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THE CHARACTERIZATION
OF IONIZING RADIATION DAMAGE
IN DNA

Final Report

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Abstract

An experimental method for the measurement of covalent DNA-protein cross-links in bacteriophage T7 based on phenol-water countercurrent distribution has been developed and a statistical model for quantitative interpretation of these measurements has been devised. It has been found that DNA-protein cross links accumulate linearly with dose in response to exposure to ^{60}Co gamma radiation at a rate .05 to .20 times the rate of accumulation of double strand breaks if phage are exposed in highly protective medium (tryptone broth). It has been found that fast neutrons also induce DNA-protein cross-linkage. Furthermore, cross-link and double strand break lesions induced by neutrons occur in multiple clusters in randomly chosen phage, in contrast to those induced by gamma radiation, which occur singly in randomly chosen phage. It also appears that neutrons induce double strand breaks in the phage with an efficiency 50 times that of gamma rays. It was found that protein-DNA cross-links occur 30 times more frequently per lethal lesion after exposure to gamma rays than after exposure to ultraviolet light. Investigations of the occurrence of double strand breaks, protein-DNA cross-links and other DNA lesions in eucaryotic cells currently being pursued are also described.

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- A. R.B. Hawkins, Ionizing Radiation Induced Covalent Protein to DNA Cross Links in Coliphage T7, Abstract from program of the International Symposium on Protein and other adducts to DNA: Their significance to aging, carcinogenesis and radiation biology. Sponsored by the American Society for Photobiology, Williamsburg, Virginia, 1975.
- B. R. B. Hawkins, The Measurement of Ionizing Radiation Induced Cross-Linkage of DNA and Protein In Bacteriophage. Radiation Res. 68, 300-307, 1976.
- C. R. B. Hawkins, Radiation Induced Formation of DNA-Protein Cross Links in Bacteriophage, (Abstract), Radiation Res. 70, 678, 1977.
- D. R. B. Hawkins, Quantitative Determination of Cross Linkage of Bacteriophage DNA and Protein by Ionizing Radiation. Submitted to International Journal of Radiation Biology. 1977.
- E. R. B. Hawkins, Statistical Theory of Chain Scission and Concurrent Damage Not Associated with Chain Scission in Linear Polymers. Biopolymers 16, 207-218, 1977.

- F. R. B. Hawkins, Double Strand Breaks and DNA-Protein Cross-Links Induced by Fast Neutrons in Bacteriophage DNA. To be submitted for publication, 1977.
- G. R. B. Hawkins, Ultraviolet Induced DNA-Protein Cross-Links in Bacteriophage, To be submitted for publication, 1977.
- H. R. B. Hawkins, Models for Recombination in Merozygotic Systems, an Appendix to H. Yen and B. L. Marrs, Map of Genes for Carotenoid and Bacteriochlorophyll Biosynthesis in *Rhodospseudomonas capsulata*. *J. Bacteriology* 126, 619-629, 1976.

I. Contract History and Goals

The USAEC awarded contract number AT(11-1)-2448 to St. Louis University for the period May 1, 1974 to April 30, 1975 for work entitled "The Characterization of Ionizing Radiation Damage in DNA" on the basis of a proposal submitted by the principal investigator (Roland B. Hawkins) in January of 1972. The goals of the research program initiated were threefold:

- A. Bacteriophage, which constitute relatively well defined complexes of DNA and protein, were to be used as model systems for characterization of the amount and types of damage induced in DNA by exposure to ionizing radiation, as well as the molecular processes by which such damage develops. Characterization was to be by extension of the methodology and conceptual framework traditional to the study of the physical chemistry of macromolecules. Special emphasis was to be placed on study of types of damage other than DNA strand breakage, such as DNA-DNA and DNA-protein cross-links, and aberrant points of flexibility in the DNA double helix, about which little was known.
- B. When appropriate, methods developed in the bacteriophage studies were to be adopted to the study of DNA damage in cells exposed to ionizing radiation.
- C. An attempt was to be made to devise a method for quantitative measurement of double strand breaks in the DNA of cells exposed to ionizing radiation.

It was intended that these studies would help in the formulation of molecular models for the development of various cellular responses to radiation; responses such as cell reproductive death, prophage induction, chromosome aberration, mutation and neoplastic transformation. It is anticipated that such models will be useful in assessing the hazards of human exposure to radiation and in manipulation of radiation exposure conditions to diminish human risk and maximize therapeutic benefit.

Support for this work was continued by USERDA under renewal contracts E(11-1)-2448 written for the periods May 1, 1975 to April 30, 1976 and May 1, 1976 to April 30, 1977. However, the principal investigator resigned from St. Louis University on August 30, 1976 to take a position working in association with Professor L. J. Tolmach in the Department of Anatomy and Neurobiology of the Washington University School of Medicine. Professor Tolmach operates a laboratory equipped for the study of the radiobiology of cultured mammalian cells. Association with Professor Tolmach was intended to facilitate extension of the studies of DNA damage in bacteriophage systems to cellular systems. The contract with St. Louis University was terminated as of 31 August 1976 and the unexpended funds were used to establish a contract with Washington University (EE-77-S-02-4300. A000), with Roland Hawkins as principal investigator, for the period 1 February 1977 to 30 September 1977. In September 1977, the principal investigator entered Washington University School of Medicine as a student

to begin studies for the M.D. degree. For this reason it was thought inappropriate to seek further renewal of the contract. However, the investigation of DNA damage in cellular systems supported by the expiring contract will be continued by the principal investigator, as time permits, in the laboratory of Professor Tolmach during the course of his student-ship.

II. Results and State of Work Still in Progress

A. DNA Damage in Bacteriophage

These studies were largely completed prior to February 1, 1977, i.e. prior to the period of the currently expiring contract. Although discussed in previously submitted progress reports (1,2), they have not been reported in definitive form and are accordingly reported here. Originally it was intended to investigate various forms of polymeric damage other than strand breakage including DNA-DNA and DNA-protein cross-links, and aberrant points of flexibility in the DNA double helix. However, the investigation focussed early in the study of DNA-protein cross links.

An experimental method for detection and assay of covalent DNA to protein cross links was developed based on countercurrent extraction of the phage in a phenol-water system. This is described and discussed in references 3 and 4, copies of which are appended to this report. This particular assay method was adopted because it was felt that the extraordinary power of phenol as a protein denaturant and solvent would insure that DNA-protein association established in such an assay would be confined to covalent cross-linkage. In contrast to radiation induced non-covalent-bond-based-associations, such bonds may be expected to occur in definitely assayable numbers, and to constitute a lesion stable to all except enzymatically catalyzed repair processes.

1. ^{60}Co gamma rays

It was found (3,4) that ^{60}Co gamma radiation does induce covalent bond formation between DNA and protein in phage T7 irradiated while suspended in .001 M histidine buffer. However, the results of these experiments also indicated that the exposure induced extensive fragmentation of the DNA and cross links of DNA and protein to themselves, which prohibited quantitative assay of either type of lesion. It appears that the radiation chemistry of protein and DNA in dilute histidine solution is prohibitively complex.

It was then found that irradiation of phage T7 in 1 per cent tryptone broth suppressed many of these reactions and gave a dose response of assayable protein-DNA cross links and double-strand breaks which permitted quantitative analysis. This is reported in references 5 and 6, which are appended to this report.

2. Statistical Theory of Protein DNA Cross Links

It was, necessary to develop a statistical theory of concurrent cross-link and double-strand break accumulations in order to analyze this response, which was accomplished and is detailed in reference 7, (appended to this report) and in the appendix of reference 6. It should be noted that this theory is fundamental to the study of any non-strand-break type of lesion created by ionizing radiation because such lesions always occur concurrently with strand break lesions. Applications of the theory to study of cross-links between sister DNA

strands, another lesion of interest in radiation and chemical damage studies, is illustrated in reference 7. This theory also may furnish an approach to the study of the geometry of nucleoprotein particles, as is illustrated in reference 6.

3. Fast Neutrons

It was decided to exploit the experimental and theoretical methods developed for study of ^{60}Co gamma ray effects on phage T7 by examining, in a comparative way, the effects of exposure to radiation with a higher linear energy transfer (LET) character. It was hoped this might suggest a basis for the variation of relative biological effectiveness (RBE) with LET. To this end, the experiments with phage T7 suspended in tryptone broth were essentially repeated, using fast neutron irradiation instead of ^{60}Co gamma rays to expose the phage. The results of these studies are reported in reference 8, which is appended to this report. As is shown in reference 8, both double-strand breaks and protein to DNA cross links appear to occur in clusters in randomly chosen virions, in contrast to gamma ray induced lesions, which occur singly in randomly chosen virions. Furthermore, when this clustering is taken into account it appears that neutrons induce double strand breaks with an efficiency (per unit of absorbed dose) of about 50 times that of low LET radiation. It is not surprising that high LET radiations produce a clustering of damage, but the magnitude of the effect seen here and its association with such a large increase in the efficiency of double strand break formation, and perhaps DNA protein cross linkage, is very surprising. The results of reference 8 should be regarded as preliminary, but they do suggest that further studies of this type with phage particles of well defined geometry, and using other high LET radiations, may be very useful in defining important molecular effects associated with variation in the microscopic distribution of energy deposition.

4. Ultraviolet Light

Protein to DNA cross links induced by exposure of biological systems to ultraviolet light have been studied for some time, generally using analytical methods different from that employed here. It was decided to examine this phenomena using the methods developed here. This brief study is summarized in reference 9, which is appended to this report. It was found that ^{60}Co gamma rays are a good deal more effective than UV light in inducing protein-DNA cross links, when doses producing comparable biological effects (phage reproductive death) are compared. This is somewhat contrary to expectation, as protein-DNA cross-linkage has received much more attention from photobiologists than from radiation biologists. Indeed, protein to DNA cross links have been very hard to demonstrate in systems exposed to ionizing radiation. Apparently the difficulty with ionizing radiation lies in the circumstance that the responses examined in study of protein-DNA cross-linking are such that they may be concealed by the effect of concurrent strand-break formation in the DNA. The extent to which this may occur is evident from examination of the theory of reference 7, where sample response curves are shown. The fraction of sample DNA which bears one or more markers (cross linked

protein molecules) is expected to plateau with rising dose at a maximum level determined by the relative rate of accumulation of markers and strand breaks, and this plateau may be at such a low level that experimentally negligible response is recorded, even though a significant accumulation of markers occurs. This effect may be particularly troublesome in the study of cross links between sister strands of a DNA molecule because the relevant competing breakage event is the very numerous, relatively benign, single strand break.

B. DNA damage in Cellular Systems

1. DNA Damage and Competence for Self Replication

Cultured HeLa cells irradiated with less than about 800 rads exhibit a dose-dependent delay in reaching their next mitosis (M_1); nevertheless, all but, at most, a few per cent divide, respread, and progress at close to the normal rate to the next mitosis (M_2). At doses from 300 to 800 rads an increasingly large majority of the cells lose the capacity to proliferate indefinitely, and by 500 rads greater than 90 per cent of the cells are reproductively dead. It has been observed (10,11) that cells exposed to up to 800 rads replicate their DNA to within experimental error (a few per cent) of totality before reaching the first post irradiation mitosis, but that they fail to replicate a substantial, measurable, fraction of their DNA in the subsequent generation. It is of interest to determine what, if any, radiation induced lesion in the DNA causes replicative incompetence of a portion of the DNA in the first complete post-irradiation generation. Such incompetence may be an important step in a mechanism by which the cells lose the capacity for indefinite replication.

Experiments utilizing equilibrium CsCl density measurements of DNA replicated during time periods defined by incorporation of labeled thymidine or bromodeoxyuridine have been carried out to determine which sectors of the DNA acquire replicative incompetence in an attempt to determine what type of lesions may be involved. The results of this work are still incomplete. Preliminary evidence indicates the extent of replicative incompetence may depend on cell growth conditions. Studies on this problem are currently underway in Professor Tolmach's laboratory.

2. Measurements of Double Strand Breaks in Cells

The double strand break in DNA induced by exposure of cells to ionizing radiation is now and has for sometime been the lesion of most interest to radiobiologists. It has been implicated as an important step in the development of chromosomal abnormalities which in turn are suspect as an important cause of cell reproductive death, mutation and neoplastic transformation. In addition, the double strand break may be important in the development of DNA replicative incompetence in the first post-irradiation generation. Studies of the

relation of double strand breaks to cellular phenomena have been hampered by inability to quantitate their occurrence as a function of dose in the lower dose range, so as to enable a dose response function in the limit of low dose to be established. At higher doses, their rate of occurrence has been studied, but the quantitative assays based on sucrose density gradient centrifugation generally employed are subject to artifacts and are of questionable reliability. Therefore, it is of fundamental importance to develop a method which is able to determine the mass of DNA molecules that are of a size comparable to the total DNA content of a eucaryotic chromosome.

This problem has been approached through use of viscoelastic recoil measurements on dilute DNA solutions prepared from *Drosophila* cells by Zimm and Kavenov (12). They found evidence that DNA of a size equal to that of the DNA content of an entire chromosome does exist in at least some cells, and persists during disruption of the protein component of chromatin by enzymatic digestion and detergent treatment. The evidence they found is very convincing. It is based on detection of a fraction of the sample DNA in the form of full chromosomal size pieces. The circumstance that this fraction is variable from preparation to preparation does not interfere with their conclusion, but it does interfere with application of their methods to the study of double strand break formation in irradiated cells. For this reason, it was decided to discontinue efforts to determine double strand breakage in cellular systems by utilizing viscoelastic measurements (1,2), and to try an alternative approach.

The alternative approach being examined is as follows. DNA is to be freed from the cell and its associated protein by gentle lysis at elevated temperature in the presence of detergent and proteolytic enzymes, using procedures similar to those employed by Zimm and Kavenov (12). The resulting solution will be very dilute in DNA, the proceeds from a few cells (the order of 10 to 1000 $\times 10^{-12}$ g) being dissolved in a few ml. The solution will be formed while it is overlaying a nucleopore membrane clamped in a plastic filter holder. On the upper surface of the membrane will be a grid consisting of overlapping drilled holes in delrin plastic. The solution of DNA will be slowly drained through the membrane, through the grid holes. DNA and some cell debris will in this way dry onto the membrane on the bottom of the grid holes. Other solutions will be applied to the grid-membrane surface to rinse most cell components through the membrane, the large DNA molecules, and perhaps some other components, will be retained on the membrane at the bottom of the well formed by the particular grid hole into which they originally lodged. The DNA on the membrane will then be stained by draining an appropriate fluorescent stain solution through the membrane, or possibly an appropriate radioactive stain will be employed. The grid will be separated from the membrane and the latter examined with a fluorescent microscope equipped for quantitative measurement of fluorescence intensity. The fluorescence of each grid spot will be

recorded, and will be taken as a measure of the DNA content of that portion of the original solution that drained through the given grid hole. Fluctuation of this fluorescence measure of DNA content will be used to determine a weight average molecular weight for the DNA, using procedures analogous to those described by Weissman and Feher (14). If conditions are employed in which there are many more grid holes than DNA molecules, e.g. if a single cell is lysed, the number of grid holes in which DNA is present will furnish an estimate of the number of DNA molecules present, and coupled with the intensity of fluorescent measurements may furnish a complete molecular weight distribution. A number of variations of this procedure are being examined. This work will continue in the laboratory of Professor Tolmach during the next year, utilizing some equipment purchased under this contract. Results of that work will be reported to the USERDA as appropriate.

3. Protein-DNA Cross-Links in Cellular Systems

In order to facilitate further measurements of DNA-protein cross-links using the phenol-water counter-current method described above (4,6,8,9), a device is under construction which will permit 48 stage counter-current distribution with less hand manipulations. A sketch of this device is shown in figure 1. Rotation of the upper cylinder with respect to the fixed lower cylinder will accomplish transfer of upper phase from one stage to another, as in other devices (15). Unlike previously described devices, this one will be able to be mounted in a basket container on the arm of a low speed (about 500 RPM) centrifuge and spun to facilitate separation of the phenol-water emulsions formed during extraction.

When this device is operational, the phenol-water countercurrent extraction procedures will be applied to cellular systems to investigate DNA-protein binding induced by ionizing radiation. The theory of reference 7 indicates that measurements of this type may be quantitatively interpreted if the weight average molecular weight of the polydisperse cell DNA is also measured. Work with cellular systems also may require some modification of the extraction procedure. Results of this work will be reported to the USERDA as appropriate.

C. Model of Genetic Recombination in Bacteria

Discussions of the statistical model of protein-DNA cross-links (6,7) and of statistical models of genetic recombination with Dr. Barry L. Marrs led to development of the model for merozygotic recombination described in the appendix in reference 13, which is appended to this report.

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3. R. B. Hawkins (1975). Ionizing Radiation Induced Covalent Protein to DNA Cross Links in Coliphage T7 (Abstract). International Symposium on Protein and other Adducts to DNA: Their Significance to Aging, Carcinogenesis, and Radiation Biology, Sponsored by the American Society for Photobiology.
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