relationship at least between the total DNA of the cell and the RNA. We have looked for this but there does not seem to be any.

PATT: On the basis of UV absorption, Mitchell believed that RNA increased and DNA decreased after X irradiation. He thought that there was a block in the conversion of RNA to DNA.

TOBIAS: I do not believe that Mitchell's experiments and our model necessarily contradict. Some of the RNA may be inactivated, but still physically present; the cell is then compelled to make new, active RNA, thus the total cellular RNA is actually increased after exposure to radiation.

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ENZYME AND RELATED EFFECTS IN THE INTACT CELL

Kenneth P. DuBois

During the course of the previous discussions, numerous approaches to the problem of the mechanism of action of high energy radiations have been explored. These have included investigations of the effects of radiations on crystalline compounds, on solutions or suspensions of biologically important materials, and on intact microorganisms, plants, and animals..

All of the approaches have contributed a great deal to our knowledge of the biological actions of ionizing radiations but, thus far, conclusive evidence for any theory or explanation of radiation damage in the intact animal is lacking.

As the accumulation of knowledge regarding the action of ionizing radiations on organ systems and on intact cells has progressed, greater interest has been manifested in the biochemical mechanisms that may be involved in the production of the injurious effects. Several phases of biochemistry have been investigated in connection with the mode of action of ionizing radiations. The possibility that disturbances in enzyme systems alter the functional activity and subsequently the morphology of irradiated cells has also received considerable attention.

Although radiation effects on enzyme systems are only partially elucidated, a sufficient amount of research has been done to permit a general discussion of the extent to which enzyme action is interrupted in radiation-injured cells.

It is hoped that the particular areas of enzymology that have been studied sufficiently with respect to radiation damage as well as those that have been neglected can be recognized from our discussion this afternoon.

Many studies have been carried out on the influence of high energy radiations on chemical compounds in vitro and have provided an indication of the relative susceptibility of various compounds to alteration by irradiation. Among the earliest studies were experiments of the type performed by Fricke and Hart (1) in which simple organic compounds were employed. One of the valuable contributions resulting from these studies was the observation that the addition of various substances to aqueous solutions of a compound could protect the compound from decomposition by irradiation. This finding is particularly noteworthy to the biochemist in his consideration of the effects of irradiation, since a simi-

lar situation in which a number of compounds are irradiated simultaneously must necessarily exist in the intact cell.

Following studies on the action of radiations on simple organic compounds, Dale (2) undertook an investigation of the action on enzyme systems in vitro, and several crystalline or partially purified enzymes were shown to be inactivated in dilute solution. Detailed studies of these enzyme inactivations demonstrated protection against loss of catalytic activity by the addition of various substances to the enzyme solutions.

The oxidizing ability of the products of ionization of water led Dr. Barron to consider the possibility that the sulfhydryl enzymes would be inactivated by radiation through conversion of their -SH groups to the inactive disulfide forms. This idea was tested with solutions of crystalline or partially purified enzymes, e. g., hexokinase, succinic dehydrogenase, phosphoglyceraldehyde dehydrogenase, adenosine triphosphatase and urease, whose catalytic activity was known to be dependent upon -SH groups. Inhibition of the activity of sulfhydryl enzymes was generally noted, and this inhibition could be reversed, as Dr. Barron explained yesterday, by the addition of glutathione after medium or low doses of X-rays. The results of these studies demonstrated the inherent susceptibility of sulfhydryl enzymes to the action of ionizing radiations.

Other experiments on the effects of radiation on enzyme systems in vitro have been conducted, and, in some cases, inhibitory effects were noted. In general, the in vitro experiments have given a good indication of the types of results which can be obtained by irradiation of enzyme systems. Factors such as the purity of the enzyme, the concentration of the enzyme in solution, and the nature of the impurities markedly affect the amount of alteration of enzyme activity. However, the increasing realization of the limitations of in vitro studies of this sort has forced investigators to turn to the more difficult task of examining the actions of ionizing radiations on enzyme systems in vivo.

Many investigators are now searching for disturbances in carbohydrate metabolism. Although research on this phase of metabolism has not yet provided an acceptable explanation for radiation damage, the information obtained to date represents a valuable contribution to the ultimate understanding.

Following the in vitro studies on sulfhydryl enzymes, attention was directed to the possible effect of radiation on these enzymes in the intact animal. Recent experiments in our laboratory (3) on tissues taken from rats subjected to high doses of X radiation illustrate the resistance of enzymes to inactivation in vivo. When animals were sacrificed at 24 hours after 20,000 r, there was no appreciable decrease in the oxidation of several substrates by liver slices. Succinate, oxalacetate, citrate, a-ketoglutarate, glutamate, fumarate and malate were all oxidized at a normal rate. Thus we see that in the intact animal, a presumably radioresistant tissue such as liver, does not exhibit a decrease in ability to oxidize several intermediates of the tricarboxylic acid cycle. Similar results were obtained in the case of kidney, heart, and brain, which are also considered to be radioresistant.

One point that I should like to make is that it is important to study biochemical mechanisms in tissues that are known to be radiosensitive.

One cannot extrapolate results obtained on radioresistant tissues to radiosensitive tissues like the spleen or the thymus. In the case of the spleen, one obtains a somewhat different picture for exposures as low as 100 and 200 r result in a pronounced decrease in the endogenous respiration. This is some-

thing that was noted by Dr. Barron several years ago and it has also been observed in our laboratory. We feel confident that, in the case of the spleen, either the enzymes involved in endogenous respiration are inhibited by irradiation or radiation produces a deficiency of substrates.

The endogenous respiration of the spleen is relatively high. When some of the intermediates of the tricarboxylic acid cycle are added to normal spleen slices, one obtains a small stimulation of respiration; radiation does not completely abolish the added respiration. We know from a comparison of the respiration of spleen, kidney and liver slices that no disturbance is produced in kidney and liver with dosages of radiation which produce marked decreases in the endogenous respiration of spleen. One cannot entirely alleviate this decrease by the addition of intermediates of tricarboxylic acid.

KAPLAN: What time is this after irradiation and what kind of doses?

DUBOIS: In the spleen, exposures as low as 100 r cause an appreciable decrease in endogenous respiration. Dr. Barron used such exposures in his work. Most of our studies have been done with 400 r. The animals were sacrificed at daily intervals for a period of 7 days and then at 10, 14 and 21 days.

PATT: Have you made observations immediately after irradiation?

DUBOIS: One day is the shortest time interval, but Dr. Barron made observations at 4, 12 and 24 hours and found decreases.

CARTER: Does not the cellular population change a great deal in this time?

KAPLAN: I wondered to what extent this is a true chemical change rather than a chemical description of the change in cell population. That is what I was getting at.

DUBOIS: That question will necessarily come up repeatedly because the biochemical changes and pathological changes follow the same pattern with respect to the time of occurrence. The parallelism of these effects suggests that they are related, but whether the biochemical and pathological changes are related as cause and effect cannot be stated on the basis of any available data.

BARRON: The only thing is, I doubt there would be any cell change from irradiation.

CARTER: How long do the lymphocytes live?

KAPLAN: The lymphocytes are destroyed as early as 3 to 6 hours after irradiation.

CARTER: The evidence for degeneration actually might be much earlier than that.

PATT: After 100 r there may be 30 or 40 percent pyknotic lymphocytes in the lymph nodes within a matter of a few hours.

POTTER: Still in the tissue?

PATT: Well, you are working with the intact animal and the situation

may be complicated by rapid removal of some pyknotic cells. This is particularly evident in the blood stream, as shown by Trowell (4).

BARRON: How many hours after irradiation can you get lymphopenia?

PATT: Within a few hours after irradiation. Maximal spleen involution or involution of lymph nodes occurs within a day or two after 100 r to the whole body.

DUBOIS: The endogenous respiration of either the spleen or thymus gland decreases markedly in one day after exposures to 400 r. It may decrease a little more in the following 3 to 5 days. There is then a gradual return so that at 14 and 21 days the rate of endogenous respiration again approaches the normal value. This is a reversible inhibition of endogenous respiration and correlates quite well in time with the reversibility of the functional activity of these tissues after 400 r.

BARRON: You have not measured the respiration in terms of DNA?

DUBOIS: No.

PATT: The curve for endogenous respiration resembles very nicely the curve for spleen involution. With 400 r, the peak would appear at about 3 days, with recovery becoming apparent during the next several days.

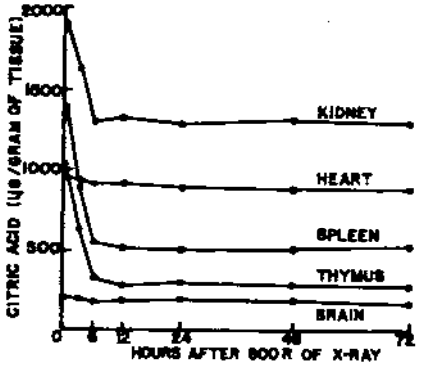

Figure 1. Effect of 800 r of X radiation on the accumulation of citric acid in tissues of fluoroacetate-treated rats. (This chart was published in a paper by K.P.DuBois, K.W. Cochran and J. Doull in Proc. Soc. Exp. Biol. and Med. 76, 422-427 [1951].

DUBOIS: In connection with these observations on the respiration of tissues of irradiated animals, I feel that it is not profitable to study the oxidative phase of carbohydrate metabolism in the liver, kidney, heart, and brain, but that there is a great deal to do in connection with radiosensitive tissues like spleen and thymus. Studies of the overall metabolic activity of tissue slices may be considered as preliminary to more definitive experiments.

To obtain further information on the gross effects of irradiation on the oxidative phase of carbohydrate metabolism, we have used the sequential blocking technique developed by Potter (5), in which fluoroacetate is employed to inhibit citric acid oxidation in tissues. This method of studying the actions of poisons on carbohydrate metabolism consists of giving fluoroacetate to irradiated animals at various times after exposure and sacrificing the animals for citric acid measurements. If radiation were interfering with the formation of citric acid at some point in the cycle, it would be revealed here by an increase or decrease in the amount of citric acid accumulated in the tissues relative to the controls.

The use of fluoroacetate technique (6) showed that there is no effect on citric acid formation in heart or brain after 800 r. There was a small inhibitory effect in the kidney; this could not be obtained with sublethal doses and, therefore, was not considered to be of any appreciable significance. There was a marked inhibition of citric acid formation in the spleen and the thymus. This indicated that some step that ultimately leads to citric acid formation in these organs is inhibited by whole-body X irradiation. After the administration of a lethal dose to rats, the effect was irreversible. The results of these experiments are illustrated in Figure 1.

After sublethal doses, the inhibition of citric acid synthesis in spleen and thymus was reversible. After 200 r, citric acid formation in the spleen showed an initial decrease to less than half the normal value. This was followed by a gradual return toward normal. At 14 days after 200 r, the ability of the spleen to accumulate citric acid was the same as in normal animals. After 400 r, the same initial type of depression occurred but it was greater in amount and the reversal took place at a slower rate. The results of these experiments and similar findings on thymus glands are shown in Figure 2. In these studies we obtained a correlation between the X-ray exposure and amount of inhibition. A correlation between the exposure and rate of reversal was also noted with the higher dose requiring a longer time for reversal.

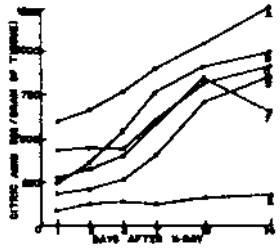

Figure 2. Duration of effects of single doses of X-rays on the ability of rat tissues to accumulate citric acid after fluoroacetate treatment. A. Spleen, 200 r. B. Thymus, 200 r. C. Spleen, 400 r. D. Thymus, 400 r. E. Liver, 200 r. F. Liver, 400 r. (This chart has been published in a paper by K.P.DuBois, K.W. Cochran and J.Doull in Proc. Soc. Exp. Biol. and Med. 76, 422-427 1951).

At this point, while we are talking about low doses of X-rays, perhaps it should be mentioned that we feel that studies using sublethal amounts of radiation are more valuable in searching for the mechanism of acute radiation damage in animals than is the lethal dose. This viewpoint is one for which there is a great deal of supporting evidence from experiments with other toxic agents. The arsenicals, for example, produce their inhibitory effects on sulfhydryl enzymes at doses far below the lethal. The agents that have strong inhibitory action on cholinesterase also are effective at doses that are far below lethal. We might expect that any action that is of importance in connection with the primary biochemical mechanism of radiation damage ought to occur at exposures that are below the LD 50. High dose studies, e.g., 800 r, are useful for exploratory work. In studying the effect of radiation on enzymes, we are inclined to first use large amounts of radiation to ascertain whether a particular reaction is affected. If the reaction is unaffected it can be discarded from further consideration, but if it is inhibited, then it seems advisable to conduct additional studies using sublethal doses. By this method, I believe that we can screen out and eliminate secondary biochemical effects. It seems probable that many of the changes that have been reported in animals after 800, 1000 or 1200 r would not be detectable after sublethal exposures. They may be secondary to bacterial infection or they may actually be due to radiation but not essential for the lethal action in animals.

BARRON: Do you think there are two different problems? One being the effect of lethal doses of X radiation and the other, to determine the initial point of action of the radiation. The approach you propose is that of using small amounts of radiation to find the initial point of damage, whereas when we are working with lethal amounts of radiation, as you pointed out, death is produced mostly by secondary infections.

PATT: Not necessarily. I have some reservation about this philosophy for screening biochemical effects that may be related to lethal action.

BARRON: I am not against his philosophy. I am agreeing with him.

PATT: I believe that DuBois implied that biochemical effects from low doses, below the LD50, might mean that these are probably intimately related to

killing of the animals or represent the initial steps. I would simply like to say that some physiologic and histologic changes, e.g., lymphopenia and lymphoid involution, apparently reflect the amount of radiation instead of the lethal effect. In other words, the inference that changes observed with low doses are essentially critical for killing does not necessarily follow.

BARRON: I think DuBois and I are in agreement because of our experience with gases as warfare agents. In order to determine the real mechanism of these gases, we had to go to doses that were not lethal.

CURTIS: I think he attempts to find the initial reaction from irradiation.

PATT: That is fine, but there was the additional qualification, namely, that the particular enzyme inactivation that you may get with 200 r, which may be a sublethal exposure, is necessarily critical for killing and to that I object.

BARRON: I was separating the lethal action of ionizing radiation from the injurious action.

DUBOIS: According to Dr. Patt's interpretation, I implied that any change that occurs from 200 r might be involved as an important factor in the lethal action of radiation. That is not exactly what was meant, but rather that if a change was found at 1000 r in the rat, for example, and could not be found at 200 r, I would be inclined to suspect that this particular change had nothing to do with the death of the rat after an LD50. In other words, any effect involved in the lethal action at the LD50 level should be detectible at doses below that amount. But this would not mean that a change, which is found, would necessarily be involved in the lethal action.

PATT: Perhaps.

CURTIS: Let me clear up this point. I gather that you don't agree with this philosophy, Dr. Patt.

PATT: I think it is fine to use screening procedures. However, the fact that a large exposure, e.g., 1000 r, does not appear to change a particular reaction does not mean that there may not be a change with lower dosages. This is arguing at the other end.

DUBOIS: In the same material?

PATT: Yes, in the same material. This is so because there may be different sorts of effects, differences in recovery, differences in the extent of cellular damage, etc. We know also, that certain pharmacological agents may have one action at low dosages and an entirely different action at high dosages.

JONES: It might be worth while studying the effect of several doses of radiation.

PATT: This is essential. The lack of an effect from a high dose does not imply necessarily that an effect will not be seen from a low dose. I will go along with your philosophy as a first approximation but I don't think we can exclude the other possibility.

PLATZMAN: No one has asserted that the primary mechanisms differ at different doses. At least, I hope not.

PATT: We are, I think, quite a few molecules and minutes away from what you probably mean by primary mechanism.

BARRON: If you irradiate protein the effect is different.

PLATZMAN: The initial chemical effects cannot be qualitatively different at different doses.

BARRON: As a matter of fact, they are different.

PLATZMAN: I am referring to the primary effects.

BARRON: The chemical effect after irradiation is qualitatively different according to the intensity of the radiation.

PLATZMAN: Not the primary effects. What you refer to arises from the kinetics.

PATT: What is primary?

PLATZMAN: The first thing that happens after irradiation.

JONES: You have been making a model perhaps too remote for us to observe it. Let us look for things that we can measure.

PLATZMAN: I only hope that no one imagines that there is any difference whatever in the qualitative initial effects.

KAPLAN: One important objection is that if you talk about observing the effect of sublethal doses, the first thing you have to be sure of is that your observation is an effect. I think this is really primary. At the risk of repetition it gets back to the question of cell population. In Figure 1, DuBois shows heart, kidney, and brain. He is dealing with relatively homogeneous radioresistant tissues there, and the cellular elements other than parenchyma that are present are negligible. There is some supporting tissue but not much. On the other hand, the spleen is really at least two different kinds of tissue. Even in the thymus, although it looks like it is all lymphocytes, if you abolish the lymphocytes you see that there are a lot of other cells.

The real question is: have you demonstrated any alteration in the citrate metabolism of those cells of the thymus or spleen that are left after the exposure that you have given? I don't see that you have any evidence for such an effect. If you are dealing with 2, 3 or 4 different cell populations, each of which metabolizes citrate normally at a different rate, and then by irradiation abolish one of the cell populations, then you will seem to get an effect on citrate metabolism. Therefore, I think we have to back up and make sure that there has been any real effect.

DUBOIS: That is right and it is in line with the whole idea that I am trying to develop this afternoon. The first thing that we have to do is to look for effects in the irradiated tissue and discard from further consideration, the tissues and enzyme systems in which no changes are found. Then we have to ascertain whether the observed effects represent actual changes in metabolism of the irradiated tissue or are a reflection of the condition of the entire organ. The pathological and biochemical changes in tissues such as the spleen might be the result of radiation injury to some other portion of the body and thus secondary in nature.

JONES: I wish to add a comment upon differences in the apparent nature of the post-irradiation response that depend upon the time selected and the dosage of radiation used. In DNA synthesis and also in the changes of spleen size after irradiation, there is general evidence for a depression that is proportional to the radiation exposure. While we think of the post-irradiation response as being a decrease in spleen size, in cell division rate, and in DNA turnover, careful examination by Dr. Lola Kelly has shown that immediately after irradiation, there is a period in which DNA turnover is enhanced. Perhaps in this case there is not a genuine transitory increase in mitotic activity; the effect noted may be due to the stimulation of cells that are already relatively insensitive to radiation inhibition because they are in the throes of mitosis.

In some contrast, there is another system, that of lipid metabolism. Alteration of serum lipoprotein is an indicator of severe radiation damage. But it is essentially an all-or-nothing type of response, whereas the post-irradiation decrease in cell division and DNA turnover is a continuously graded response dependent upon dosage.

Rabbits that die from irradiation show alteration of serum lipoprotein metabolism. At the same exposure, rabbits that survive do not show the change. The typical response is an elevation of the higher molecular weight classes of B-lipoprotein associated with a lack of removal of neutral fat from these lipoproteins. Thus, the general statement of the effect of irradiation upon lipid metabolism is that lipoprotein utilization is halted by irradiation. Nevertheless, such animals, examined immediately after irradiation, show a 16-fold enhancement of lipoprotein utilization for about 5 to 10 minutes. It is after this that the 48-hour period of lack of lipoprotein utilization ensues. On the third day after irradiation, the system again reverts to a 16- to 20-fold enhancement of the normal functional level of lipoprotein interconversion and utilization of their neutral fat content.

CARTER: By lipoprotein interconversion you are referring to the shift in the ultracentrifuge peaks.

JONES: Yes, essentially it is the shift in the flotation rate with a lowering of the S_f number which accompanies the decrease in density brought about by the removal of neutral fat from the lipoproteins. Starting with lipoproteins that may be of chylomicron size, which have a flotation rating of S_f 40,000, there is a progressive reduction approaching S_f 6 by enzymatic hydrolysis of neutral fat.

POTTER: All in plasma?

JONES: Yes, the whole process seems to take place in the plasma. I must apologize for this discussion which deviates from the specific topic but I suppose that the circulatory system or the whole mammal can be considered a cellular unit.

DUBOIS: Returning to the subject of the influence of radiation on citrate synthesis, I would like to mention some effects that we have observed in studies on the liver. Following irradiation, the liver of the male rat acquires the capacity to accumulate large quantities of citric acid after fluoroacetate treatment whereas, that of the normal male rat is unable to do so. However, the normal female rat can accumulate citric acid after fluoroacetate treatment. The effect of radiation here is essentially to change the metabolism with respect to the response to fluoroacetate so that the liver of the irradiated male rat resembles that of the normal female rat in its ability to accumulate citric acid.

Figure 2 shows the effect of radiation on citric acid formation in the liver. Although one does not observe any effects of radiation on the ability of liver slices to oxidize a number of different substrates, the fluoroacetate technique indicates that there is a disturbance in citrate formation in this organ. Although we do not know the exact cause of this biochemical change, we suspect that a factor that normally regulates citrate formation in the liver of the male rat is altered or destroyed by radiation.

This change in the metabolism of the liver is not associated with the lethal action of radiation because the effect is irreversible after 400 r in contrast to the reversible effect on citrate synthesis in the spleen and the thymus. The effect on the liver persists for at least 3 months after 400 r.

KAPLAN: What happens to the female?

DUBOIS: The values in the female are about normal after irradiation. There is quite a wide normal range, but both normal and irradiated female animals accumulate large quantities of citrate in the liver after fluoroacetate treatment.

PATT: Does citrate accumulate in the male castrate?

DUBOIS: Yes, without radiation. We suspect that it might be due either to interference with or the prevention of synthesis of androgenic compounds and/or adrenal cortical hormones. We have done a considerable amount of work along this line, which indicates that castration will produce an effect like radiation and that treatment of female animals with testosterone will decrease citrate formation to the level seen in the male.

CARTER: Could this not also be due to the failure of the liver to inactivate the estrogenic hormone after irradiation?

DUBOIS: Yes, that is possible.

CARTER: This is commonly seen in liver disease.

DUBOIS: Estrogens do not have any stimulatory effect in normal animals. If one gives estradiol to normal male animals, citrate formation does not increase appreciably.

CARTER: That may be due to the normal livers' capacity for inactivation.

DUBOIS: Our experiments with adrenal and sex hormones are in line with the idea that there is a hormone involvement in the radiation effect on citrate formation. However, the experiments do not prove it.

KAPLAN: This is just after irradiation of the liver?

DUBOIS: The effect is not detectable immediately after irradiation but rather requires several hours to become pronounced. The nitrogen mustards will produce, (7) qualitatively and quantitatively in most respects, the same type of response as radiation on citric acid formation as shown in Table I. Citrate formation in the spleen and thymus is markedly depressed by doses of methylbis (β-chloroethyl) amine in the LD50 range, and the amount of citrate formed in the liver is markedly increased just as it is in irradiated male rats.

TABLE I

INFLUENCE OF NITROGEN MUSTARDS ON CITRATE ACCUMULATION IN SPLEEN, THYMUS, AND LIVER OF IRRADIATED RATS

Mg./kg. of Ethyl Bis (β-chloroethylamine)	Liver	Spleen	Thymus
	(μg. citric acid/g. fresh tissue)		
Control	64	1425	1045
2.5	729	744	710

POTTER: May I ask a question about the mechanics of an experiment like that? When you do that, obviously you do not take all the rats at once and inject them.

DUBOIS: We usually injected one dose and killed groups of 4 animals each day. All animals were not necessarily injected at one time.

POTTER: But on any given day would you give them all the same dose?

DUBOIS: Yes.

POTTER: You would tend to give them one dose and then the next day give them the next dose?

DUBOIS: Yes.

POTTER: No, I just mean in the statistical operation of the experiment would you randomize your dosage in each case or not?

DUBOIS: No. However, the doses given on successive days were not always the next highest doses used in the study. The dose was either raised or decreased on successive days depending on the outcome of the previous experiment. The doses actually were randomized in this respect but this was not done purposely.

SHERMAN: Did you run controls each day?

DUBOIS: Not each day. There was no reason to do so, because they were always the same. Controls were run at intervals, say, of 2 weeks. I don't think I got the point of Dr. Potter's question.

POTTER: I am just raising the question of how many days the rats in the bottom row had been on a given diet in comparison with the rats in the top row.

DUBOIS: They were all on the same diet for the same period of time throughout the study. All animals weighed close to 225 g. and were the same age.

One might expect that many of the biochemical effects of nitrogen mustard would resemble those of X-rays, and for some types of experiments, the nitrogen mustard might be more useful than X-rays.

KAPLAN: In that connection, I do not know how your doses compare with the sublethal but toxic doses of mustard that we have used in mice. In our experiments, they produced essentially no decrease in thymic weight. Comparable doses of whole-body X radiation, in terms of lethality, cause a decrease in thymic weight to perhaps 15 or 20 percent of normal. So that this apparently "radiomimetic" substance is not always radiomimetic. It is interesting that it should produce a similar biochemical effect in the thymus of rats. It would be interesting to check to see whether the rat's thymus is equally sensitive to the drug in terms of weight response.

DUBOIS: That is a point worthy of mention. Lethal doses of the nitrogen mustard produced effects equivalent to, or resembling, those obtained by 200 r X radiation.

BENNETT: I should like to ask -- I suppose there are technical reasons -- why the spleen and thymus are studied and only seldom the bone marrow?

DUBOIS: The spleen and thymus are used as examples of radiosensitive tissues. In many types of experiments the amount of tissue required is a factor that limits the choice when animals such as the rat or the mouse are used.

Now to proceed with our main discussion. The data in Table 1 show that carbohydrate metabolism in the liver is not interrupted by doses of nitrogen mustard that will produce a marked increase in citrate synthesis. The oxidation of pyruvate and fumarate by liver homogenates prepared from rats given 1 mg. per kg. (a lethal dose) of methyl-bis (β-chloroethyl) amine takes place at a normal rate. Nor is the oxidation of acetate or pyruvate or the formation of acetoacetate affected. The absence of effects on these reactions resembles the lack of effects following doses of whole-body X irradiation on tissue respiration. By measurements of specific enzyme concentrations, it is quite well established that the activity of cytochrome oxidase, succinic dehydrogenase, and of malic dehydrogenase is not inhibited or increased by lethal doses of radiation in most tissues, including spleen and thymus. There have been reports of the activity of succinic dehydrogenase being decreased in the spleen after irradiation, but this amounted to a decrease of only about 25 percent after as much as 800 r. A great deal of further work has to be done, especially on tissues which are sensitive to radiation, in which systematic investigations of the enzyme systems are conducted.

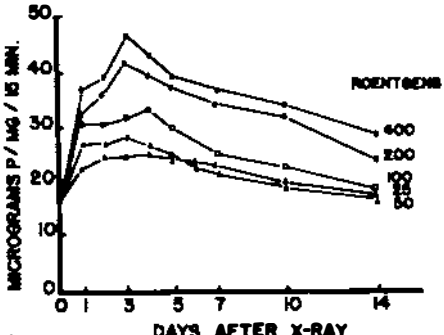

Figure 3. Adenosine triphosphatase activity of the spleens of rats at intervals after various exposures to total-body X radiations. (This chart appeared in the paper by K.P. DuBois and D. F. Petersen, American Journ. of Physio. 176, 282-286 1954).

One of the groups of enzymes which does show a change when specific assays are used is adenosine triphosphatase (8). The data in Figure 3 show the results of adenosine triphosphatase assays on spleens of irradiated rats. Twenty-five r produces a response and 50, 100, 200 and 400 r produce further increases in enzyme activity. The maximum was about 2.5 to 3 times the normal activity in this tissue. This is not an in-

hibition but an increase in the rate of hydrolysis of ATP. After these exposures, which are sublethal, the effect is reversible. You will note that at 14 days, the activity is again approaching the normal level with the higher doses and is back to normal with the lowest. This represents a change that is detectible at about 1/20 of the X-ray LD50 for this species.

JONES: When was the first observation?

DUBOIS: At three hours after X-ray exposure.

CARTER: This is done on a homogenate of spleen?

DUBOIS: Yes.

CARTER: Are the nuclei intact or disrupted?

DUBOIS: Disrupted. The maximum increase in activity of this enzyme occurs after 400 r. After 600 or 800 r there is no further increase in enzyme activity. The only difference when a lethal dose is given is that there is no reversal of the effect during the survival time.

This change in not restricted to the spleen. It also occurs in the thymus (Figure 4). In the thymus, the dose required to produce an equivalent amount of increase in terms of percentage is somewhat higher than in spleen. A very small effect is observed at 50 r, a little greater effect at 100, and a pronounced increase at 200 and 400 r.

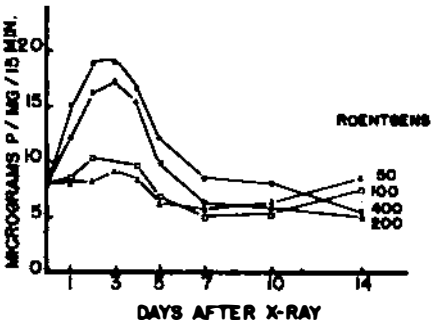

Figure 4. Adenosine triphosphatase activity of the thymus glands of rats at intervals after various exposure to radiation. (This chart appeared in the paper by K. P. DuBois and D. F. Petersen, American Journ. of Physio. 176, 282-286, 1954).

It may be of interest to note that there is no increase in enzyme activity in the thymus after 20,000 r, although activity does increase in the spleen. Nor is there any decrease in thymus weight 24 hours after 20,000 r. There is a case in which the response to high dosage is strikingly different from that at low dosage, which calls to mind the previous comment along these lines. In other words, if the initial test had been done using 20,000 r, we would have concluded that the ATP-ase activity is not changed in this tissue, whereas at the lower dose there is a marked increase.

CARTER: Do you have any data that would indicate that the substrate for this reaction existed at higher or lower levels than normal? Does this expression of enzymatic activity have some counterpart in the concentration of the substrate?

DUBOIS: No, not on the basis of the in vitro system in which ATP was added in excess.

CARTER: Or in the cell?

DUBOIS: In the cell the ATP concentration after 400 r of X-ray is reduced to $\frac{1}{2}$ of normal.

MAZIA: In the case of the phosphatase group of enzymes, an increase in activity could very well indicate a degenerative change. When you are purifying alkaline phosphatase from horse kidney, a standard operating procedure is to permit the tissue to autolyze. Before autolysis, you find much less activity. Activity increases as the tissue rots. There are some cases where phosphatase activity is increased by digesting the tissue with trypsin.

DUBOIS: This increase, which you are mentioning, concerns glycerophosphatase activity. In the case of ATP-ase, degenerating spleens which have been ligated in situ or removed and kept at 38°C, show no increase but rather a marked decrease in activity. There are several other points that should be mentioned. One is that as the dosage of X-rays is increased above 400 r, the activity per mg. of tissue remains at the same level as after 400 r.

Fractionation of the spleen done by Maxwell and Ashwell (9) indicated that 50 percent of the ATP-ase activity is confined to the microsomes and the rest is distributed throughout the other fractions, with the supernatant generally having only a very small fraction of the activity. But if one does a crude fractionation of the whole organ and separates it into pulp and connective tissue, there is an increase in both of the fractions. The total increase in the organ cannot be accounted for in this case by depletion of the population of any one type of cell. Activity in the connective tissue fraction increases just as well as in the remaining portion. The relative amount of connective tissue remaining in the framework structure does not account for the rise in the total organ.

MAZIA: My point was that this sort of result might not indicate enzyme formation at all, but liberation of activity by changes that one could consider degenerative.

DUBOIS: If this were enzyme synthesis, it would represent a 3-fold increase.

SPIEGELMAN: You can get much more than a 3-fold increase in enzyme synthesis. You can get a 1000-fold increase. The point is to decide whether this is synthesis or if it isn't, and this can be done, perhaps by use of suitable analogues.

MAZIA: There is another way in which you can do it. Working with the mammal, C.H. Li has observed that hypophysectomy will prevent the formation of tryptophane oxidase. One might get at the question of synthesis of new enzyme protein by such a procedure.

CARTER: Do you mean adaptive enzyme formation?

MAZIA: Yes.

DUBOIS: This increase is not due to the presence of an enzyme activator in the spleen or at least to an excess of activator, because the activities of spleen homogenates from irradiated and normal animals are completely additive. The possibility of increased enzyme synthesis is worthy of study but with due consideration to the fact that we are dealing with mammalian tissues where enzyme syntheses to the extent of 1000-fold increases are not often observed.

CARTER: Would you consider that this might be analagous to the situation that Dr. Potter and others have described in the liberation of latent ATP-ase from the mitochondrial system?

DUBOIS: No, the characteristics of the enzyme splitting ATP in spleen seem to be different from those of liver. For one thing, the activation curves with calcium and magnesium are identical. If we replace calcium with manganese the activity is much lower.

Analysis of the residue after incubation shows that the ATP is converted mainly to ADP. The phosphorus liberated has come from the single reaction consisting of the conversion of ATP to ADP. There is no appreciable kinase present and as a result, very little adenylic acid is formed. The sum of the residual ATP, ADP and adenylic acid accounts for the original amount of substrate.

POTTER: I don't think you have latent ATP-ase in the spleen. If you consider that this effect is not due to a changing cell population, then you have to start considering an increased amount of enzyme.

DUBOIS: We do not believe that this is entirely explainable on the basis of cell population but we still need to do additional experiments on that aspect of the problem. This effect does not occur in liver, brain, heart, kidney or other radioresistant tissues regardless of the X-ray dose.

A point of interest is that the rate of hydrolysis of ADP by homogenates of irradiated spleen is not increased to nearly the same extent as the hydrolysis of ATP.

CHARGAFF: You have based it all on the amount of tissue. If you did it on the nuclear count or the DNA would it look the same? Did you get the same type of increase if you took another base line?

DUBOIS: It has been done on the basis of nitrogen and the increase is still obtained. That was done by Ashwell and Hickman (10). It has not been done in terms of DNA.

KAPLAN: This is a very pertinent question because Leonard Cole has recently shown that if you compare splenic weight reduction on a mg. basis with the reduction in DNA content of the whole spleen following radiation, there is a far greater decrease in DNA content than one can account for by change in weight. In other words, the number of nuclei left are far fewer than the weight change would lead you to believe because the most radiosensitive cells are the smallest cells. There are a lot fewer nuclei and fewer cells in those spleens 1, 3 and 5 days after irradiation than there were before.

SPIEGELMAN: If this is true, the other enzymes should go up. It does not seem likely that this type of explanation is going to be the answer.

KAPLAN: No, but this is still a better way of expressing it.

SPIEGELMAN: Yes, I will agree with you, but I think that probably you will not iron out this difference if he does not see it with other enzymes.

DUBOIS: And we don't with the enzymes that have been studied. To proceed with the discussion, the question of the influence of radiation on oxidative phosphorylation by the spleen should, perhaps, be mentioned. Although there is a decrease in phosphorylation by spleen homogenates and preparations of spleen tissue, I think the increase in adenosine triphosphatase activity is a complicating factor and that we should now re-examine the phosphorylation picture. One reason for this is that in the phosphorylation setup that is ordinari-

ly used, fluoride is added to inhibit the ATP-ase activity. Addition of the quantity of fluoride oridinarily used will decrease enzyme activity by about 50 per cent in both normal and irradiated spleen. However, even in the presence of fluoride, the ATP-ase activity of a phosphorylation system is about twice as high in irradiated as in normal spleen. Therefore, it might appear that oxidative phosphorylation was inhibited when actually there might only be increased hydrolysis of the phosphate esters formed.

The breakdown of 5-adenylic acid is also reversibly increased in the spleens of irradiated animals at doses that are below lethal; after 800 r, there is an irreversible increase in the 5-nucleotidase activity of spleen and thymus. The characteristics of this effect with regard to time of onset, magnitude, and duration are very similar to those that I have just described for ATP. The breakdown of adenylic acid due to increased nucleotidase activity occurs in the spleen and thymus but not in most other tissues of irradiated animals.

The administration of protective agents will decrease the amount of enzyme change in tissues of irradiated animals. Para-aminopropiophenone (PAPP) a methemoglobin-forming agent, shown by Storer and Coon (11) to protect animals against lethal doses of radiation, partially prevents radiation-induced increases in nucleotidase and adenosine triphosphate activity. For example, when 400 r was given to PAPP-treated rats, only a very small increase in the ability of spleen to split adenylic acid was noticed.

Even though we still have a great deal to do in connection with determination of the exact reason for the increase in enzyme activity, this biochemical change can be used for studying the action of agents which are known to protect against radiation lethality. PAPP does prevent this biochemical change from occurring. This does not mean that the ability of PAPP to prevent mortality is due to its protection against the phosphatase increase but, it does illustrate a method of detecting whether a chemical agent is protecting against radiation-induced damage to the spleen.

KAMEN: Is there any theory as to how this agent operates?

DUBOIS: Probably by its ability to produce tissue anoxia, because of methemoglobinemia.

KAMEN: What concentrations are used?

DUBOIS: Thirty mg. per kg.

POTTER: Incidentally, is it ineffective if you give it 30 minutes afterwards?

DUBOIS: Yes.

PATT: Does para-aminopropiophenone have any action on isolated tissue or on other in vitro systems?

DUBOIS: No, insofar as is known.

KAMEN: How long do you have to wait before it stops being protective?

DUBOIS: The radiation should be administered within 2 hours at least after the drug is given.

PATT: I think the prevailing evidence indicates that the PAPP probably works through an anoxic type of effect.

SPIEGELMAN: Can you get the same effect by choking the animal?

PATT: I am sure that you could. Some years ago, Titus Evans strapped the chests of young rats to mechanically retard breathing and he observed certain protective effects. As I recall Storer and Coons' original work, the maximal methemoglobinemia occured at about 30 to 45 minutes after injection; yet apparently the maximal protective effect occurred when the material was injected immediately before irradiation. This is the only fact I am aware of that does not quite jibe with a resolution in terms of anoxia, although I think that it is due to anoxia.

DUBOIS: Since their animals were irradiated for a period of approximately 20 minutes, it is possible the effective methemoglobinemia was achieved during the middle of the radiation period.

BENNETT: This would affect the LD50 by a factor of only 50 percent. It is not a major effect, though.

DUBOIS: The effect of this prophylactic agent on ATP-ase was to reduce the amount of rise in enzyme activity after 800 r to a level that would have been seen after 200 to 400 r in the unprotected animal.

As a practical use of this particular finding, one can employ this relatively simple assay system to screen potential prophylactic or therapeutic agents.

KAPLAN: Is this actually cheaper than weighing the tissues or getting histological sections?

DUBOIS: No, it would not be advantageous if one could get results with a few animals by weighing the organs, or if the difference between 400 and 600 r at 24 hours could be detected by organ weights. However, we do not feel that organ weights are that reliable, and a larger number of animals are needed. Therefore, enzyme assay has been a faster method and more reliable with fewer animals, at least in our experience.

Mercaptoethylamine and cysteine also protect against the increase in nucleotidase activity of the spleen after 400 r. Nearly equal protective effects were obtained with mercaptoethylamine at the maximum tolerated dose which is 175 mg. per kg. and with cysteine at 1000 mg. per kg. given I.P. I think this agrees with Dr. Patt's mortality findings with these agents. Again we have here a demonstration of two agents that will protect against a biochemical change induced in the spleen by radiation and will also protect against radiation mortality.

In connection with the problem of the biochemical mechanism of radiation damage, I have mentioned some of the experiments that have been done to point out progress that has been made and areas that need study. I think it has probably become apparent that ionizing radiations do not produce widespread inhibition of enzyme reactions in living animals and that a great many of the important reactions in intermediary metabolism go unharmed after relatively large doses of radiation. Furthermore, the larger effects that have been obtained are noted only in the so-called radiosensitive tissues and even there, a great deal of work is needed, as several discussants have pointed out, to show definitely whether these are primary effects in the sense that they are radiation-induced

changes in the enzyme itself, secondary to some other change within the radiosensitive tissue or the result of injury to some other tissue.

I think that it is necessary to continue a systematic investigation of various enzyme systems so that we can perhaps eventually arrive at a definition of radiation damage in terms of certain biochemical pathways.

CURTIS: I gather you feel that there is a certain amount of hope that biochemistry will eventually find one key enzyme that is hit by radiation. Or, do you feel that this approach has been pretty well worked over and it is time to try other approaches such as the precursors of the enzymes or DNA or something of that sort.

DUBOIS: I feel that both approaches should be pursued with equal vigor. I don't think that the DNA aspect should be studied to the exclusion of the one under discussion. I feel that the enzyme changes precede the cellular changes, but at the present time, we are not far enough along to say that there is any conclusive evidence in support of this opinion.

CARTER: Does not your work actually tend to show that it is not the enzyme but the tissue that is specifically sensitive? That is, liver certainly has 5-nucleotidase, heart has and spleen has, but the spleen manifests its sensitivity to radiation by increasing the apparent activity of this enzyme.

DUBOIS: Yes, the work certainly shows the difference in tissue susceptibility. Although enzymes with similar catalytic properties exist in the resistant tissues they may differ markedly in their susceptibility to poisons. Naturally occurring protective substances, differences in requirements for activators, and even differences in their chemical constitution cause enzymes in various tissues to respond differently to toxic agents. Thus, concluding that proteins which catalyze a particular reaction in all tissues should be affected similarly by a toxic material is not necessarily a valid conclusion.

PATT: I quite agree with Dr. Carter and have very little to add except to re-emphasize that even if one can relate a change in a particular enzyme to subsequent changes in the cell population, this may still be a rather indirect manifestation of the initial biochemical injury. In other words, this enzyme need not necessarily represent the immediate locus of radiation action.

SPIEGELMAN: In what cases can you relate any changes to the killing of cells?

PATT: Massive X irradiation, e.g., 50,000 r, can embarrass respiration of liver and kidney or of avian red cells in vitro, presumably because a certain number of cells have been killed.

SPIEGELMAN: That is many times the lethal dose.

PATT: Yes, in terms of the whole animal. In general, it takes a rather large exposure to embarrass respiration.

BARRON: But DuBois has shown that he can inhibit the respiration of the spleen.

SPIEGELMAN: It seems to me that this does not mean that the radiation did anything to the enzyme because he looked at them a long time afterward. It must be a secondary response to some other event.

BARRON: No, because we did the same experiment during the war and found respiration inhibited 4 hours afterwards.

SPIEGELMAN: It isn't like a needle where you stick it in and pull it out. That is my point.

BARRON: I think it is.

PATT: How do you interpret Altman's findings of an increase in respiration of rat marrow homogenates immediately after irradiation.

BARRON: We found the same thing.

In single cell irradiations, whether there is an increase or a decrease depends on whether the action is on the glutathione content of the cell, which serves to inhibit respiration, or on the cellular enzymes. We showed with both sea urchin egg and sea urchin sperm that a small dose of X-rays could increase respiration. With fertilized sea urchin eggs, I think we could get an increase with 100 r; with unfertilized eggs 200 r were required; and with sperm 100 r.

PATT: I am referring now to mammalian tissue. I think that Altman found that there was first an immediate increase in respiration and then a decrease on the next day after an exposure to several hundred roentgens. I offered Altman's work only in response to the inference that the decrease in respiration at 4 hours would probably also be observed immediately after exposure.

BARRON: That was not on spleen.

PATT: No, it was marrow which should probably react similarly to spleen.

BARRON: No, because we were unable to find any inhibition with marrow.

MAZIA: This is an important question. Someone ought to set up the experiment in such a way that the bone marrow respiration could be measured immediately or even during the irradiation.

CARTER: We did this with bone marrow at one time and found that immediately following large amounts of irradiation there is no change in the respiration of bone marrow. That is, just immediately afterwards.

BARRON: We found the same thing. We found inhibition in bacteria immediately after irradiation with exposures as low as 500 r. This was done by irradiating the bacteria in their own culture medium.

POTTER: It seems to me that the experiments on enzymes and carbohydrate metabolism will, in the end, be found to be intimately related to nucleic acid synthesis, and it is to our advantage to try to bring these together and to try to find a common denominator. For instance, in the case of the fluoroacetate phenomenon, I think this is the result of whether the tissue can still activate acetate. We are carrying out studies that suggest that whether you can activate acetate is a sort of barometer of the ATP-ADP ratio. This is something that you cannot get at experimentally by any direct measurement because of its highly dynamic nature, but by indirect experiments, we have a number of indications that the capacity to activate acetate is a barometer of that ratio. So that I think that

what goes on in these tissues may eventually come back to some of these common denominators between nucleotide metabolism and nucleic acid synthesis.

Regarding Dr. Patt's and Dr. Carter's comments, I should like to say, that this may be a matter of tissue damage and that I think the same enzyme can be knocked out by X irradiation in all of the tissues. The reason you have sensitive tissues is simply because those tissues contain less of the enzyme that is hit by the radiation and that the effect that Dr. DuBois sees is the reflection of the knockout of that enzyme present in such small amounts.

We have examples in our own experience where the enzymes in the various tissues can be shown to be susceptible to highly specific agents, and when you hit the whole animal with these agents, some tissues are not affected at all. They are the ones that have large amounts.

PATT: Is there any way of increasing the concentration of these enzymes other than with irradiation? It should be possible then to test this hypothesis by irradiating at a time when the levels are already increased.

POTTER: While this discussion is on protection against X irradiation, I should like to ask what you can add that potentiates irradiation.

PATT: High oxygen tensions may enhance effects on tissues that are ordinarily somewhat anoxic but oxygen will not effect enhancement generally.

PLATZMAN: Has pure oxygen ever been tried?

PATT: Pure oxygen does not alter the sensitivity of animals as judged by lethal effects. Returning to Dr. Potter's question, certain agents, e.g., nitrogen mustards, can synergize with X-rays but the effects are complex. One can potentiate or enhance the killing of animals by imposing a variety of stresses or traumas but I think these may be very far removed from the sort of reactions that we are thinking about here.

TOBIAS: Hypophysectomy prevents formation of some enzymes, and it is known that many hypophysectomized animals are more radiosensitive also. Should one use hypophysectomized animals to study enzyme activity?

KAPLAN: We have data that indicates that hypophysectomized animals are not appreciably more radiosensitive, at least in terms of lymphoid tissue response. I think you have to admit that a number of these animals are so markedly starved to start with that the increment that one gains after irradiation is a rather meaningless increase in mortality that you would get if you half-killed them with any other agent.

PATT: We studied hypophysectomized rats some years ago and also observed an increased sensitivity to lethal action but not to atrophy of lymphoid tissue.

KAPLAN: I have been thinking about trying to find some experimental way to get around this problem of cellular selection in a radiosensitive tissue with respect to biochemical determination at some interval following irradiation. You really have several possibilities. When you irradiate a radiosensitive tissue, a lot of cells die. The first question is to what extent are the biochemical changes that you see, a reflection of alterations in the cells that die? The second question is that there are cells that are left behind. You really don't have any way of knowing whether their initial biochemistry is the same as that of the

cells that die or not. Finally, you have the problem of estimating whether the death of some of the cells does not do something to alter the metabolism of the cells that stay behind.

Any susceptible tissue is going to have cell death after irradiation, and unless you study radiosensitive tumors and radioresistant tumors, and a spectrum of tumors in between, where you can get a high degree of cell homogeneity, I see no way to get around this problem. Even there I am not sure that you can.

SPIEGELMAN: If you have very sensitive assay procedures for the biochemical changes you are following, one way of deciding such a question is to compare the answer you get from many small samples with a lumped sample. If there is heterogeneity, it will show up very quickly.

KAPLAN: I don't think the small samples would work. These various kinds of cells are all interwoven in the same tissue. There are some geographic relationships, with cortex and medulla in the thymus, and white and red pulp in the spleen. But there is actually no proof, even within the white pulp, where there are large lymphocytes and small lymphocytes and stem cells, that the metabolism of each of these classes of cells is the same with respect to the things that are being measured. So that in any tiny area of these organs, you are dealing with different classes of cells. The problem in highly radiosensitive tissues, if you give a dose sufficient to get a measurable effect, is that some classes of cells are simply not present in the early post-irradiation period. They are no longer there to be sampled.

POTTER: Would 15 minutes be soon enough?

KAPLAN: There, of course, you are dealing with still another kind of problem because some of the cells are in the process of dying. What you are really asking is, what is the biochemical change that characterizes the cells that have been hurt? Well, some of them have been hurt and are dying and others apparently have not been hurt and may even have been stimulated to heightened activity because of injury to the other cells present in the tissue.

BENNETT: Do you feel that this difference in behavior depends on their condition with regard to when they are going to undergo mitosis or anything as general as that?

KAPLAN: It apparently has nothing to do with it as far as lymphocytes are concerned.

POTTER: We cannot rule out these cells that are dying. I mean, it is all right to talk about a change in composition, but presumably if we produce a biochemical reaction which has something to do with their dying, then it is legitimate to look for that effect because that is part of the dying.

KAPLAN: Yes, but you don't have any way of separating out the changes that exist in the cells that are dying from the changes or lack of change in the population that is not going to die.

SPIEGELMAN: There is one technique that we have employed that may or may not be applicable to this case. For example, we wanted to ascertain whether during induced formation of enzymes all the cells of the population are making enzymes uniformly or whether some are making a lot of enzymes fast, and others very slowly.

The technique depended on the fact that when a cell has its full enzyme content, it never expresses more than about 5 percent of it .... that is, if you assay the intact cell you get 1/20th of what you obtain on assay of a cell lysate. Now let us consider, say 100 cells that are being induced to form enzymes. Suppose that the enzyme-forming capacity is discontinuously distributed throughout the population, so that, for example, 10 cells make enzymes extremely rapidly in the first 10 minutes and another 10 cells begin making enzyme in the next 10 minutes, and so on. If you examine the ratio of activity of the lysed cells to the intact-cell in the early course of the induction, you should find the expected 20-fold increase. If on the other hand, all the cells are acting alike and forming enzyme uniformly, the ratio will start out as 1:1 and then gradually climb to the 20:1 characteristic of full enzyme content. The results obtained agreed with the last statement.

Thus, if you could find a biochemical activity easily assayed that behaved cryptically in this fashion, and that was lost as a result of irradiation, you might be able to determine whether the loss is uniformly occurring among all the cells of your population or is discontinuously distributed.

DUBOIS: Going back to Dr. Potter s point, I too feel, that all of these things may have a common denominator because the changes that I have discussed, and probably a number of others, are similar insofar as their onset, duration, etc., are concerned. If we consider changes occurring in the spleen after 400 r, i.e., the inhibition of citric acid synthesis, the increase in phosphatases, and the decrease in ATP, they all follow the same pattern. The decrease in endogenous respiration does also. I have purposely avoided talking about nucleotides because of Dr. Carter's discussion tomorrow, but there are some interesting relationships between nucleotide changes and those that I have reported.

KAPLAN: Hydrocortisone is a very powerful lympholytic agent, particularly on the thymus. It will wipe out the lymphocytic population of the thymus just as effectively as a good-sized dose of X-rays.

One way in which one might get at this question, I think, is to see whether there is an increased activity of some of these enzymes in the cell population left after treatment with hydrocortisone where radiation is eliminated as a factor. If such an increase occurs, then I think it would be fair to say that the cells that are not destroyed by these two agents have as their inherent level of activity one that is appreciably higher than that of the population before the lymphocytes are taken out of it. Perhaps a comparative experiment with hydrocortisone might be a useful way to approach this.

JONES: I should like to ask this question for information. It seems to me that with most of the substrates used by Dr. DuBois there was some depression of respiration. Two interpretations of this could be possible: (1) That this would then reflect a possible change in enzymatic functional reserve that was far greater than the shift in the steady state concentration of the cells tested has revealed, just as Dr. Spiegelman suggested a moment ago and (2) Shouldn't we explore the matter of total cell damage as being a summation of many little fragments of change?

DUBOIS: In this case, a very small depression could be a reflection of a change in one enzyme system because the whole cycle is operating in the oxidations described. The dose used was very high, and we have other data with lower doses that show no effect. With exposures lower than the LD50 range and with

800 r, there was no appreciable change in the respiration of those particular tissues.

CURTIS: To get back to the second part of your question, if I understood it, it was that a 5 or 10 percent change in all the enzymes might somehow add up to the lethal effect.

JONES: Yes, I also anticipate a difference from cell to cell. There may be great individual differences in the enzyme content of each cell and parts of cells for any particular type of enzymes; on a random basis, some should go up and some should go down, but in this case you have shown us that every enzyme is somewhat depressed. Could this in itself describe a total change that may be of greater consequence to cell function than the apparent average 5 to 10 percent depression of all the effects?

CURTIS: Do you think that this is the case?

JONES: No, I just brought up the question. What do others here think about it as a possibility?

BENNETT: This is being investigated by Miss Hughes in our laboratory. Acetate-C^{14} is given to animals that have been irradiated, and the rate of excretion of $C^{14}O_2$ is determined. The experiments have really just been initiated, but the rate appears to be closely comparable to that of the normal animal.

If irradiation is affecting the Krebs cycle and all the rates were decreased to 90 percent of normal, one might expect that excretion of CO_2 would be down to something like a quarter of normal. This does not appear to be the case in the whole animal. But there are limitations. One might not be observing the metabolism at the critical time, etc.

CURTIS: I think we might come back to a point that Dr. Pollard brought up this morning. That is, that very few molecules are disrupted by a lethal dose of radiation. In that case, we were talking about large molecules. Is it not true that this afternoon we are talking about much smaller molecules?

DUBOIS: Yes.

CURTIS: If that is correct, then the smaller the molecule, the less is the probability that enough of these molecules will be affected by a lethal dose of radiation to be of importance. This means a lower probability that we are dealing here with a fundamental aspect of the whole radiation problem.

POLLARD: I have just made a few scratch-pad calculations that might be interesting. Take a cell of a length of 5μ and a radius of 1μ. Roughly speaking, there are 9,000 RNA molecules in it and 900 DNA. This is calculating that DNA molecules have a weight of 10^6 and RNA molecules of 10^5 and that their proportion is that given for yeast this morning.

If you suppose that 100 r are distributed proportionately to the area that these things cover -- incidentally, this is assuming the effect to be purely indirect -- if you assume it direct, it goes in volume -- for this type of action, you can say that you are going to get 1/3 of a DNA molecule inactivated per cell, 10 RNA per cell and 90 protein per cell. You started out with 900,000 protein molecules. So you have 1 in 10,000 protein molecules inactivated. You started out with 9,000 RNA and you have 10 of those inactivated. So you have about 1 in 1000 of those and maybe 1 in 1000 of the DNA.

What you can conclude from this I don't know. The conclusion I come to is that 100 r does not do anything. To me it is clear that there must be additional factors operating which are multiplicative in character, and what they are I don't know. You can see some of them as physical. For instance, take the matter of area; the fact that the DNA is long and thin whereas I have assumed it to be more or less spherical for this calculation. If DNA is long and thin it will mean that it stands a better chance of being inactivated. This will give a multiplicative factor of perhaps 4 or 5. But it still does not get us past the fact that we have only about 1 in 500 of the DNA molecules inactivated.

SPIEGELMAN: If each one of these is unique and uniquely necessary.

POLLARD: I didn't say that. If I had said it, I would have been hopped on. You said it.

SPIEGELMAN: I think that is something that we can reasonably assume to be true.

POLLARD: I think so, too.

TOBIAS: Assume that the RNA is distributed somewhat like the DNA so that for synthesis of a given protein you need, say, 9000 RNA molecules. Assume further, that synthesis in the cell proceeds one step after the other, perhaps on the surface of the RNA. If radiation then inactivates any one RNA molecule , e.g., No. 455, it may be that from then on the rest of them don't count, and protein synthesis is broken down. Under these conditions, you would have a high probability of damaging RNA molecular chains.

POLLARD: I think there is another point (I believe it is Dr. Barron's) that could make quite a difference. In making the effect in proportion to the area, we have used the smaller molecules that occupy most of the area and we have assumed that nothing happens. Suppose the smaller molecules are actually carriers of radiation energies that are then communicated to the other molecules, which is essentially your point. They may be sort of symbiotic in action. Then you can have a factor of 5 in addition.

But I feel sure that whatever you do you can never get enough effect in the smaller molecules to account for the effect in the cell. They may be intermediary in their action, but the effect you have to look for, I feel sure, is in the larger molecules. I agree, of course, with Dr. Spiegelman, that they are critical. I believe they are in the chain, and I suspect we ought to be looking for those kinds of things.

HOLLAENDER: This is what I brought up this morning when I said that 1 r would interfere with the rate of mitosis. It might be possible that you do interfere only with the function of a few enzyme molecules.

KAPLAN: You have to remember that there are other kinds of cells that are easily knocked off by 100 r. This forces us into some further complications because all the cells in the animal body, with relatively few exceptions, have a normal complement of chromosomes and they have about the same amount of DNA. Even if you postulate that there are 10,000 kinds of DNA, each of which is unique, presumably all the cells have all of these kinds of DNA because they all got them from a common source.

This forces us to postulate some kind of amplification system peculiar to certain kinds of cells, in which, so to speak, many roads lead to Rome. Ra-

diation injury to many biochemical roads in certain kinds of cells might lead convergently to damage of certain kinds of vital molecules. What this common denominator is in radiosensitive cells is really the core of what we are looking for.

CARTER: The amplification factor must even go outside of the cell, because Dr. Jones has shown very nicely on many occasions that there is a distant effect; that something is transported from the area of radiation to affect cells in another site.

KAMEN: Would this be the explanation for why you don't find effects immediately in a given organ that you take out of the carcass after whole-body irradiation? Maybe something is being affected that is sending out that hormone and it has to wait.

KAPLAN: I think some of the remote effects could be due conceivably to release of adrenocortical steroids that do have an inhibitory effect.

PATT: I think we are getting a little far afield from Dr. Pollard's hypothesis.

MAZIA: It seems to me that one of the things we should not overlook -- and this applies to Dr. Pollard's calculations -- is that the molecular units we are speaking about are clustered structurally with other units, and the clustering is rather important. In the system that Dr. DuBois discussed, we are dealing with mitochondria. Suppose, instead of hitting the big molecules that are clustered, you hit the "holes" between them. The "hole" might have a diameter 0.01 that of the enzyme molecule. What happens to the enzyme?

POLLARD: That would be a pretty big hole.

MAZIA: What would happen? Say that the mitochondrion is the target, and we are, at a given dose level, bombarding the structural cement without damaging directly any of the enzymes. We just blow up the mitochondrion. Isn't this an approach to the amplification Dr. Pollard was speaking about.

POLLARD: Well, I think it goes back to this question of the charge running around in the molecule. It might run around the whole structure until it came to a particularly sensitive spot, and if that particularly sensitive spot is an enzyme that has very few representatives, then you will have scored a hit pretty easily.

PLATZMAN: I don't think that this running around should be thought of in terms of such a structure, and I doubt that you intended it that way.

POLLARD: The point is that if it hits a critical enzyme, it does not have to look around. If it just hits that, it will produce a considerable effect. It is the enzymes that are not critical that are the ones that matter. In fact, if you suppose that of these 900,000 enzymes many are duplicated and many in a sense, are not essential, then obviously any inactivation of those that have a multiplicity isn't important. Dr. Mazia's point is that if you overlook the ones that are critical, you will probably find peculiar radiation effects.

MAZIA: The idea is to look upon the mitochondria as an integrated system. If you knock out one or a few enzyme units you put the whole structure out of business. Or we can turn to another structure effect. Irradiation can cause rearrangement of parts in a chromosome without at all affecting the qualitative character of the DNA. We know that such a rearrangement will have a very

significant biological effect. Or we can go to the cell surface and think of what a few "holes" in the surface would mean for the function of the total cell. I am thinking here of some of the things that Dr. Curtis has done on the possibility of conduction from cell to cell. A few holes in the surface of a small cell might mean the end of that cell -- would you agree, Dr. Curtis.

CURTIS: Yes, that is true, but you have to think of repair processes. You know that very large molecules can pass across cells. If you like, you can say that they punch holes in the cell membrane that are repaired immediately. It may be that they punch different kinds of holes than you are thinking about.

MAZIA: I am not thinking of holes literally. I am thinking still of the amplification problem. So much of our discussion of radiation effects deals with them in terms of direct action of functional units such as enzymes. What I am proposing for consideration are effects that alter the conditions of action of the enzymes or other molecular units. Three of these have been brought up. One is the case where a group of enzymes is clustered tightly in a functional unit; if you knock out one, all others become useless. The second is where the radiation action is not on the molecules at all but on the links -- or cement, if you will -- holding them together in a functional cluster. The third is where the environment is altered in a minor way which affects the activity of the large molecules in a major way. An example of this would be a small change in the surface of a cell or a nucleus or a mitochondrion, which, in turn, would permit a small flux of some ion (H^+ for instance) to which many of the molecules would be sensitive. All of these effects are possible and all would be examples of amplification as long as we choose to relate the radiation exposure to its large-scale physiologic consequences.

KAPLAN: Especially the interrelationships of some of these large structures in certain cells and not in others. That is a nonselective, irreversible damage to one molecule -- one or a very few molecules -- of such a large structure could lead to inactivation biologically of the entire structure.

That is a better thesis, I think, than the one that you started on tentatively, which would force you to the notion that the radiation somehow could tell which is the special, unique enzyme in the radiosensitive cells and pick it out selectively, whereas it couldn't do this in any other cell. It seems more logical to postulate that the radiation can nonselectively hit any kind of large molecule, but that in certain kinds of cells there is a much more vulnerable interrelationship between molecular structures.

POLLARD: One of the things that has always affected me is the fact that chromosome breaks are so easy to produce. When one thinks of a chromosome, it almost certainly consists of 50 or a 100 nucleic acid molecules lined up alongside one another. It is often said, for example, that one α-particle passing through this will cause a break. One α-particle may put a lot of energy in there but actually does not put a 1:1 relationship in each of the molecules it goes through. Perhaps an α-particle will, but a deuteron or a slow electron won't.

MAZIA: The situation with regard to chromosome breaks may not be as difficult as it seems. I think that we are dealing with the dissociation of fairly large nucleoprotein particles that are held together only by ionic bonds. The structural stability of the chromosome may be a little deceptive in the sense that we usually encounter the chromosome under conditions where these bonds are most effective. We have some evidence to support this.

ZIRKLE: Do you mean to say that you are forsaking the whole idea of a protein chain skeleton for the chromosome?

MAZIA: Yes, I am thinking of the protein continuum of which I was a supporter. For the time being, I have had to give it up in favor of a particulate structure.

POLLARD: We ought to get that in writing.

MAZIA: I can discuss it a little if you wish. The source of the trouble is the fact that when we looked for methods of taking chromosomes apart -- I mean literally trying to put them into solution -- it always seemed to be very difficult. We had to use methods that broke down the proteins, and therefore came to the notion of a continuous protein backbone. But we never had paid attention to the ionic environment of the chromosomes. When we finally did experiment with this variable -- a lot of the basic information was in the literature but not much attention was paid to it -- Bernstein and I, working with sperm cells, found that we could disperse the nucleus completely into a solution of desoxyribonucleoprotein particles. These were, in the case of sea urchin sperm, about 4000 $\mathring{A}$ long and 200 to 300 $\mathring{A}$ wide, judging from what we saw with the electron microscope.

The chromosomes dispersed easily enough once the conditions were met, but these were rather exacting.

First of all, in the material we work with, we have to introduce a chelating agent that will remove calcium and magnesium. After this, we have to bring the chromosomes to an ionic strength below that of 0.05M NaCl. If you just remove the Ca and Mg nothing happens. If you treat directly with distilled water, the chromosomes swell but do not come apart. But if you apply the two treatments in sequence, the chromosomes may be completely dissolved. I have studied this phenomenon cytologically on salivary gland chromosomes and grasshopper spermatocyte chromosomes. Bernstein and I made the chemical studies on the nuclei of sea urchin sperm. More likely than not, the exact requirements for dispersing chromosomes will vary from one kind of nucleus to another.

From this information on how a chromosome may be taken apart, let us try to organize a picture of how it is put together. Let us say that these nucleoprotein particles are the basic units. You might picture them as being held together by bridges of divalent ions. Once these were removed, the particles could separate. But they would not necessarily separate unless they repelled each other sufficiently. This may account for the fact that even after removing the Ca and Mg, it is necessary to go below a certain ionic strength. Electrolytes would tend to swamp out the repulsions; removing the electrolyte would enable the charged particles to repel each other effectively and go into solution.

Now what I would like to ask the panel of physicists here, is how one can picture a primary radiation event as acting on this kind of ionic bonding. I am suggesting that radiation-induced breakage of chromosomes is the result of an action that permits these ionically bonded particles to come apart and not the result of damage to the molecules within the particles.

PLATZMAN: Do you mean how can a single ionization in one of the particles snap the calcium?

MAZIA: The question is what the radiation can do to the situation between the particles.

PLATZMAN: I would tend to bet at the moment on heat. That is, the energy of the initial ionization or excitation would be dissipated as heat inside one of these units, a small enough unit, so that breakage would occur at the weakest point.

MAZIA: I should think so, since the procedure I have described will alone split the chromosomes.

CHARGAFF: What would put these particles together? Each of your particles is much smaller and thinner than the chromosome.

MAZIA: I am postulating these ionic bridges plus the fact that under the conditions of ionic strength, which we believe to exist in the cell, the nucleoprotein is insoluble -- the particles interact and do not separate readily.

CHARGAFF: The nucleic acid has many primary phosphoric acid dissociations, and I think those probably would be taken care of in each of the protein particles. There would not be much ionic force left over -- I don't know how many of these particles go to form a chromosome and to keep it together before it has to split. You see you must have a factor that assures regularity.

MAZIA: So far as their holding together is concerned, it is a fact that in vitro, at the ionic strength that we consider to be reasonable for the cell, the material is insoluble.

CHARGAFF: Oh yes, there is no question about that.

PLATZMAN: Would you complete the picture as to how the small chromosome builds up?

MAZIA: There is evidence that these two variables are involved. There is no evidence as to where the two kinds of interactions are located.

PLATZMAN: If we don't speculate, we probably never will get anywhere. Do you have a lot of those going up and down and also sideways? Is that what you have in mind?

MAZIA: Yes. Let's try a picture in which the divalent ions serve as end-to-end bridges for the particles, for the sake of speculation.

POLLARD: van der Waals' forces will hold things of that size together to some extent.

PLATZMAN: I still think that heat is the thing that finds our weakest bond.

POLLARD: There is not enough heat in these things.

PLATZMAN: I am not thinking of general heating.

I have in mind a high vibrational excitation of the molecule (probably produced via internal conversion of electronic energy), -- you called it prepartitioned heat last year, I believe. The physical question devolves on whether enough of this energy can find the critical bond before dissipation outside of the molecule has proceeded too far.

POLLARD: I prefer my moving charge. Still it does not matter. It is

the same thing. If the calcium is the weakest point then the radiation will certainly find the calcium and that could lead to a break.

PLATZMAN: It is really not the same thing. However, at the moment I cannot think of a clear criterion for distinguishing between the two in the case before us.

MAZIA: The reason why I am bringing up this whole question is because I have not seen any discussion of the primary radiochemical action on bonds of the type we have been discussing. They might be very important in the cell.

PLATZMAN: In the new book edited by Dr. Hollaender, you will find a few sentences that Professor Franck and I wrote on just this point (12).

POTTER: Can you centrifuge out those little elementary particles?

MAZIA: Do you mean in solution?

POTTER: Yes.

MAZIA: Yes. I am told that it will be a little difficult to study them in more detail by ultracentrifugation because they are not soluble in salt solutions.

SPIEGELMAN: Suppose you have done this. How do you bring the ionic strength up again and what happens to your material?

MAZIA: Once in solution, it can be precipitated by raising the ionic strength with NaCl and can be redissolved by removing the NaCl. If no Ca is around, precipitation and solution seem to be a matter of the conditions for interaction and repulsion of the particles, which is determined by the ionic strength.

PLATZMAN: Can ultraviolet cause the same type of chromosome breakage?

HOLLAENDER: To a slight degree only.

PLATZMAN: Disregarding frequency?

HOLLAENDER: There is much less chromosome breakage.

PLATZMAN: Do you still observe the same type of break?

HOLLAENDER: Yes.

PLATZMAN: Then it seems to me that might be supportive of heat rather than of ionization. Not that it makes any difference at all at this stage.

MAZIA: Isn't it still considered to be a fact, Dr. Hollaender, that there is a difference between ultraviolet radiation and ionizing radiation with respect to the chances of producing a break versus the chances of producing a change within the gene?

PLATZMAN: I would be impressed with that evidence only if someone really did experiments in the vacuum ultraviolet. In fact, however, most experi-

ments have used 2537 $\overset{\circ}{A}$. This is not enough evidence.

HOLLAENDER: Dr. Mazia is correct that there is a different type of effect produced by ultraviolet and X-rays in regard to chromosomes. You have a larger number of chromosome breaks produced by X-rays and a greater predominance of gene effects with ultraviolet. The difficulty with ultraviolet, of course, is the question of penetration and how much the cytoplasmic material protects the nucleus from radiation damage. Of course this is no problem with 250-kv. X-rays.

ALLEN: This would seem to suggest that the effect of ionizing radiation on this calcium bond might be via an indirect effect, whereas the ultraviolet having a direct effect, is manifested more inside the organic part.

There were some old experiments in which it was shown that the mobility of colloidal particles of gold and graphite, dispersed in water, was affected by extremely small doses of X radiation.

ZIRKLE: These particles are a little long to be genes, are they not?

MAZIA: They are about the length of one DNA molecule.

This brings in some interesting work. In recent years, there have been a lot of developments in the study of suballeles or sub-genes, members of a genetic locus that have related effects but that can function independently. I understand -- and I could be wrong -- that Stadler has done some work showing that while these genetic subunits can change independently of their fellows in spontaneous situations, when you irradiate them, they all are affected together; there is an all-or-nothing result. If this is so, it might be concluded that radiation affects the grosser discontinuities in the genetic system. Such a result would make sense if we supposed that the groups were held together to form a chromosome by bonds that were different from and more radiosensitive than the bonds holding together the subunits within each group.

KAPLAN: Do these large particles exist in the resting cell?

MAZIA: We think so. At least we can extract particles having similar properties from resting cell nuclei and from condensed nuclei such as we find in sea urchin sperm.

POTTER: How do you see them?

MAZIA: With the electron microscope.

BENNETT: Is there any way that you can determine if radiation causes breaks in these, or aren't the breaks of that nature?

MAZIA: Dr. Bernstein, who is now working with Kauffman at Cold Spring Harbor, has been studying X-ray effects on solutions of these particles. I know that he finds them very sensitive but I do not know the nature of the effects he observes. I'm pretty sure that the nucleoprotein as a whole is sensitive at doses that are biologically interesting. The question I am raising is whether breaks might not occur between relatively large subunits of chromosome structure, rather than within them.

CHARGAFF: But you could, of course, have them stacked so that the units are not next to each other but in different positions. You really have to

make a lot of assumptions.

MAZIA: Oh, yes. All I am proposing -- and it seems to be reasonable -- is that there is a kind of discontinuity in the structure of the chromosome that will make the chromosome sensitive to variables -- such as the ionic environment -- which we ordinarily would not think of when we consider the effects of irradiation of nucleic acids or proteins.

CHARGAFF: Except that this would make it even more mysterious how a chromosome can split so regularly.

PLATZMAN: Why do you say that?

CHARGAFF: If you assume a simple ionic binding between blocks it is very hard to visualize it mechanically.

PLATZMAN: Wouldn't you say that about any picture whatsoever?

CHARGAFF: That is why I hesitate to put mechanisms on the blackboard. That is where you have to be very careful.

PLATZMAN: Crystals reproduce each other. They grow.

CHARGAFF: Crystals grow in a saturated solution, it is true.

MAZIA: Dr. Chargaff, we are not putting down a mechanism to account for how the chromosomes do anything positive. We are considering how they can be broken apart, which is a little easier problem.

SPIEGELMAN: How about the chemistry? Is there evidence for the regular spacing of divalent ions.

CHARGAFF: I don't know. You will have to ask Dr. Mazia.

MAZIA: There is a lot of evidence showing that the concentration of divalent ions in the nucleus is high. Some workers, such as Allgén, have stressed the finding that it is very difficult to get rid of these in the purification of DNA. Dr. Chargaff may have had some experience with this.

CHARGAFF: Magnesium is really everywhere. It is a good assumptior that it is also in these proteins. Some divalent metals are easy to find anywhere.

ALLEN: What about depolymerization of these acids on irradiation? Could that also be connected with the calcium bridge?

MAZIA: I don't know. For the moment, I am trying to focus attention on the situation between the macromolecular units; on whether the chromosome is discontinuous.

CURTIS: I think it would also be worth mentioning that Dr. Steffensen at Brookhaven National Laboratory has been growing plants in media which are deficient in calcium, magnesium, and other ions. He finds that there are many more spontaneous chromosome breaks than normal in plants grown on calcium-deficient media.

MAZIA: He has obtained a 19-fold increase in spontaneous chromosome breaks by raising plants on calcium-deficient media. The Ca-deficiency cannot be too severe, or the plants will not grow at all. Holding the Ca-supply at a marginal level, he still observes the 19-fold effect.

PLATZMAN: Is it spontaneous?

MAZIA: Yes. I don't know whether there are any data on radiation effects yet.

CURTIS: Not yet.

POLLARD: If you measure the dependence of this effect, then you know what the bond is. If you can show that this is spontaneous, at a rate which is not very high, you have an ideal bond. It overlaps.

ZIRKLE: Chromosome breaks by high-energy radiation are highly variable among different kinds of cells. In newt cells we tried like the dickens to break chromosomes, but got scarcely any breaks. This failure may have been because with our methods we could see only relatively gross changes, but at any rate, it is very suggestive. Or, maybe our calcium relations in the cultures did not favor breaks.

MAGEE: Does this thing include carboxylate?

MAZIA: I don't know. Dr. Chargaff points out that we have phosphate groups.

CHARGAFF: I don't know. Of course, you can disperse nucleoproteins without a chelating agent. The first modern preparations of DNA were made by extracting thymus with distilled water. This way you got a very nice solution of material. If the solution is brought to 0.15 molar -- physiological saline -- and precipitated, the particles can be redissolved more easily in medium strength sodium chloride, but you are not sure under these conditions that whatever divalent metal was there will be left.

MAZIA: Curt Stern revived the method of isolating nucleoprotein at low ionic strength a few years ago, and we were following up his work. In the case of sea urchin sperm, we could not put the nucleoproteins into solution by washing the nuclei in distilled water. They swelled tremendously, but nothing came out. We noted that he had used arsenate to inhibit desoxyribonuclease. We tried citrate, and found that after washing with citrate, we could dissolve the nucleoprotein in distilled water. In our experiment, the citrate was not functioning as a desoxyribonuclease-inhibitor at all, but as an agent complexing divalent ions. The chelation step was necessary in the case of the sea urchin and was definitely necessary when we tried to dissolve the formed, visible chromosomes in the salivary glands of Drosophila and in the cells of the grasshopper testis.

We have not worked with calf thymus. It could well be that there are cases where the divalent ion bridges are less important, and that the repulsions introduced by lowering the ionic strength would be adequate not only to swell the chromosomes but to take them apart.

CHARGAFF: In salt solution, you can certainly do without the chelating agent, for instance, in the Pollister-Mirsky procedure.

MAZIA: Is there a possibility that preliminary washing in physiological salt solution is enough to remove the divalent ions by straightforward ion exchange?

SPIEGELMAN: One experiment would be extremely interesting here. That is to take purified transforming principle, put it through your procedure, and bring it back again to its precipitated form and then see if it is still active.

CHARGAFF: The transforming principle does not have the protein in it.

POLLARD: Suppose you did not purify it and you used it just as nucleoprotein?

CHARGAFF: With very few exceptions, you cannot get nucleoproteins from bacteria. There is only one described in the literature. You cannot get a real nucleoprotein from pneumococcus. At least no one has been able to do so. No one has isolated nucleoprotein.

MAZIA: Has anyone tried working deliberately at low ionic strength?

CHARGAFF: I think so but cannot be sure. We have tried other materials, not thinking of the transforming principles, to prepare nucleoprotein and so far, have only succeeded in the case of tubercle bacilli.

KAPLAN: It is interesting the calcium apparently has an important effect on mutual adhesiveness of cells. Coman showed some time back, that tumor cells can be separated from one another with relative ease and that their intercellular matrix contains a decreased amount of calcium. If you treat normal tissues with agents that deplete calcium, you can show a similar decrease in adhesiveness of cells and an increased capacity of the cells to wander away from one another.

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CHANGES IN NUCLEIC ACID METABOLISM AS A RESULT OF RADIATION

Charles E. Carter

After discussing the effect of radiation on nucleic acids for over two days, there is little left to be said about this subject. Nevertheless, I will try to pick up a couple of loose threads. These remarks will be confined largely to three aspects of the problem: (1) the effect of radiation upon the metabolism of nucleic acids delineated through the incorporation of isotopic precursors into desoxyribose nucleic acid; (2) the effect of radiation upon the structure of nucleic acids; (3) the effect of radiation upon activity or specific function of the nucleic acid insofar as we know it.

In the first two of these categories our knowledge is approaching a satisfactory state of affairs. In the last category, that of function or specific biological activity, we cling to data that are provided by the cytogeneticists and to the studies of bacterial transforming activity of high molecular weight desoxyribose nucleic acid preparations. Obviously, this is the area in which knowledge of nucleic acid biochemistry is deficient.

There is one fact I think we might establish before we get started that imposes a limitation upon us. I think it can be stated this way: At this time I do not believe that any one of us is certain of the molecular identity of a nucleic acid. Is that right, Dr. Chargaff? Do you know that you have a molecular species when you work with a nucleic acid?

CHARGAFF: There you come to the definition of a macromolecule. What you really can't tell is what you mean by molecular weight of a protein. The same thing goes for nucleic acid. But you are correct. One can't be sure.

CARTER: This should not be an obstacle to research, rather, it should promote it. But nevertheless I think that we should admit that limitation.

First, we will discuss certain types of experiments that have employed low molecular weight isotopic precursors to provide a matrix for discussion of the problem of the metabolism of nucleic acid and how it is influenced by radiation.

I want to introduce this by a brief comment upon some of the assumptions that are made in this type of experimentation. Again, you can be so crit-

ical about these assumptions that you can prevent yourself from doing the experiment. That is not what is intended. It is merely that when we evaluate these experiments we must do so in a context of certain limitations that I think are obvious.

When we talk about a precursor entering a high molecular weight component, we envision many intermediate steps, but as a simplification we can say that the precursor enters a pool; i.e., a labeled precursor enters an unlabeled pool with which it mixes. The assumption is made that it mixes homogeneously in that pool and it is extracted from that pool into an essentially homogenous high molecular weight compound.

Certain modifying factors have to be introduced here. This pool may have subcompartments. The assumption is usually made that the precursor equilibrates rapidly through all of these compartments, so that an essentially homogeneous pool is established, and from this pool it is then extracted to make a high molecular weight nucleic acid.

Certain modifying factors can be introduced into the size of the pool and into the equilibration between the compartments that will radically modify the specific activity of this precursor. For instance, if the pool is small in size (the same amount of activity goes into a pool that contains much less of the precursor), then the specific activity is going to be higher.

Another modifying factor is that the precursor may enter this pool but it may not equilibrate in all the compartments.

The other problem that we meet is that the precursor may enter a pool that is much greater in size, then the specific activity of the precursor will drop.

How homogeneous is the composition of the high molecular weight nucleic acid? If labeled glucose is injected into an animal and the glycogen is isolated from liver at various times, there are areas of the glycogen molecules that are labeled heavily and some that contain little or no radioactivity. This modifies to a certain extent our interpretation of nucleic acids. We don't know with certainty, although here again we have some pretty good evidence that is beginning to accumulate, whether a precursor assimilated into a nucleic acid is distributed homogeneously throughout that high molecular weight material.

With respect to phosphorus, we have some good data from Heidelberger's laboratory that shows that there are subgroups within the nucleic acid molecule with different rates of incorporation of radiophosphorus.

COHN: Would it be fair to point out that in the case of glycogen you have essentially a spherically expanded molecule, whereas in the case of the nucleic acid you have a linearly expanded molecule.

CARTER: This is perhaps one of the first times that Dr. Cohn has gone on record as giving up the branched chain hypothesis of ribose nucleic acid structure.

CHARGAFF: Not necessarily. He has not gone that far.

CARTER: Well, we have introduced quite a few reservations into the interpretation of these data. I think it is apparent that the experiments that will tell us most about the influence of radiation upon the metabolism of labeled nu-

cleic acid precursors will be those that describe most completely the fate of the precursor after it is administered to the animal, not only in terms of nucleic acid metabolism but into other metabolic areas as well. And I think, in assessing nucleic acid metabolism with these techniques, those experiments that employ several different labeled precursors - precursors of the heterocyclic ring structure, e.g., ribose and phosphorus, will be of greatest value.

There is a large body of literature on the effect of radiation on incorporation of isotopic precursors into nucleic acids. I am not going to attempt to review it. I am going to introduce some of the evidence gathered by Harrington and Lavik[1] and use that as a starting point for this discussion. This evidence is based upon experiments in which rats were given 100 r of x irradiation. Soon after the exposure the labeled precursor was given. After an interval, I believe it was 24 hours in these cases, the thymus gland was removed. The desoxynucleic acid molecule was degraded, and the incorporation of activity into the various fragments was determined. They assumed that the precursor given to the animal was the precursor of the high molecular weight nucleic acid. And they expressed results in terms of a ratio of the molar specific activity of the isolated compound and the administered precursor.

All the factors that we discussed briefly, of course, have to be taken into consideration in the interpretation of the results. But some interesting data emerge from an experiment of this type.

TABLE I

EFFECT OF X IRRADIATION ON INCORPORATION OF RADIOACTIVE PRECURSORS INTO THYMUS DNA

Precursor	% Inhibition	Product
I. P.^{32}	53	DNA-P
Orotic - C^{14}	66	Cytosine
	75	Thymine
Adenine - C^{14}	-25	Adenine
	-10	Guanine
Formate - C^{14}	48	Guanine
	14	Adenine
	-23	Thymine

With P^{32} they found that following irradiation less radio-activity was incorporated into the phosphorus of the desoxynucleic molecule. P^{32} was inhibited 53 percent in the irradiated rats as compared with the normal controls. This type of experiment had been done previously by Hardin Jones and others.

Orotic acid, as a pyrimidine precursor, was inhibited to the extent of 66 percent into cytosine and 75 percent into thymine. Incorporation of adenine labeled in the 8 position was not inhibited. As a matter of fact, the specific activity in the irradiated sample was higher. So that Harrington and Lavik[1] expressed the adenine data as minus 25 percent inhibition into desoxynucleic acid adenine, and minus 10 percent inhibition into desoxynucleic acid guanine.

Formate is the precursor of the 2 and 8 position of purine and goes to the methyl group of thymine. Formate assimilation into desoxynucleic acid guanine was inhibited 48 percent; into desoxynucleic acid adenine it was inhibited 14 percent; and into thymine it was not depressed. Actually the specific

activity was higher - minus 23 percent.

Well now, you are faced with interpretation, and this poses some problems.

BENNETT: I think before anyone tries to interpret such an experiment, account has to be taken of the different rates at which these compounds will go through the pools that you are discussing. It is very important in such an experiment to know if the radioactive compound was present in the animal 2 hours, 24 hours, 4 days or whatever length of time.

CARTER: And of course this information must be evaluated in the light of the now well-established complexity of metabolic paths leading to nucleic acid anythesis. If we take phosphorus, for instance, although many of the intermediate steps between inorganic phosphorus and pentose phosphate may be written, we don't know the mechanism whereby phosphate enters the polynucleotide molecule. This uncertainty extends to the mechanism of pentose assimilation.

POTTER: You are absolutely right when you say that pathway is not known. We have experiments showing that carbon 1 labeled glucose eventually will get there, and it is hoped that some time in the next 10 years the pathway might be known, but you cannot extrapolate from the studies on enzyme processes of which many alternatives occur in the animal.

CARTER: In the case of the metabolic construction of the purine and pyrimidine bases, the work of Greenberg, Buchanan and Kornberg has placed us on sound ground. But to date, when we look at the studies of radiation effects on incorporation of low molecular weight precursors into nucleic acid, we are unable to say whether inhibition or acceleration of the formation of the purine and pyrimidine intermediates plays any role in radiation effects on nucleic acid metabolism. This is asking for a lot of data, but whoever undertakes to describe the effects of radiation upon nucleic acid metabolism must give us a more complete statement about the fate of the precursor molecules. In the case of radioactive formate, we need to know something about the effects of radiation upon the complex that forms enzymatically between the 1 carbon unit and the coenzyme form of tetrahydrofolic acid; we need to assess the experimental findings on nucleic acid metabolism in terms of formate assimilation into serine and protein as well as in terms of pool size and rates of excretion.

Examination of the metabolism of some of these intermediates takes on added importance in the light of some of the findings of Dr. Potter. He isolated the pyrophosphates of all of the 5' nucleotides that occur in ribonucleic acid and by this time probably has some that occur in the desoxynucleic acids. It is tempting to believe that these compounds are the immediate precursors of the nucleic acid. I say it is tempting because there is as yet no evidence that proves it.

POTTER: It is a matter of opinion, I guess. I think it is important that in the first paper with Hurlbert, the quantitative yield from the acid soluble pool to the acid insoluble pool is 80 percent or better. I will say that it is a matter of opinion whether you say they must have been the precursors or not. I don't think it adds anything to the discussion to go into it.

CARTER: There is certainly no evidence from these experiments that would argue against the 5' nucleotide being the precursor.

BENNETT: We have data on adenine with respect to how much of it goes into DNA, RNA and the soluble nucleotide pool that you are speaking about[2][3]. I might say that at the X-ray dosages we have given, these data compare with the P^{32} - phosphate data for similar tissues and dosages that have been obtained by Dr. Kelly at the University of California. It appears that in a tissue, let's say such as bone marrow, which might be a good one to discuss, the amount of adenine-4, 6-C^{14} or P^{32} - phosphate incorporated into DNA is about 5 percent of normal when a mouse is exposed to 1000 r x irradiation, whereas the change of amount of adenine incorporated into the RNA or the adenylic acid nucleotides is small -- a factor of 2 at the most.

CARTER: In other words, if you were to reconstruct your data you would say that adenine enters a pool of intermediates, and that this reaction is essentially uninfluenced by the irradiation?

BENNETT: A pool of intermediates as determined by nucleotides, such as 5 - adenylic acid, ADP, ATP, etc., and incorporation into RNA is uninterrupted while DNA incorporation is inhibited extensively. The amount of adenine removed from this pool and incorporated into RNA in some tissues is actually increased, but it is not changed to anywhere near the same degree that the amount incorporated into the DNA is changed.

DUBOIS: 1000 r is a pretty big dose.

BENNETT: We have not done experiments using any smaller dosages which may be why our data do not agree with that of Lavik and Harrington. Other reasons for the discrepancy might also be suggested.

DUBOIS: What time after irradiation?

BENNETT: We have done experiments at numerous times from 2 hours to 3 days afterwards. The time interval during which the adenine or phosphorus was in the mouse was 2 hours; in other words, almost as short a time as possible. The effect of irradiation changes with time; if one does a long-term experiment, one is integrating results in a fashion which, because of all these pool factors, one just does not know about. So it seemed advantageous to us to do the experiment over as short a time interval as possible and at as many intervals as feasible.

CARTER: You see the dilemma you are faced with in any interpretation; that is, either you must say that the desoxynucleic acid is metabolically heterogenous --

CHARGAFF: That has been shown by Bendich and others, at least to a certain extent.

CARTER: That is the point to be made here. There are types of molecules or regions in the molecules that turn over at fantastically different rates. Bendich has shown by a fractionation technic two clear desoxynucleic acid fractions that turn over at widely different rates --

CHARGAFF: The trouble is, his fractions are not analyzed.

SPIEGELMAN: How are these separated?

CARTER: They are separated by salt and alcohol fractionations. I

think Dr. Chargaff has gone much further than this in the characterization of the DNA molecules by extraction technics from the thymus gland. He has shown that, depending upon the salt concentration, one can isolate several desoxynucleic acids of high molecular weight of different composition.

CHARGAFF: We have fractionated practically everything that you can lay your hands on with the exception of phage where the fractionations are not very easy, but you can get up to 10 fractions.

CARTER: The chances are if you had the patience you would have more.

CHARGAFF: I think there can be 100,000.

CARTER: This is extremely important.

COHN: It looks like a continuous spectrum.

TOBIAS: How are these differences characterized?

CHARGAFF: By composition. There is a certain similarity which I don't want to go into. But you have a spectrum which begins on one end with a high guanine and cytosine content of the nucleic acid and goes to a very high adenine and thymine and very low guanine and cytosine, and from the distribution curves you can figure out that you must have a very large number of individuals.

CARTER: This actually lays very firm ground work for our interpretation of data bearing upon structure of nucleic acids. I am sure that most people in the room have never heard of Walter Jones, but he has contributed a classical statement which probably will survive much longer than some of his scientific contributions, and that is "a nucleic acid is a method of preparation."

COHN: He said that about 1920, didn't he?

CARTER: Way back. But this is being amplified continuously.

CHARGAFF: There are all sorts of proteins. This is a very general statement.

COHN: There is an inconsistency in speaking of proteins as plural substances and of RNA and DNA as single substances. This is often done rather loosely in conversation.

CARTER: At any rate, we have many factors which must be considered in the modification of the interpretation of these isotope incorporation data, and perhaps now what we really must do is not only have the characterization of these fractions but we must make all of our correlations in terms of biological activity. At this stage of the game we cannot do that for most of the nucleic acids. Where the activity brings about inheritable transformation in a few select bacteria this can be done. It is obviously the most fruitful area in which to study desoxynucleic acid, because the criterion of biological activity can be employed in the study of the high molecular weight components.

MAZIA: Except that it is a criterion of whether it is native or not, but it can't possibly be, the way it is set up now, a criterion of purity.

CARTER: Can it be by the same methods we use in the purification of the virus?

MAZIA: I think not, because the biological tests are set up so that you can measure one activity if it is present in the mixture.

CARTER: What I was thinking of was the interpretation in terms of one infection per particle.

COHN: You can say whether the characteristics you are looking for in the nucleic acid are present or absent, but you cannot say that there are not others present also. There could be a plurality of things in this preparation.

SPIEGELMAN: The point is, it has been done by Stocker. It is possible to design the experiment so that you know whether a single event is sufficient to get the transformation. This is the thing that you really want to know. Undoubtedly it is a mixed population.

COHN: It is not what the chemist wants to know.

CARTER: But he has to have that information. With these molecules, we must attempt to identify in some way structure with activity, and our interpretation of structures will have to be made within that framework. Here again, I think that Dr. Chargaff is going to provide us with the information we want.

Just reviewing briefly this business of the study of the metabolism of nucleic acids, in an attempt to equate activity with metabolism and in an attempt to evaluate radiation effects in terms of the assimilation of isotopic precursors, we can see many factors which invalidate the usual interpretations that have been made and many factors which compel us to insist upon more rigorous standards for future interpretations.

I would guess that the data that Hardin Jones has on the incorporation of P^{32} in nucleic acid will hold. I think it is solid, and the mere fact that you don't have pool sizes and a lot of things that we are beginning to insist upon here, does not detract from its use. I think that perhaps the greatest use to which these data have been put is in the elucidation of humoral factors, working at a distance from the irradiated site. Jones has used this approach to search for agents which can alter the rates of incorporation of phosphorus into the desoxynucleic molecule.

Would you like to modify that to any extent?

JONES: I did want to bring out one point which I think gives some validity to the general interpretation that is dropping back again on these biochemical data. Everything that Dr. Kelly and I tried to do in the way of relating P^{32} turnover of the DNA fraction with mitotic counts checks if we talk about times greater than 15 minutes after irradiation. There is good agreement between this index of depressed DNA turnover and the other DNA labeling methods, namely, C^{14}-glycine, C^{14}-formate, C^{14}-adenine, and so on. I still think that a more useful understanding of the disturbance of the mitotic process will come from knowing how the separate pathways and pools of these substances can be involved in depressing labeled DNA formation.

From the Harrington data you have just shown us, it is quite obvious that if the whole picture is one of consistency, there must be some metabolic

pools that have decreased and other pool sizes that have increased after irradiation.

CARTER: Or some nucleic acids that turn over more rapidly or some areas of the molecule that exchange rapidly. It is conceivable, for instance, that formate may exchange into the thymine moiety without net synthesis of the molecule.

I think the other problem that we must consider here is the effect of radiation upon structure and how structure may influence the expression of activity. In all of these discussions of structure we can only go so far as information of the kind that Chargaff and Cohn have provided is complete, and I don't believe that even they will admit that it is complete. But nevertheless it is upon our fundamental knowledge of structure that we must proceed in these analyses.

The effect of radiation upon the biological activity of nucleic acids may be due to a disruption of structure in the high molecular weight compound as well as to failure of its synthesis, and that disruption of structure is expressed in subsequent reactions. So that I don't believe that we should consider synthesis and turnover to the exclusion of structural factors.

PLATZMAN: You said, "may be due to disruption of the structure." What are the alternatives?

CARTER: Well, the structure of the enzymes and the structure of the co-factors in the system, etc., that are not related to the high molecular weight structure of the nucleic acid.

PLATZMAN: It must be some structure?

CARTER: Yes, I don't believe in ethers. I am trying to separate the events. The point I want to make is that this transforming principle, disrupted by ionizing radiation so that it looses its activity, will have the net expression of failure of synthesis, but that the immediate event may be that which takes place on this high molecular weight structure, completely independent of the enzymes, the coenzymes, and the low molecular weight substrates that go into the constitution of the newly synthesized compounds.

The effects of ionizing radiation upon structure that have been described fall into two categories. There is the fry-and-fall-back school of radiobiochemistry where many roentgens are dissipated into solutions containing solutes of biological interest. Then there are the studies done at relatively low absorption of energy in solution. I believe that both of these approaches give interesting and valuable information.

I don't believe that the fact that it takes a lot of energy to produce certain detectible changes in the structure of the nucleic acids argues against this being important or an event that is involved in radiobiological response. I think that in a suitable system with suitable amplification such as Dr. Pollard talks about, and with the use of suitable enhancement factors, such as Dr. Barron has demonstrated, it may take place. Scholes and Weiss[4] and Butler and Conway[5] have recently, at least, done most in this area and they have shown that there is actual disruption of the high molecular weight structures with high dosage of irradiation; even the heterocyclic rings break.

CHARGAFF: Is there evidence in this approach that the nucleic acids

are more sensitive than proteins or any other biological structure?

CARTER: No, there isn't, and, as a matter of fact, as Dr. Barron pointed out, there is fairly good evidence to indicate that they are less sensitive.

Inorganic phosphate can be formed from the organic phosphoryl esters, and ammonia can be liberated from these solutions. All of these events take place with high energy absorption. A lot of radiation is necessary to accomplish this.

Perhaps the more immediately interesting radiation effects in this area are related to changes brought about in the high molecular weight desoxyribonucleic acid by the various forms of radiation. This work probably begins with that which Dr. Hollaender did with ultraviolet radiation, and then was continued in cooperation with Taylor and Greenstein employing X radiation. The fundamental experiments are there. The effects are all described. They have provided a fertile area for re-investigation and for amplification. It was found that when the polymerized desoxyribonucleic acids were exposed to X radiation, they rapidly lost their properties of high viscosity, and that not only this primary event could be shown, but that if the irradiated nucleic acid were kept in solution after irradiation, viscosity tended to decrease continuously for a long period of time thereafter.

These effects have been re-investigated by Butler and Conway and Scholes and Weiss, and their investigations have brought forth several interesting additional findings. In the first place, Butler and his colleague found that this after-effect is largely absent when the nucleic acid is irradiated in nitrogen. That is, if the nucleic acid is irradiated in the absence of air, viscosity does not decrease significantly after the initial ionizing event. Scholes and Weiss, under slightly different conditions, found that irradiation in nitrogen produces about one-third of the after-effect of irradiation in oxygen, and this has modified to a considerable extent their interpretation, but nevertheless oxygen has a profound influence upon this drop in viscosity of the nucleic acid.

KAMEN: About a week ago, there was another article in Nature by Conway[6] more or less taking Scholes and Weiss to task and upholding Butler's and Conway's original notion.

CARTER: That is based on a difference in techniques. Butler, after irradiation, takes the nucleic acid out and dilutes it by a factor of 2. There is, he says, an immediate reorientation of molecules after dilution. Whereas Scholes and Weiss do their viscosity studies in the same solution, and the after-effect which they get in the presence of nitrogen, Butler and Conway say is merely a slow reorientation that takes place after the ionizing event. If they take the solution and dilute it out by a factor of 2 the event is no longer seen.

CHARGAFF: But the original viscosity decrease during the irradiation is not influenced by absence of oxygen.

CARTER: To a very small extent. The primary event is influenced to an extremely small extent.

PATT: Is oxygen added immediately after irradiation in all instances?

CARTER: They have done it immediately after.

PATT: In other words, the solutions are irradiated in nitrogen and then equilibrated with oxygen.

CARTER: The interpretation has usually been (and I think there is general agreement here) that the presence of oxygen during irradiation gives rise to an increased amount of oxidizing radicals, but there is, in addition to that, another factor which is apparent from Butler's data. If one takes a nucleic acid and puts it in an aqueous solution which has been irradiated with 10^4 r, there is a slow, slight drop in viscosity of the high molecular weight nucleic acid. If one takes a nucleic acid that has been irradiated in nitrogen and then adds to it water that was irradiated in oxygen, one finds a more extensive, more rapid drop in viscosity.

Finally, this can be compared with the considerably more extensive drop in viscosity of solutions of nucleic acid irradiated in oxygen.

The formulation that arises from these data is as follows:

Not only the oxidizing radicals formed in the aqueous solution by the absorption of radiation participate in this phenomena, but irradiation gives rise to oxidized groups upon the nucleic acid which can participate synergistically or additively with the radicals that are formed in solution. So that there are two concepts to be considered in the interpretation of these data:

1. The radicals produced in water by the irradiation.
2. The radicals that are formed on the high molecular weight nucleic acid.

ALLEN: Is this effect produced if you put the thing into a solution of peroxide?

CARTER: This effect is produced. This slow, small decrease in viscosity.

ALLEN: I wonder if peroxide out of a bottle will produce the same effect.

CARTER: Yes, but not nearly as efficiently.

BARRON: I was going to ask whether the authors have measured the amount of peroxide formed in water.

CARTER: Yes, they have.

BARRON: And this effect is not equal to the amount of hydrogen peroxide from the bottle?

CARTER: No, it is not.

BARRON: That has been our experience also. The peroxide obtained from manufacturers is different from the peroxide obtained after irradiation.

CARTER: This is understandable, isn't it Dr. Allen?

ALLEN: It is not understandable if the peroxide is obtained from a good manufacturer.

BARRON: This was 99.5 percent peroxide obtained from Niagara Falls.

HOLLAENDER: Most of the commercial peroxide has a stablizing substance incorporated in the suspension.

BARRON: No, this is pure peroxide.

ALLEN: It is supposed to be inhibitor-free.

TOBIAS: Are these effects identical with X rays?

CARTER: They have used peroxide plus UV and very extensive degradation is produced. Whether it is identical, I don't know.

KAMEN: Radiation has the same effect.

CARTER: Yes, it does but is it identical?

KAMEN: It is identical with low radiation doses. With high radiation doses it is different.

CARTER: Well, the point that I want to bring out from this discussion is that apparently peroxide radicals are formed on the nucleic acid which actually participate synergistically in this phenomenon.

POLLARD: Has anybody ever tried dry nucleic acid?

CARTER: Yes, it has been done.

POLLARD: What happens?

HOLLAENDER: You have to go much higher.

CARTER: I think, too, that we must include the factor of protein in the high molecular weight structure. I don't know of any evidence which would indicate that nucleic acids exist as free nucleic acids. They are polyelectrolytes, and undoubtedly they exist in combination with protein. Is that all right with you, Dr. Chargaff?

CHARGAFF: Sure.

CARTER: So that actually when we study effects of radiation upon these high molecular weight compounds, the studies should be conducted on a nucleoprotein if we want data applicable to cell biology. This problem has been studied by Anderson in Hollaender's laboratory and he finds that there is much greater sensitivity as measured in terms of viscosity drop when a nucleoprotein is irradiated compared with a nucleic acid. I believe this is because the factors of high molecular weight orientation are probably preserved more closely to the native state in these nucleoproteins, and that actually you have an increased degree of sensitivity in your measurements simply because of high molecular weight orientation.

There may be another interpretation. Nevertheless, there is now adequate evidence that doses as low as 25 r will produce extensive depolymerization or at least extensive changes in terms of viscosity alteration in the mole-

cule.

Scholes and Weiss[7] contend that ionizing radiation attacks the pentose moiety, or in the case of the desoxynucleic acid, the desoxypentose moiety.

They argue from analogy of the irradiation of the low molecular weight organic phosphoryl esters. Ethyl phosphate irradiated with about 9×10^4 r can be transformed into acetylphosphate.

Glycerophosphate irradiated at about the same level also undergoes an oxidative type reaction, which they formulate as an attack by the perhydroxyl radical which converts the secondary hydroxyl to a ketone. The net effect of converting the hydroxyl to a ketone is to labilize the phosphate.

They argue that the same sequence of events will take place in the desoxypentose moiety. That the attack of the oxidative radical will be on the lactone, with a rupture of the furanose ring and with a labilization of adjacent phosphate. Consequently the chain will rupture, and the high molecular weight characteristics of the molecule will disappear.

Most people argue against this interpretation because of the inability to find traces of inorganic phosphate or other degraded components and the fact that changes in viscosity can take place without finding these components.

Concomitant changes in molecular weight have been studied by other criteria. While viscosity changes may be extensive, fundamental di-ester linkages have been unaffected and sedimentation behavior may be unchanged. What has been affected is the high molecular weight orientation that is based upon association or aggregation or weak bonding of some nature that cannot be identified with fundamental particle size.(5)

However, Scholes and Weiss[7] find that if they take a nucleic acid and do a very mild acid degradation, they get virtually no phosphate. From the irradiated nucleic acid they get 15 times the amount of inorganic phosphate.

CHARGAFF: I have had the feeling that there is something wrong with these experiments.

PLATZMAN: Why would they be so hard to repeat?

BARRON: We have attempted to repeat them. We have never found phosphoric acid or ammonia.

CARTER: Have you ever tried the acid labile phosphate experiment?

BARRON: No. We irradiated nucleic acid with 50,000 r, and we could not find either ammonia or phosphate.

CARTER: But nevertheless we have to keep these experiments of Butler in mind, that something has taken place on the nucleic acid molecule which makes it more susceptible to attack by peroxide and the oxidizing radicals, and somehow or other we have to come up with an explanation, not to satisfy us or to make us happy but so we can do experiments.

ALLEN: Do I understand that the acid labile phosphate is increased by radiation?

CARTER: Yes.

ALLEN: Other people say that free phosphate is not increased by radiation.

CARTER: That is right.

ALLEN: Then what is the contradiction?

CARTER: I don't think there is a contradiction on that point because Dr. Barron has not looked for acid labile phosphate but Weiss does say that he gets inorganic phosphate as well during this operation. On that point there is disagreement.

COHN: Do you mind if I raise a point that I wanted to raise about 20 minutes ago? That is, a certain way of looking at phosphorus or ribose. Phosphorus is a more important constituent of the macromolecule than purine and pyrimidine because it is a double link in the chain. There has been a tendency by some to look on these as less important than adenine or even of formate experiments. This may not be justified in terms of the macromolecules.

ALLEN: Could you go over again the question of the molecular weight of this irradiated material? The viscosity is greatly decreased in solution, I understand, but you say that other criteria of the molecular weight indicate no decrease. What are those other criteria?

CARTER: Sedimentation.

MAZIA: Isn't it true that when you have molecules of such high asymmetry, the sedimentation depends only on the width not on the length?

CARTER: What it depends on is orientation in the fields just like the problems of anomalous viscosity.

MAGEE: You are talking about sedimentation velocity?

CARTER: Yes.

MAGEE: Don't you make sedimentation equilibrium measurements?

CARTER: You can but I don't know that anybody has good data on nucleic acid, and that would be the information that would give you the important data here.

There are just one or two points that I think may be added here, and to be fashionable we have to draw the Watson-Crick model. The Watson-Crick model is based upon no new evidence. It encompasses a lot of analytical data, and I think the work that Chargaff did in determining the composition of nucleic acid is among the most important features of these data.

The DNA molecule is looked upon, based upon the data of X-ray diffraction crystallography, as composed of 2 intertwined helices developed about the same axis, and I believe it draws out something like this: the length of turn is about 34 $\mathring{A}$. The feature that is somewhat new compared to the Pauling diagrams is that the phosphorus groups are on the outside. Incidentally, they say that a structure like this cannot be described for ribose nucleic acid be-

cause of van der Waals' forces.

CHARGAFF: Another one, not dissimilar, can be described. Not this particular one. Because of the pitch of the helices, it is not possible to accomodate 2' hydroxyls.

CARTER: The novel feature here is that it utilizes very nicely Dr. Chargaff's data which showed that there is always a ratio of adenine and thymine approaching 1, and similarly guanine and cytosine.

This helical structure is held together by the phospho-di-ester linkage and also by hydrogen bonding between purines and pyrimidines, adenine and thymine, guanine and cytosine, hydrogen bonding that develops about the 1 and 6 positions in the purine and pyrimidine rings. The hydrogen bonding gives orientation, specificity, and rigidity to this structure. The sequences develop in opposite directions in the 2 chains.

The double helical model provides a mechanism for replication or reduplication of the structure.

It is an apparently satisfactory statement of structure, and a formulation upon which experiments can be based. It is a phenomenon that is almost unparalleled in modern scientific publication. Watson and Crick formulated this structure and within a few months 8 or 10 people have rushed into the literature to prove that they are right. That does not happen very often.

CHARGAFF: No, people rushed in 5 years before they published it to prove they were right. Actually, Wilkins, Franklin and Gosling had excellent X-ray data which were published.

CARTER: It was their data that Watson and Crick used.

CHARGAFF: I think you might say that Watson and Crick were confirmed 5 years before they published their hypothesis.

POTTER: Could you comment on the Watson-Crick structure from the standpoint of how the experiment fits biological specificity?

CARTER: There is specific orientation.

POTTER: It is all on the inside, isn't it?

CARTER: The chain comes out of the helix and there is an opportunity for replication on each strand.

SPIEGELMAN: There is one piece of information which was given at the National Academy meeting. The titration curves do not completely fit the Watson-Crick model. There are too many free titratable phosphate groups. And the suggestion was made by Dr. Schachman, that there are breaks along the chain so that not every phosphate group is di-esterified along the chain. Stent has done some ingenious experiments, which would really take too long to describe, in an attempt to see if the breaks are distributed at random along the molecule. The data he had just before the Academy meeting were still preliminary, but they fit beautifully with the idea that the molecule is not continuous.

CARTER: Yes, in their formulation they talk about chains having 2100 units or something higher than that. This business of end groups is extremely

important in the formulation of nucleic acid structure and action, and the data we have are not very good on the number of end groups in one of these chains.

TOBIAS: According to Stent and Schachman's idea, only one arm of the chain is broken in irregular places and not as far as 2100 units but maybe 25 units.

Stent thinks that half of the nucleic acid chain might be broken in various places along the nucleic acid molecule. When the molecule is irradiated, ion pairs can cause other breaks along the chain, usually in a single arm only. If a radiation-induced break occurs in a place where there is only a single chain intact, the molecule is completely broken.

When phage are irradiated at various temperatures, it turns out that the radiosensitivity of the phage (and I think Dr. Pollard has done similar experiments and similar experiments were done on cells by Wood as well) shows a very striking increase with temperature. Sensitivity may go up as much as 5 times within a few degrees centigrade.

The interpretation there is that some of these hydrogen bonds, statistically speaking, break with increase in temperature, and Stent thinks that if maybe 20 of them are broken simultaneously, then the defect is as effective as a defect caused exactly opposite the already open places. This is really quite an intriguing idea to me at least, though I must admit that the explanation of thermal increase of radiosensitivity does not require the Watson-Crick model.

CARTER: Actually I think that you have to implicate some kind of a staggered structure to get these long chains. Do you feel that way?

CHARGAFF: Yes, unfortunately there are no calculations available. I think that you want to calculate how long a chain can exist without snapping. There are limits to a continuous polymer chain of this type, but I can think of no criteria.

PLATZMAN: If you envisage that, the position has already changed with time. It is a dynamic, changing thing.

CHARGAFF: The desoxynucleic acid is really supposed to stay where it is, in the resting cell. It does not turn over at all.

PLATZMAN: Certainly these fluctuations in hydrogen bonds are dynamic.

CHARGAFF: I think a bridge builder would understand more about the effect of these hydrogen bonds on 2 parallel chains than I do. But I have a feeling that you have a mutual strengthening of 2 different types; (1) a covalent type of linkage and (2) a secondary valence one. It is possibly true that if one of the types breaks in several places, you get automatic snapping of the other type. I think if you break a few covalent bonds, many of the hydrogen bonds will be disrupted.

CARTER: Incidentally, there is some supporting data from infrared spectroscopy. Frick found in the 3.1 - 3.2 micron region a shoulder which corresponds with the nitrogen-hydrogen bonds. Upon titrating his nucleic acid he found that when viscosity fell, the shoulder disappeared.

CHARGAFF: I think the Watson-Crick structure describes probably

very well what goes on in the stretched fibers in the crystalline structure which has been subjected to X-ray. I am not sure that one should not distinguish between the two parts of their hypothesis; namely, the one that describes the crystallography of what can be measured and the one that postulates this biological model, because I don't know that there is any evidence that nucleic acid looks like that if it has not been subjected to this stretching. I don't know of any evidence except our chemical data, and they could be interpreted differently.

POLLARD: Yes, Wilkins has evidence of that.

CHARGAFF: That is poor evidence. I think that you do not see the same type of details that you see in the stretched structure.

POLLARD: Still, it is the only evidence and it is positive.

CHARGAFF: Yes, but he has too few details to say they are compatible with such a structure, and I don't think he shows it.

POLLARD: There is nothing incompatible.

CHARGAFF: No, but there is quite a distance between something not being incompatible and being true. I mean there is a long way to go.

CARTER: At any rate, this has served as an extremely useful point of departure for many discussions, and probably about it a considerable amount of solid investigation will be built.

POLLARD: It has completely licked the problem of biological cell duplication.

CHARGAFF: I don't share your enthusiasm. There is a need for a peculiar enzyme which enters these huge coils in such a way that you can get duplication. As a matter of fact, it has really taken away some of the fun, because you expose surfaces that are extremely identical. The polyribose backbone is toward the outside, and you have to go inside to build one chain and the other.

POLLARD: Start with the ends and work down.

SPIEGELMAN: That is the way they imagine it; that is, the thing is really unwound.

CHARGAFF: We call the enzyme "unscrewase."

SPIEGELMAN: They have what can be considered as not a completely implausible picture of the function. But I think if Stent's reasoning is correct it is going to simplify matters tremendously because of the possibility of not having to do the whole thing at once but in sections if these breaks are real.

CHARGAFF: Except I would like to know what these sections really mean. Is each section a gene or what?

PLATZMAN: Has anyone ever made completely deuterated proteins or nucleic acids?

CARTER: I don't think so.

PLATZMAN: Has anyone tried it?

JONES: In the biological system? I don't think that metabolic functions would operate sufficiently with such a change in reaction properties induced by hydrogen replacement.

SPIEGELMAN: Pure deuterium? I thought they stopped when you get above a certain level.

PLATZMAN: The function of zippering would be rather different with deuterium bonds, i.e., the role of the dynamic distribution of breakages.

CHARGAFF: I don't know anything about what a deuterium bond looks like as compared with a hydrogen bond.

PLATZMAN: It would be much weakened. It would have a completely different kind of temperature effect.

CHARGAFF: Then you really could not expect to grow these structures.

PLATZMAN: Well, it would be a question whether you could or not. That in itself would be important.

CHARGAFF: You would have to know whether you can grow bacteria in deuterium.

MAZIA: The Watson-Crick formulation regarding replication is experimentally testable independent of any concept of structural details. Stent is doing it with phage and with chromosomes.

KAMEN: What are these experiments?

MAZIA: The principle of them is that if in the system replication works, then the material of the parent molecule is distributed between the daughter molecules. The experiments are essentially to determine whether the parent molecule as such survives and the daughter molecules consist entirely of new matter or whether the 2 daughter molecules consist of half parental matter and half new matter.

CURTIS: It has to be half?

MAZIA: Yes.

POLLARD: It has to be done twice. You need both generations to be sure.

SPIEGELMAN: That has to be checked. It really disagrees with a lot of other experiments.

POLLARD: Luria and Human also showed that multiplication by splitting takes place. We also know that it is nucleic acid synthesis which takes place first. There is no intervention of proteins first.

CARTER: What is the evidence for these things?

POLLARD: Simply that no new protein whatsoever develops until so

late in the cycle.

SPIEGELMAN: It has been shown that there is no new protein synthesis for a period during which what is referred to as the vegetative DNA pool is formed. There is a considerable amount of DNA synthesis prior to the appearance of any phage specific protein.

CARTER: This is high molecular weight DNA?

SPIEGELMAN: This is high molecular weight DNA.

POLLARD: Also Luria and Latarjet's radiation experiments showed something like this.

CARTER: That DNA synthesis proceeds the period of protein formation?

POLLARD: Yes.

POTTER: What you are saying is that DNA synthesis requires only DNA.

SPIEGELMAN: No, it does not mean that. All it means is that you do not make mature phage simultaneously with DNA.

MAZIA: Can we be sure that DNA produced during the first period is phage DNA? This can mean merely that the pieces are being made.

SPIEGELMAN: These pieces were big enough to participate in genetic exchange.

CARTER: How big are they?

SPIEGELMAN: I don't know, but they certainly cannot be at the nucleotide level.

CARTER: A problem arises because isn't it conceivable that low molecular weight units can be transferred in and out of the polynucleotide chains to give new sequences, and that actually you don't need to have a complete chain to get a specific structure to bring about a specific event.

KAMEN: Are you saying that you can start with one of these chains in the Watson-Crick model and peel a hunk off the outside?

CARTER: Right.

CHARGAFF: The model then requires that you put in the complementary piece on the other side. This requires a lot of ingenuity.

CARTER: The enzyme might do it.

Well, I should like just to put the lid on this thing so we can get out of here.

There are a couple of consequences of splitting a polynucleotide chain. The ratio of the end group to di-ester linkage is important in a variety of en-

zymatic reactions. What we need is evidence in this field. Of course, speculation is good too. Actual experiments that will show us what a nucleic acid does or what it influences are extremely important to any interpretations we make in this area.

One thing that I think we can be fairly sure of, is that a nucleotide sequence terminating in an end group can determine to a great extent the specificity of this group of substrates for several enzymes. We know that when this end group is removed from a low molecular weight desoxyribonucleic acid chain the low molecular weight chain may become a substrate for desoxyribonuclease. So the breaks in these sequences may have metabolic significance as well as structural significance. Evidence of this sort is badly needed.

Running through all of these discussions, of course, has been consideration of the transforming principle. I believe that we have exhausted most of the immediate possibilities in this discussion. But I think that one action of radiation upon nucleic acid metabolism has escaped discussion and I think that it may represent one of the most important actions. By this I refer to the work of Lwoff (8) on the induction of lysogeny.

This area of bacterial physiology and biochemistry is extremely complex and I cannot even attempt to make a short rational discussion of it. But the phenomena which Lwoff has studied opens up an area of great fundamental importance in biology, the phenomon of phage production in a strain of bacteria that has carried the phage, or the ability to produce the phage in a non-infective stage. Lysogeny then is described as the phenomenon of inheritable transmission of host-producing phage.

Actually this is a thread that has run through microbiology for many years. There are many early observations, one of the most interesting being that of DeJong (about 1900) in which it was found that spores of B. megatherium, heated to 100 degrees, were lysogenic. That is when the spores grew out, they produced a phage which caused lysis and death of the organism.

The modern counterpart of this experiment has been performed by Lwoff and his co-workers. Strains which are susceptible, that is, which are known to carry the genetic characteristics that will permit the development of phage, become lysogenic upon X irradiation or exposure to ultraviolet light. Desoxynucleic acid synthesis and phage synthesis increase so that X radiation actually is an agent that has re-orientated the metabolism of the nucleic acid. The net effect is actually to induce a burst of nucleic acid synthesis. A phenomenon of this nature, I believe, is just as important as any inhibition of nucleic acid metabolism that can be produced.

As a contribution to fundamental biology I think it is of infinitely greater importance. It strikes very close to some of the basic problems: the nature of virus, the production of neoplasia, and the relation that neoplasia may have to an abnormal particle metabolism of this nature. I think this is an area that should be investigated extensively by the radiobiologists.

Sol, you are working in this field. What importance do you place upon it?

SPIEGELMAN: Well, I think it is very important. I think, however, that it is not likely that radiation is going to tell us what it is all about.

CARTER: Radiation is an inducing mechanism. We have been talking

about the inhibition of nucleic acid synthesis in metabolism and the degradation of large molecules. Apparently radiation can also induce nucleic acid synthesis.

SPIEGELMAN: It, perhaps, might be worthwhile to describe briefly some extremely fascinating series of facts which have recently emerged concerning lysogeny. In the first place, it is important to recognize that one can have lysogenic and non-lysogenic varieties of the same strain. The distinction between them can be easily exhibited in many cases by induction with UV. The doses that induce are extremely low and of the order of 5 percent kill for normal cells. Exposing lysogenic cells to this radiation however, leads to virtually a 100 percent kill, as the result of the subsequent development of the virus.

If one exposes cells of a non-lysogenic strain to what is called temperate virus, the cells become infected apparently without any marked effects on their metabolism and without effect on viability. Once the infection with temperate viruses has taken place, one can subsequently demonstrate that every cell contains at least 1 virus particle. It is important to note however, that the virus is not present in a recognizable state before induction, since upon breaking open such cells, one cannot detect the presence of many infective agents.

These observations raise the question of where the virus is and in what form it exists. This stage of virus existence has been called the prophage stage. I cannot at this time, detail the experimental evidence in support of the conclusions to be mentioned, but I think it worthwhile to simply state them. One can say, with a large amount of certainty, that immediately subsequent to infection the virus does behave like a cytoplasmic particle and is transmitted in a random manner. However, in a few divisions it disappears from the cytoplasm and takes up a position on the chromosomes. It can be further demonstrated that the position taken up by a particular temperate virus is always the same since it can be localized by means of crossing experiments. Thus, if you cross a lysogenic strain with a non-lysogenic one, you will find in the progeny an association of the transmission of the prophage character with several closely linked markers controlling other normal metabolic processes.

In some instances, it has been possible to demonstrate that 2 alternative positions are possible, and in these it has been demonstrated that 1 cell can be infected simultaneously with 2 viruses in the prophage stage.

We have here indeed, a most amazing situation. One takes a self-duplicating unit, puts it into a cell where, instead of behaving like an independent entity in the cytoplasm, it incorporates itself into the genetic apparatus of the host. In this manner it is transmitted from 1 cell generation to the next. It will be noted further that this mechanism guarantees that every cell contains the infective agent.

One other feature of extreme interest is that these viral agents carry in not only the ability to produce more viruses but also other genetic characters. For example, if one has a lysogenic galactose positive cell and induces with UV so that mature virus particles are produced, one can then infect non-lysogenic galactose negative cells with these virus particles. One finds that along with establishing lysogenicity, one also transforms the galactose negative cells into ones capable of fermenting galactose, and the newly acquired character is permanently inherited.

A most dramatic case of this type of "Transduction phenomenon" has been exhibited in the last few years with respect to toxin-producing diphtheria

bacilli infected with a virus. Elimination of the virus, which can be done by a variety of methods, leads to production of a non-toxicogenic or non-pathogenic strain. A large amount of experimental data leads to the conclusion that there is a 1 to 1 correspondence between cells carrying the virus and those able to produce the toxin.

CHARGAFF: Who did this work?

SPIEGELMAN: It was first observed by Freeman in 1950. Groman in Seattle has done the most extensive work and recently, Foxdale and Pappenheimer in New York have contributed to the problem. The most recent paper by Groman (9) presents the most conclusive evidence on the question.

KAMEN: Is that in the Journal of Bacteriology?

SPIEGELMAN: Yes, most of this work has been published in the Journal of Bacteriology.

I think that most of us who have been close to this area in the last several years, have found it difficult to digest the amazing amount of fundamentally new and unexpected information that has emerged from these researches. Certainly the ability of exogenous agents to incorporate themselves completely into the genetic apparatus of host cells and confer not only the obvious property of lysogeny but, in addition, other genetic properties is most unexpected.

Let me describe one ingenious experiment that demonstrates clearly that for a while the viral agent remains as an independent entity in the cytoplasm These experiments were carried out by Lederburg and Stocker. They possessed a non-lysogenic strain that was also non-motile, lacking flagella. They exposed this strain to a viral agent derived from an organism that was motile. If the viral agent were to incorporate itself into the genetic apparatus of the host immediately and also carry over the genetic ability to produce flagella, then all of the cells produced from such an infected cell would be capable of producing flagella. Should the virus remain in the cytoplasm for a time, however, and be transmitted in a random manner from one cell generation to the next, then some cells would come off lacking the viral agent and therefore, also the genetic ability to produce flagella. These possibilities were tested by placing virus-infected non-motile organisms on a moist agar plate. As soon as a cell produces flagella it will start to move and in the course of changing its position, it will divide. If one of the daughter cells comes out lacking the viral particle, it will lose its flagella and, therefore, be unable to move. The daughter cell possessing flagella will move on. Under these circumstances then, you would expect to obtain a trail of non-motile ancestors along the path of the flagellated organisms. This trail eventually disappears when the virus agent becomes fixed in the chromosomes. Such trails were indeed observed in these experiments and they represent a beautiful demonstration on a cellular basis that there is a lag before these agents become fixed into the genetic apparatus.

KAMEN: Well, if I wanted to concoct (which would take us into the noon hour) a theory of radiation it would be that in every cell population you have a certain percentage that is radiosensitive by virtue of the fact that you have incorporated into the chromosomal apparatus a unit that is lysogenic.

SPIEGELMAN: That is where Pollard's magnification theory may come in. We must bear in mind the possibility that these represent very special biological groups that have evolved this symbiosis.

CARTER: As a matter of fact, Mellinick isolated something like 30 or 50 viruses which man lives with perfectly happily. It is a nice relationship. When the viruses undertaken from man were given to mice, the results were disastrous. So that I don't think we have to confine our speculation too narrowly.

MAZIA: There is a variety of genetic responses that may not be found in higher organisms because higher organisms could not have evolved if they had such sloppy genetics.

SPIEGELMAN: I doubt very much whether the genetics of microorganisms is "sloppier" that that of the higher forms. The ' sloppiness" referred to by Dr. Mazia, is more apparent than real. It stems primarily from the great precision with which one can perform genetic experiments with microorganisms. The microbial geneticist can, and routinely does, deal with 10^9 individuals and with hundreds of generations. Further procedures have been evolved that permit him to select easily a particular genotype even though it be present in only 1 out of 10^9 individuals. The point is that the range and the precision of the observations which can be performed with microorganisms are several orders of magnitude above that.

KAMEN: Do you think that anything in humans suggests that they don't have sloppy genetics?

SPIEGELMAN: I was the one who raised the voice of caution as a matter of fact.

KAMEN: We will put that on the record.

POLLARD: I don't believe that these things have much to do with nucleic acid as such. It seems to me that what is important is how nucleic acid is related to the cell.

CARTER: I think that nucleic acid metabolism is an expression of one of the most integrated activities of the cell and it is the area that is the most attractive to study.

POTTER: This phenomena may represent chunks of nucleic acid coming out of the molecule, may it not. Just as an amino acid can come out of the middle of a polypeptide chain, and an intact polypeptide chain cannot go to pieces because of its hydrogen bonds to other chains, so here you may have disrupted units of nucleic acid backbone which can come and go because the structure as a whole is held together by hydrogen bonds.

I mention this in connection with Dr. Cohn's statement about a line of reasoning which is drawn from the idea that the phosphate is bonded on each end and, therefore, is in there tighter than anything else. If one includes the hydrogen bonding then this is no longer so. I think that we have to draw our conclusions about what goes in and what comes out in terms of metabolic experiments in which we determine whether certain precursors go in and stay in and other precursors go in and come out. I think only by this metabolic experiment can we get at this question of whether the structure is as simple as pictured in the absence of hydrogen bonds.

This sort of thing suggests that if you break them out, the whole thing does not go to pieces functionally. So I think those two concepts are closely related. It makes me think that the structure as a whole can carry a certain num-

ber of enzyme precursors or functional precursors. You made the statement that when it does go in under certain circumstances, it can only go in by bumping something out.

SPIEGELMAN: Well yes, it can stay in only by bumping something out. However, this replacement process is probably part of the mechanism of getting the material fixed properly in the chromosomes. The picture that most of us have at the present time of these so-called transduction phenomena is somewhat as follows: Imagine a chromosome of the host recipient cell and a small segment carried in by the transducing virus agent. We know that homologus chromosomes tend to synapse. It is not therefore, unreasonable to suppose that the small piece carried in by the viral agent will pair off with the corresponding portion of the homologus chromosome. If you now have a double cross-over, then the only thing that can happen is that the piece which had been in goes out and the new piece is inserted in its place in the chromosome. I think it is true that most of us are disturbed by one consequence of this type of explanation. It requires a very high frequency of double cross-overs in very small regions. However, at the present moment it explains all the facts thus far available.

KAMEN: Do you see any loss of any property as a consequence of incorporation of this foreign material?

SPIEGELMAN: You can get both losses and gains depending upon where you take your genetic area.

KAMEN: That is what I mean. How are these losses absorbed? You gained a galactose positive trait, but what did you lose?

SPIEGELMAN: You lose the galactose negative locus -- well, to give you another case, streptomycin resistance can be lost and you can convert by such transduction phenomena into streptomycin-sensitive. This has also been accomplished by transformation.

BENNETT: Is it at the same place presumably?

SPIEGELMAN: There is no cell yet, and the big search is on now in a lot of laboratories to get a cell in which you can do transformation, transduction and classical recombination and then find out what the relation is between the three.

BENNETT: You mean as far as absolute site is concerned?

SPIEGELMAN: That is right. Recently in our department, Lennox has demonstrated transduction in coli K-12 which is able to recombine. So we have two of those now. If we can get a transformation for E. Coli, then we will have all three in one organism, and this is really what is needed to study the relationship.

KAMEN: Isn't Salmonella a good candidate for this purpose?

SPIEGELMAN: Salmonella can be transduced but not transformed.

POTTER: From the standpoint of nucleic acid metabolism after irradiation, can you speculate on why the transduction is always of one character? Are there any exceptions to this?

SPIEGELMAN: Yes. Stocker has a case which is very strong for

multiple transduction. Apparently they are hard to find. This again should not disturb us. One of the first things that happens as a result of active phage infection or induction of an active phage synthesis is fragmentation of the nuclear apparatus.

A very simple picture of what transduction is all about is that accidentally during the formation of mature phage, one of these fragments gets incorporated into the phage particle. When it gets into the cell, then this phenomenon occurs. The chances of getting 2 characters transduced will then depend upon how close they are together on the chromosome. We have few characters which are very close together; our map, thus far, is sparsely dotted with loci. As we add more loci we should increase the frequency of multiple transduction.

KAMEN: That has very little to do with irradiation.

KAPLAN: There is one possible analogy here to the material from bone marrow and spleen that seems to exert some effects on X irradiated mice and possibly other species. There is now fairly good evidence that the activity resides in the nucleus of the cell.

There is a little evidence, which Cole has put forth, which would indicate that the activity is destroyed by DNA-ase and by trypsin but not by RNA-ase. This material, if injected in the form of differentially centrifuged nuclei (but thus far not extractable from the nuclei) acts remotely on radiation-damaged cells that have been blocked from going through mitosis. Very shortly after it is administered, there is a release from this mitotic block and the cells start to proliferate in the thymus, the spleen, and in lots of other places where they have been unable to recover. Concurrently with this, a whole host of attributes of the animal that have been knocked out, suddenly come back. Its ability to combat infection, for example, is restored almost overnight.

This material may be analogous to the transforming principle, as Cole has pointed out, although the analogy is a rather remote one at this time.

MAZIA: Must the nuclei be of the same genetic strain as the animal into which they are injected in order to be effective?

KAPLAN: Well, we are not completely certain of that. There is a little evidence that Lorenz published, that would suggest that there may be a small heterologous effect but in order to demonstrate such an effect one needs a much larger amount of material.

It is interesting that this material is found only in bone marrow and in the spleen of the mouse and not in any other tissue.

It appears in rat marrow and it is probably going to work in marrow of all species. If you look at it biologically, the spleen of the mouse is unique in the sense that it functions very actively as extra-skeletal bone marrow and this is not true of other species; therefore, if you talk about the spleen of the mouse you are really talking about bone marrow and to become too narrowly concerned with spleen as spleen is not important.

DUBOIS: How much protection has been obtained in the rat?

KAPLAN: About 50 percent. The difficulty is that in the rat, there is another unique event, namely the sensitivity of the intestines, which is apparently not related to the marrow factor. The hematopoietic injury can be, in a large

part, corrected by marrow injection in the rat, according to Bond's recent work.

POTTER: If I get your point correctly, you might think that in X-ray damage you have blasted a piece of the nucleic acid out of the total unit which has not lost its integrity because of hydrogen bonding. And now, if you put in plenty of pieces, say, from marrow nuclei, you may supply at random a piece that can fill the gap in the chain and heal the damage. Is that the point you are making?

KAPLAN: No, I would not go that far. I would only say that the cells in some of these hematopoietic tissues seem to be incapable of recovery after X irradiation that is about midlethal for the mouse -- they are not capable of recovery, and by virtue of the impairment of function which results, the animals die because they are vulnerable to infection or hemorrhage, or they lack certain vital functions. This is not important to the discussion. The point is merely that they are unable to recover on their own. If you give them this material, almost overnight the hemotopoietic tissues recover the ability to start dividing rapidly. When we shield the spleen or the thigh, or inject bone marrow immediately after irradiation -- of course, the shielding would be during irradiation -- then, within a few days after irradiation, we can show very significant differences in the weight of the thymus between treated and untreated animals. This is a very significant change. Something has happened to those thymus cells which suddenly relieves the effect of irradiation and lets them start working again.

HOLLAENDER: We have conducted similar studies in regard to recovery from X irradiation with E. coli. I believe much more is known about this system.

SPIEGELMAN: What did you supply ?

HOLLAENDER: E. Coli B/r can be made to recover from X-ray damage to a considerable degree by growing it on yeast or meat extract as mentioned before. A synthetic medium consisting of inorganic salts, glucose, glutamate, uracil, and guanine, will produce as good a recovery at 37°C as yeast extract.

SPIEGELMAN: We had an idea along similar lines. It was based on the assumption that demonstration of transformation with certain bacteria stemmed from the fact that the wrong characters were being used. We started by subjecting the cells to lethal doses of X-rays and then exposing them to DNA from unirradiated cells. The idea was to reverse the lethal effect with uninjured DNA. The results, however were completely negative.

HOLLAENDER: We have tried to feed irradiated cells DNA or RNA but no effect was noticed. Of course, one difficulty might be that these materials can not enter the cell.

The active material actually was found first by isolating spleen extract by paper chromatography. We found three areas on the paper, extracts of which if combined, could simulate the spleen effect. It appears now that these three materials might be glutamate, uracil, and guanine. Further experimentation is necessary to make this certain (10).

SHERMAN: In your experiments did it make any difference which kind of spleens you used; whether they were rat spleens or rabbit spleens?

HOLLAENDER: Well, actually we used calf spleens for our experiments. Of course, we know why the calf spleen did work.

The spleen tissue was ground up and a water extract was made. This was purified by alcohol extraction.

We have made a survey of different tissues of the rabbit in regard to the recovery factor, and we found that the spleen contains more of this material than any other tissue tested. It is possible that synthetic media other than the one mentioned before may work also, but that we just do not have the right combination.

KAMEN: Is there any pertinence to the idea of taking some of the postulated precursors for DNA that are available and trying them, just inching up the scale a bit?

KAPLAN: Cole has done some of that work already. I imagine others have done it too. With the purest DNA-protein preparations that he can get, for example, by using low ionic strength extraction techniques, he gets very lovely nucleoprotein which does not work.

PATT: Will thymus extract facilitate the regeneration of thymus?

KAPLAN: Thymus does not work. As I said, marrow is the only thing that works.

PATT: We have a situation here of blood-forming tissue rejuvenating blood-forming tissue. However, thymus, which may be considered a part of the blood-forming scheme, does not facilitate the recovery of its kind, i.e., lymphoid or of other blood-forming tissues.

KAPLAN: Well, it is more complicated that that. Actually marrow, as you know, is an erythroid and a myeloid tissue, and thymus is essentially a lymphoid tissue. Without getting into a discussion of blood formation, it is still rather striking that myeloid tissue should cause regeneration of lymphoid tissue. Well, in following that idea up (and Cole has done this too), we thought it would be interesting to find out whether one could show that the activity is associated with one or another cellular series in the marrow. If you inject turpentine subcutaneously a sterile abscess is formed within 2 to 4 days. This produces an overwhelming myelocytic response in the marrow. This marrow, however, does not have greater activity than ordinary marrow.

The same is true if you use phenylhydrazine to produce transient anemia, which causes tremendous erythroid hyperplasia in the marrow. Therefore, we can conclude that the factor is not concentrated in the more mature cells of either series, since neither an induced erythroid nor an induced myeloid hyperplasia has a differential effect. I am not sure, but would suggest, at least, that the material in the marrow is derived from a very primitive cell form that is not differentiated along either line. That is a perfectly good lead, but we have not proven it as yet.

CURTIS: I am afraid our time is up and we must adjourn. I want to thank all of you on behalf of the National Research Council for coming and participating in this discussion. It has been a stimulating meeting and I hope each of you has profited from it as much as I have.

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