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MASTER

REPAIR OF DNA TREATED WITH γ-IRRADIATION AND CHEMICAL CARCINOGENS

COMPREHENSIVE REPORT OF ENTIRE PERIOD OF ERDA SUPPORT FROM 6-1-75 to 1-15-78

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I. Main Research Accomplishments

At the time of the initial ERDA application in 1975, this laboratory had partially purified an enzyme from E. coli which was named Endonuclease II of E. coli. This enzyme had been purified 1600 fold (1) by ammonium sulfate fractionation, chromatography on phosphocellulose, Sephadex G-100, and DNA-cellulose. The assay was based on the liberation of radioactive fragments of DNA treated with methylmethane sulfonate (MMS) from a gel. This enzyme preparation was then shown to be active on T-4 depurinated-reduced DNA (2) and on T4 DNA treated with methylnitrosourea (MNU) (3). In the latter paper, the release of 3-methyladenine as well as 0-6-methyl guanine was observed by chromatography on Dowex 50 as well as on TLC plates. Evidence for base release rather than the breakdown of DNA to nucleosides was the finding that 0-6 methyl guanine rather than 0-6 methyldeoxyguanosine was present in the enzymatic digest by TLC. Thus, it was concluded that the enzyme preparation recognized both 3-methyladenine and 0-6 methylguanine, that these were liberated by an N-glycosidase and that a phosphodiesterase was also present. It was stated that it was not clear whether the enzyme preparation was a single protein with two activities, or was a mixture of two proteins--an N-glycosidase and an apurinic endonuclease.

Work with the aralkylating agent 7-bromomethyl-12-methylbenz(a)anthracene subsequently showed that the 1600 fold purified preparation would also recognize DNA treated with this substrate and would release purine adducts, as well as cleave phosphodiester bonds (4). The release of base rather than nucleoside derivatives was shown by migration on Sephadex LH 20 as well as on TLC. Both the phosphodiesterase and the N-glycosidase activity on the dimethylbenz(a)anthracene DNA was observed in a fraction separated on DEAE from the main activity on depurinated DNA. This will be discussed below. Besides these activities the 1600 fold purified preparation of Endonuclease II was also shown to be active on γ-irradiated DNA at loci which were other than depurinated or depyrimidinated sites (5).

Thus, at the start of the present contract period two and a half years ago, a partially purified enzyme fraction had been isolated from E. coli which showed an N-glycosidase activity as well as a phosphodiesterase activity on DNA treated with methylnitrosourea, and with 7-bromomethylbenz(a)anthracene and a phosphodiesterase activity against γ-irradiated DNA. Both 0-6 methyl guanine and 3-methyladenine were released from DNA treated with MNU; the adenine and guanine derivatives from the DNA treated with 7-bromomethyl-12-methylbenz(a)anthracene were also liberated. All of the work was done by Dr. Kirtikar with the occasional assistance of Mrs. Irene Ukstins and Mr. Jerry Slaughter.

The original application requested support first for further work on this enzyme-specifically: (1) to purify further, (2) to develop an assay for the loss of alkylated bases, (3) to attempt to separate the N-glycosidase activity from the phosphodiesterase activity, (4) to define the site of cleavage, (5) to define other chemical carcinogen substrates, (6) to define the substrate residues in γ-irradiated DNA. Besides the work on E. coli enzymes, support was requested for further work on a calf thymus enzyme, for work starting on a human placenta enzyme, and if the mammalian work was successful to support work on the location and distribution of such repair enzymes in mammalian tissue.

This progress report will cover four different aspects of the work in this laboratory: (1) Endonucleases II and VI and Exonuclease III of E. coli; (2) methods for assay and for synthesis of substrates; (3) attempts at purification of repair enzymes from mammalian tissues; (4) β-propiolactone reactions with deoxynucleosides and with DNA.

1. Endonucleases II and VI and Exonuclease III of E. coli

Dr. Kirtikar, who had done the work on the 1600 fold purified fraction from E. coli, did all of the ensuing work unless stated otherwise.

(a) Purification

For purification, the ammonium sulfate fraction after phosphocellulose was applied to DEAE and a rather typical pattern was obtained when fractions were assayed in the presence of 8-hydroxyquinoline with native DNA, depurinated-reduced DNA, or DNA treated with methylmethane sulfonate (MMS) present in acrylamide gels. Assays were also done for exonuclease III in which $^{32}P_i$ release was measured from ^{32}P labeled DNA treated with micrococcal nuclease to produce 3' phosphates. Because several activities were separated, the ammonium sulfate fraction was applied directly to DEAE. A typical pattern is shown in Fig. 1. Peak I contained activity against depurinated-reduced DNA in the gel as well as exonuclease III activity. It should be noted that these two activities did not correspond exactly to each other. The activity against MMS treated DNA in peak I was approximately 10% of the activity against the depurinated reduced DNA. The activity against the depurinated-reduced DNA was purified extensively and was designated endonuclease VI. Dr. Kirtikar purified this enzyme approximately 11,900 fold through ammonium sulfate, then DEAE, phosphocellulose, DNA cellulose and two Sephadex columns. The purified material on gel had a single band but only a small amount of material was available to put on the gel. On SDS gel electrophoresis a molecular weight of 31,000 was obtained while on gel filtration a molecular weight of 31,500 was observed. Exonuclease III contaminated the endonuclease VI preparation and was only completely removed by the two Sephadex columns. Exonuclease III, purified in our laboratory, and also provided by Dr. Richardson, had a molecular weight by gel filtration of approximately 26,000 daltons. The experiments done to distinguish between exonuclease III and endonuclease VI have been reported from this laboratory (6).

Peak III of the DEAE cellulose column contained activity against MMS-treated DNA, and against depurinated-reduced DNA but the level of the latter was approximately 10% of the former. The activity against MMS treated DNA was purified approximately 17,000 fold through ammonium sulfate and DEAE, phosphocellulose, DEAE, DNA-cellulose, and Sephadex columns. On an SDS gel there was a major band at MW 34,500, which accounted for 90% of the protein and a minor band at MW of approximately 28,000 daltons. On Sephadex, the activity was observed at 33,000 daltons.

(b) Assay and properties of endonuclease VI

A study was made of the conditions for depurination both with the DNA-gel and with DNA in solution. Temperature at pH 3.5 was varied with the former. With DNA in solution, the release of ^{14}C-labeled purine bases at pH 3.5, 5.0, and 7.0 at varying temperatures was tested, as was the degree of single strandedness of the DNA. Alkali labile breaks were also measured at pH 3.5 and at varying temperatures. The enzyme was inactive on single-stranded depurinated-reduced DNA, did not require divalent metal, but was stimulated 1.8 fold by Mg^{++} or Mn^{++}. It was inhibited 75% by EDTA at 5×10^{-4} M, but not at all by the same concentration of 8-hydroxyquinoline. It was inhibited by p-chloromercuribenzoate and by 0.32 N NaCl but not by t-RNA or phorbol ester. It did not recognize any non-alkali labile sites in UV or Y-irradiated DNA, or in DNA treated with MMS or 7-bromomethylbenz(a)anthracene. It made 3' hydroxyl and 5' phosphate groups.

(c) Assay and properties of endonuclease II

A series of experiments was done to determine the optimum degree of alkylation of the DNA in the gel as well as in solution with the purified enzyme. The gel assay

was used to determine various properties of the phosphodiesterase. A pH optimum of 8.2-8.4, a 1.9 fold stimulation by Mg^{++} or Mn^{++}, and an inhibition by 10^{-3} M EDTA, p-chloromercuribenzoate and 0.32 N NaCl, but not by t-RNA were observed. Cleavage to make 3'-hydroxyl and 5' phosphate groups was found by analysis with snake venom phosphodiesterase and bovine spleen phosphodiesterase. The phosphodiesterase activity on depurinated-reduced DNA was only one tenth that on MMS treated DNA. The enzyme was not active on UV treated DNA. Incubation with MNU treated DNA released both 3-methyladenine and O-6-methylguanine as shown on high pressure liquid chromatography, and the purine base methylbenz(a)anthracene derivatives were observed on TLC. Using the methylbenz(a)anthracene DNA and observing release of alcohol soluble counts, 3-methyladenine was found to be a competitive inhibitor with a Ki of 7×10^{-4}. Phorbol ester was also a competitive inhibitor of base release with a Ki of 3.4×10^{-8} M while the 4-O-methyl derivative had a Ki of 3.3×10^{-6} M. Several lines of evidence were presented to support the concept that both the phosphodiesterase and the N-glycosidase were present on the same polypeptide.

All of this work by Dr. Kirtikar was written up in three manuscripts with the intent of submitting them the Journal of Biological Chemistry. However, they are being held as will be discussed below.

(d) Mutants of E. coli altering an apurinic endonuclease, endonuclease II and exonuclease III and their effect on in vivo sensitivity to methylmethane sulfonate

The summary of a paper recently published in Biochemistry (7) is as follows: The levels of endonuclease II, an apurinic endonuclease, and exonuclease III in the parent strain (AB 1157) of Escherichia coli and in various mutants were determined by chromatography on DEAE-cellulose. AB 3027 and NH 5016 lacked endonuclease II and exonuclease III. BW 2001 lacked the apurinic endonuclease and exonuclease III. While BW 2008, BW 9093, and BW 9059 lacked only exonuclease III. Deletion mutants BW 9101 and BW 9109 lacked all three enzymes. The latter mutants locate the genes for the two endonucleases in the region of exonuclease III (xth) of 38.2 min (White et al., 1976). All of the mutants which were sensitive to methylmethanesulfonate in vivo lacked exonuclease III, but not all mutants lacking exonuclease III were MMS sensitive. The deletion mutants and NH 5016 were the exceptions.

The work on the enzymology of the endonucleases was done by Dr. Kirtikar, the enzymology of the exonuclease by Mr. Cathcart and the in vivo sensitivity by Mr. White. Mrs. Ukstins grew cells and prepared Fraction III, the ammonium sulfate cut.

The present status of all the work on endonuclease II is in question. Several people in my laboratory have been trying unsuccessfully to prepare fraction IV of endonuclease II. This is the Peak III material obtained from DEAE (Fig. 1). On two occasions two different investigators have obtained DEAE patterns similar to the one shown. However, multiple other attempts have been unsuccessful. We have varied a series of factors to try to reproduce the pattern, such as cell strains, time of harvest, storage of cells, cell washing, the $(NH_4)_2SO_4$, a PEG step to avoid streptomycin, different batches of MMS, etc. We are unable to get consistent results such as were obtained in the large scale column preparations done by Dr. Kirtikar with the assistance of Mrs. Ukstins, or in the mutant studies. Also we find no N-glycosidase activity in some of the partially purified fractions made by Dr. Kirtikar. An examination of the 3-methyladenine N-glycosylase described by Lindahl has located this in the peak I region of the DEAE fractionation although this has not been characterized further. The nature of our present difficulties is not clear and several members of the laboratory are working very hard to try to clarify the problem.

2. Methods of Assay and Synthesis of Substrates

Since the original observation of base release from MNU treated DNA, it has seemed logical to try to devise a good assay for an N-glycosylase. The use of alcohol soluble counts is very dangerous and obviously exonuclease can be a problem. A back-up system for this first line of investigation has been devised and involves the use of mini-DEAE columns and then the analysis of the effluent by high pressure liquid chromatography. A major part of the apparatus was purchased with funds from the ERDA contract. A system has been worked out in the past year which gives good separation of 7-methylguanine, 3-methyladenine, and 0-6 methylguanine. This type of assay, where the products are carefully characterized, is essential in order not to purify for example an exonuclease plus phosphatase which would produce nucleosides rather than free bases.

A second assay which has been introduced is the PM-2 assay with which we can now look at conversion of twisted circles to nicked DNA by sucrose gradient or by filter binding.

Because of the cost of substrates we have worked out two syntheses. We now make our own [^{3}H] MNU from [^{3}H] methylamine by a straightforward synthesis described by Lawley and Shah (8). The yields on three preparations have been around 65%. We have also had methylbenz(a)anthracene tritiated for use and then have brominated this by the procedure described by Dipple (9).

All of the work with the chemical carcinogens until after they have been reacted with DNA is done in the isolated biohazards hood purchased with funds from the ERDA contract.

3. Search for Mammalian Enzymes Involved in Repair of DNA

(a) An endonuclease for calf liver specific for apurinic sites in DNA

A report of this material by Kuebler, J. P. and Goldthwait, D. A. appeared in Biochemistry (10). A summary is as follows: An endonuclease specific for apurinic sites in double-stranded DNA has been partially purified from calf liver extracts. The enzyme has a pH optimum of 9.5, is only slightly stimulated by low concentrations of Mg^{2+}, and has a molecular weight of 28,000. Inhibitors of the endonuclease include Ca^{2+}, EDTA, p-hydroxymercuribenzoate NaCl, and tRNA. The enzyme introduces single- and double-stranded breaks in depurinated DNA. High concentrations of the enzyme preparation degrade untreated single-stranded DNA, but not ultraviolet (UV) irradiated DNA or DNA treated with methylmethanesulfonate or 7-bromomethyl-12-methylbenz(a)anthracene. Enzymatic incisions produce 3'-hydroxyl and 5'-phosphate end groups. Some of the properties of the calf liver apurinic endonuclease differ from those of a similar endonuclease obtained from calf thymus by S. Ljungquist and T. Lindahl [(1974)(11)] and in this laboratory.

(b) Search for endonuclease in human placenta which recognizes DNA treated with MMS

A large amount of time was spent looking at various fractions of human placenta for an enzyme active on MMS treated DNA entrapped in acrylamide gel. Ammonium sulfate fractions were made of the supernatant fraction centrifuged at high speed and these were then examined on phosphocellulose, DEAE, or DNA cellulose columns. The patterns were always complicated and assays included MMS treated DNA, depurinated-reduced DNA, and native DNA. We were searching for a peak of activity which recognized MMS treated DNA but not the other two substrates. Twice on DEAE columns we found such peaks, but they could not be stored and were not found on several other runs. Several peaks active on depurinated-reduced DNA were observed and these enzymes have since been reported by another group. This approach was dropped in favor of looking for N-glycosidase activity.

(c) Possible isolation of a 3-methyladenine N-glycosylase in rat liver nuclei

A 3-methyladenine N-glycosylase activity has been identified in rat liver nuclei, and appears to be localized in the non-histone protein fraction. Although the activity is quite low, no detectable amounts of 7-methylguanine, O^6-methylguanine, adenine or guanine are released. However, approximately 5-6% of the 3-methyl adenine residues present are excised. To date, efforts to segregate this activity from enzymes acting on native DNA have been unsuccessful, due to the small amounts of protein obtained. Because of the instability of the glycosidic bond of 3-methyladenosine we are still cautious about artifacts which might give us this result.

MNU[^{3}H] DNA, containing 70% 7 methylguanine, 13% 3-methyladenine, and 6% 0-6-methyl guanine was used as the substrate. At the conclusion of the reaction, the reaction mix was passed over a small DEAE column, equilibrated at pH 8.0, and washed extensively. This procedure binds unreacted DNA, oligonucleotides and mononucleotides to the DEAE, while free bases are eluted. After the addition of markers, the eluant was analyzed by high pressure liquid chromatography.

The non-histone protein fraction of purified rat liver nuclei contains several enzymes capable of activity on unmodified DNA. This fraction, while not able to liberate free purines from native DNA, does remove a small, but significant amount of 3-methyladenine from DNA reacted with [^{3}H] MNU. Furthermore, the activity can be fractionated by ammonium sulfate, precipitating near 40% saturation. Metal ion does not appear to be an absolute requirement for activity, and in fact, only a slight reduction in activity is observed with 0.1 mM EDTA in the reaction. EDTA does inhibit nuclease activity on native DNA by about 50%. It is therefore unlikely that the excision of the modified adenine is the result of native enzyme(s), although this possibility cannot be ruled out.

Although the results at present are not conclusive, they do suggest that rat liver nuclei are capable of excising 3-methyladenine. The properties of this activity are similar to a 3-methyladenine N-glycosylase which we have recently identified in E. coli, and in fact is probably the same enzyme purified by Lindahl. Both the E. coli and rat liver enzymes are active in the presence of 0.1 mM EDTA, and do not release 7-methylguanine or 0-6-methylguanine.

Attempts are now in progress to purify the activity from rat liver.

Prior to the work with rat liver, extensive unsuccessful experiments were done with ascites tumor cells and calf liver.

4. β-Propiolactone Reactions with DNA and Its Derivatives

The reactions of β-propiolactone with DNA and its purine deoxynucleosides is under investigation. The reaction of β-propiolactone with deoxyguanosine yields 7(2-carboxyethyl)guanine (12). This compound has been synthesized and purified by HPLc to use as a standard.

The reaction products formed between β-propiolactone and adenosine and deoxyadenosine have been studied. β-propiolactone (7.5 mM) was incubated with the adenine derivatives (0.5 mM) in 2 ml of water at 45° for 3 minutes. Excess β-propiolactone was removed by repeated ether extraction. The deoxyadenosine reaction mixture was chromatographed on DEAE and three separate peaks were obtained. One was deoxyadenosine. One had a spectrum similar to 1-methyladenosine with comparable shifts in acid and alkali

and a maximum in neutral solution at 258 mμ. This compound is considered to be 1-carboxyethyldeoxyadenosine. The retention time on high pressure liquid chromatography of this compound in a methanol-phosphate buffer system was 9.6 minutes. Hydrolysis of the nucleoside with 0.01 N or 0.1 N HCL at 100° for 3 minutes gave a compound with a retention time on HPLC of 7 minutes and with 1 N HCl of 6.05 minutes. The adenosine derivative, with a retention time of 7.5 minutes, gave on hydrolysis with 0.1, 1.0 and 3N HCl, retention times of 7, 6, and 5.5 minutes respectively. The retention times and the absorbtion spectra of the adenosine and deoxyadenosine derivatives were similar after hydrolysis with 1 N HCl. Thus hydrolysis of both these nucleoside derivatives under specific conditions yielded the same base derivative.

The third compound obtained from the DEAE column had an unusual spectrum with a peak at 305 mμ. This compound could be separated from the "258" compound on HPLC. On acid hydrolysis, a compound was obtained with spectral properties at pH 2, 7, and 12 similar to the hydrolysis porudct of 1-carboxyethyldeoxyadenosine, and with a similar retention time on HPLC. On gentle acid hydrolysis a compound with spectral properties and migration on HPLC similar to 1-carboxyethyladenosine was observed. The exact nature of the "305" compound is not known, but it may be a lactone. This remains to be proven.

Preliminary studies of the compound presumed to be 1-carboxyethyldeoxyadenosine, the "305" compound and deoxyadenosine by nuclear magnetic resonance show shifts in the protons at C-2 and C-8 as well as appearance of lines due to protons in the carboxyethyl moiety. Initial studies suggest a greater shift in the C-2 proton which would be expected if the substitution were on the N-1 position. Considerably more work needs to be done in order to confirm the structure of the "258" and "305" compounds. Preliminary studies on the ratio of 1-carboxyethyladenine to 7-carboxyethylguanine in DNA alkylated with different amount of β-propiolactone have been done.

Some time after these studies were started, a paper by Maté, U., Solomon, J. J. and Segal, D., (13) appeared which defined the alkylated adenine in DNA as 1-(2-carboxyethyl) adenine. This was done by UV spectra and electron impact and chemical ionization mass spectra.

II. Plans for Future Research

The objectives for the next several years are as follows:

1) Isolation, purification and characterization of DNA repair enzymes in E. coli.

2) Isolation, purification and characterization of DNA repair enzymes in mammalian tissue.

3) Study of the action of repair enzymes in vitro on chromosomal structures and substructures.

4) Removal of alkylated bases from cells reacted with MNU.

More detailed plans are described below under these headings:

1) Isolation, purification and characterization of DNA repair enzymes from E. coli.

a) Endonuclease II of E. coli. As indicated in the accompanying progress report, a great deal of work has been done on this enzyme by Dr. Kirtikar. As characterized by her, the enzyme, with a molecular weight of 34,500, had a phosphodiesterase activity and an N-glycosidase activity on DNA treated with MMS, MNU and 7-bromomethylbenz(a)anthracene, and a phosphodiesterase activity on Y-irradiated DNA. As has been pointed out in the first portion of this report, we have been having difficulty repeating Dr. Kirtikar's work. Our aim has been to get an active peak III on a DEAE column (Fig. 1). The steps required to do this are very time consuming and require approximately a week and a half per run. Therefore the number of variables that can be checked per unit of time is limited. We have tried to devise short cuts, but these have not been successful. At present we are first concerned with the substrate. We have found that the MMS obtained from Eastman Kodak is of variable quality. We have examined different batches by GLC and find that the most recent batch has a high percentage of impurity. This batch also gives less alkylation of DNA as measured by the sucrose gradient technique after depurination of alkylated groups and treatment with alkali. More work to define the quality of MMS and the conditions for making substrate is underway.

An examination of different strains of cells is required. Most of the recent work has been done with J.C. 4583, an endonuclease I minus strain. Although in the original work by Dr. Kirtikar, three peaks of activity on DEAE were routinely seen with this strain, we are now seeing only the first peak. Part of the second peak is endonuclease I and we plan to make fractions from AB1157 in order to have a peak II marker in the chromatograms. The problem is whether peak III is being thrown away on an earlier step of purification or is being degraded. We plan to vary the streptomycin batch and concentration to see if this is a factor. We have tried proteolytic inhibitors to no avail but there are several other variations to be tested. It is possible that endonuclease II is an induced enzyme and that some agent in the previous growth medium was responsible for induction. Growth of cells with a low level of MMS might test this hypothesis. Once we can obtain an active peak III, we can then continue this purification and check activities of the phosphodiesterase and N-glycosidase on MNU and 7-bromomethylbenz(a)anthracene treated DNA.

b) Endonuclease VI of E. coli. Because of our major focus on the endonuclease II problem, we have not had time to try to repeat the endonuclease VI results of Dr. Kirtikar. We do know that by the DNA-gel assay with depurinated-reduced DNA, there is activity in the peak I region which parallels fairly closely the activity of exonuclease III. From Dr. Kirtikar's studies, these activities can be separated only by Sephadex on the basis of different molecular weights. The material in peak I will be subjected to

chromatography on Sephadex to check for separation of exonuclease III and endonuclease VI. We wish to also check some of the properties of endonuclease VI to be sure it is not exonuclease III, or endonuclease IV or V.

c) 3-Methyladenine N-glycosylase. We have observed the activity described by Lindahl in peak I of the DEAE column. We would like to purify thi s further to study some of its characteristics and establish whether it recognizes other substrates.

d) 0-6 Methylguanine N-glycosylase. The endonuclease II preparations of Dr. Kirtikar were able to remove 0-6 methylguanine. A great deal hinges on our being able to repeat her work and show that there is such an activity Since there is removal of 0-6 methyl guanine *in vivo* (14) there must be a system for this and that would remain to be clarified. Search for this repair system will be difficult but could be pursued in the fashion which led to identification of the 3-methyladenine system using MNU treated DNA and the techniques we have developed, for specific base release. Most of the work by Dr. Kirtikar on 0-6 methylguanine removal was done with a 1600 fold purified enzyme which had not been purified with DEAE. Early fractions of this preparation can be tried.

e) Exchange reaction with alkylated bases. The work of Hennings (15) suggests that there is a repair system for NMU treated or β-propiolactone treated DNA (16) which removes possibly only the damaged base. Such an exchange system has been seen with guanine and a guanine derivative base in t-RNA (17). Probe experiments could be done to search for the exchange of bases in alkylated DNA, or their introduction into depurinated DNA.

f) Enzymatic removal of β-propiolactone derivatives from DNA. We are developing the technology to examine the removal of the adenine and guanine derivatives formed by reaction of DNA with β-propiolactone. We have defined the adenine derivative, have reasonable quantities of both the adenine and guanine derivative in pure form which we can use as carrier, we know how these derivatives migrate on HPLC, and we have started studies of conditions for alkylating DNA. It is possible that the repair system for MNU treated DNA is the same as that for β-propiolactone treated DNA as suggested by the work of Hennings (16). This would provide a more stable substrate than MNU treated DNA. We plan to examine both *E. coli* extracts and mammalian cell extracts.

2) Isolation purification and characterization of DNA repair enzymes from mammalian tissue

Presently an extensive search for an N-glycosylase activity for 3-methyladenine and/or 0-6-methylguanine is underway in rat liver. This search is based on the assays developed which involve DEAE mini-columns and high pressure liquid chromatography. What has become apparent is that exonucleolytic systems complicate the search. If a mammalian N-glycosidase is not inhibited by EDTA for example, then the exonuclease problem may be eliminated. Current experiments with fractions from rat liver have given very suggesti results regarding base release, but much work needs to be done to define the nature of any release observed to be sure it is not due to sequential reactions, spontaneous breakdown, etc. We plan to explore enzymes in rat liver, human placenta and possibly calf thymus.

3) Study of the action of repair enzymes *in vitro* on chromosomal structures and substructures

It is proposed to examine first the reaction of MNU with DNA of nucleosomes compared to free DNA to determine if there is any alteration in the bases attached by the MNU. This could provide some information regarding the protein-DNA interactions and whether they are preferentially in the major or minor groove. A series of controls with varied salt concentration will be necessary. Next, a comparison of nucleosome reactivity

with chromatin reactivity will be examined to determine if the DNA between nucleosomes reacts differently to alkylation. Finally, an examination of the activity of a purified N-glycosidase on alkylated bases on nucleosomes will be examined. Originally, this was proposed with endonucleae II. Depending on results of experiments described above, this enzyme or the 3-methyladenine glycosylase will be used.

4) Removal of methylated bases from cells reacted with MNU

With the high pressure liquid chromatography system it is now possible to analyze very rapidly the loss of bases from DNA in a cellular system. It is planned to screen a series of cell types to determine whether there is once cell line in which there is rapid and efficient removal of alkylated bases from the DNA through an enzymatic procedur rather than through spontaneous hydrolysis. If so, this may provide a system for analyzing the enzymatic mechanisms of removal. Another series of experiments which we would like to do is to examine the removal from normal vs. Xeroderma pigmentosa cells where it has been reported by other workers that the removal of O-6 methylguanine may be diminished in the mutant cells.

Up to several months ago, it appeared that our program was moving ahead and that with the enzyme endonuclease II, we would be able to do a great many interesting experiments. However, with the realization that we could not seem to repeat the isolation and purification of endonuclease II, the laboratory personnel has been working frantically trying to determine what some of the factors may be in this problem. The requirement for reapplication comes at a very difficult time because we are in the midst of trying to find out what is wrong, and as yet we do not have the answers.

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III. Graduate Students and Post-Doctoral Fellows

No graduate students working on this project have yet obtained degrees. Mr. Jerome Lang and Mr. Richard Cathcart are still in training.

Dr. Dollie M. Kirtikar has completed post-doctoral training here and left the laboratory in September, 1977.

Dr. Suzanne Polmar has completed post-doctoral training here and left the laboratory in September, 1976.

IV. Publications from this Laboratory of Material Supported by ERDA

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V. Evaluation of Knowledge in the Area, Its Significance and Needs for the Future

Our effort is focussed on enzymatic subcellular mechanisms of DNA repair procaryotes and eucaryotes. Progress is being made in this whole field, but knowledge is far from complete. A brief examination of efforts in this area can be divided into work in bacterial systems vs. work in animal systems. Subdivisions involve the type of lesion in DNA to be repaired. In bacteria DNA damage due to U.V. light involves repair of dimers and of other pyrimidine lesions. Dimer repair enzymology is being carefully examined by Grossman and his associates, and recent papers describing the enzymes in M. luteus are an example (1). The repair of DNA alkylated with MMS has been under investigation by our group and also by Lindahl and by Laval (2, 3). The latter two groups have described in E. coli and M. luteus respectively an N-glycosylase which liberates 3-methyladenine from DNA. This enzyme has no phosphodiesterase activity and does not recognize 0-6 methylguanine. Endonuclease V of E. coli described by Gates and Linn (4) recognizes

alkylated DNA, but probably because it recognizes single stranded areas. Enzyme activities which recognize γ-irradiated DNA have been described in M. luteus (5) and E. coli (6, 7). Activity on depurinated DNA has been described for exonuclease III of E. coli (8, 9), for endonuclease IV (10) for endonuclease V (4) and for endonuclease VI (11). Enzymes specific for polycyclic hydrocarbons have not been described except by this laboratory.

In animal tissues, an enzyme has been partially purified from lymphoblasts which recognizes UV treated DNA (12). Lesions in γ-irradiated DNA and UV irradiated DNA are recognized by an enzyme in calf thymus (13). Lesions in DNA treated with UV, AAAF, and 7-bromomethylbenz(a)anthracene are recognized by an enzyme present in rat liver (14) which may be similar to endonuclease V. Evidence has been presented by Brent of activity in human lymphoblasts which recognizes alkylated DNA and acts as an N-glycosidase (15). Enzymes specific for apurinic sites have been isolated from calf thymus (16) from calf liver in this laboratory, from human placenta (17) and from human lymphoblasts (18). A number of other investigators have also isolated enzymes active in repair.

A complete understanding of DNA repair in the cell requires first a knowledge of the chemical nature of the alteration of the DNA, then a knowledge of the precise enzymatic mechanisms responsible for removal of the altered moiety and the subsequent repair, an understanding of the more complicated events involved in repair of DNA associated with protein in chromosomal structures, knowledge of the sequence of events in vivo both in cell cultures and in the whole animal, and a correlation of the subcellular with the cellular events. Eventually this knowledge must be applied to human cell systems to determine the ability of specific tissues to respond to specific chemical agents. One would hope to be able to measure levels of a specific enzyme in a tissue and predict the susceptibility of the tissue to a chemical carcinogen. Knowledge as to whether a specific enzyme is induced and if so under what conditions would be important.

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VI. Division of Federal Support for Overall Research Program

My program is supported by two NIH grants and the ERDA contract. One of the grants is for study of chemical carcinogens which interact with DNA and for enzymes involved in repair of DNA (CA-11322). The other grant is for the effect of X-rays on DNA and DNA repair mechanisms (CA-18747). Prior to the ERDA contract, it was necessary to spend a great deal of time writing grant applications to obtain support for various aspects of the entire program. The ERDA contract has obviated this. The work with chemical carcin-

ogens, particularly the synthesis of [^{3}H] MNU and [^{3}H] 7-bromomethylbenz(a)anthracene as well as the reactions of these materials with DNA must be done in a biohazards hood. Prior to the purchase of the hood through the ERDA contract, there was no place in the old medical school building where this work could be done. The contract has also provided funds to purchase the major portion of a high pressure liquid chromatography apparatus which has been invaluable in the work on N-glycosylase enzyme activity in mammalian cells. In the description of work in progress, an attempt was made to emphasize the difficulties in distinguishing a specific N-glycosylase from more non-specific reactions. For this, it is necessary to have a rapid reliable way of measuring alkylated bases and this HPLC apparatus provides this. Part of the program envisages work with cells in tissue culture and the loss of alkylated bases from these. The need for culture equipment to be used in the isolated laboratory is being met by the contact.

Working on different aspects of the entire program are three post-doctoral fellows and two graduate students. A training grant which we have had in the Department which supported graduate students ends this year and these must be supported on grants since there are no departmental funds for them. There is some common ground between the chemical carcinogen NIH grant, the X-ray grant and the ERDA contract. The work on Endonuclease II and VI has been supported primarily by the NIH chemical carcinogen grant. Many of the projects planned for the ERDA proposal and the X-ray proposal were based on the availabili of endonuclease II. Because of the difficulties described in this summary, most of the members of my laboratory have been involved in working on various aspects of enzyme purification and identification. This has involved Dr. Philip Yang supported by the X-ray grant, and Dr. Ru Fang Chen supported by the ERDA contract. Both graduate students, Mr. Lang and Mr. Cathcart, supported by the training grant, have also been involved. Until we can clear up the status of Endonuclease II, we will be unable to proceed. For this reason it is difficult to draw clear lines between specific parts of the project. The work on mammalian enzymes is at present less dependant upon present ERDA support but requires the use of equipment purchased with the ERDA contract. The work on β-propiolactone is totally supported by theERDA contract at the present time.

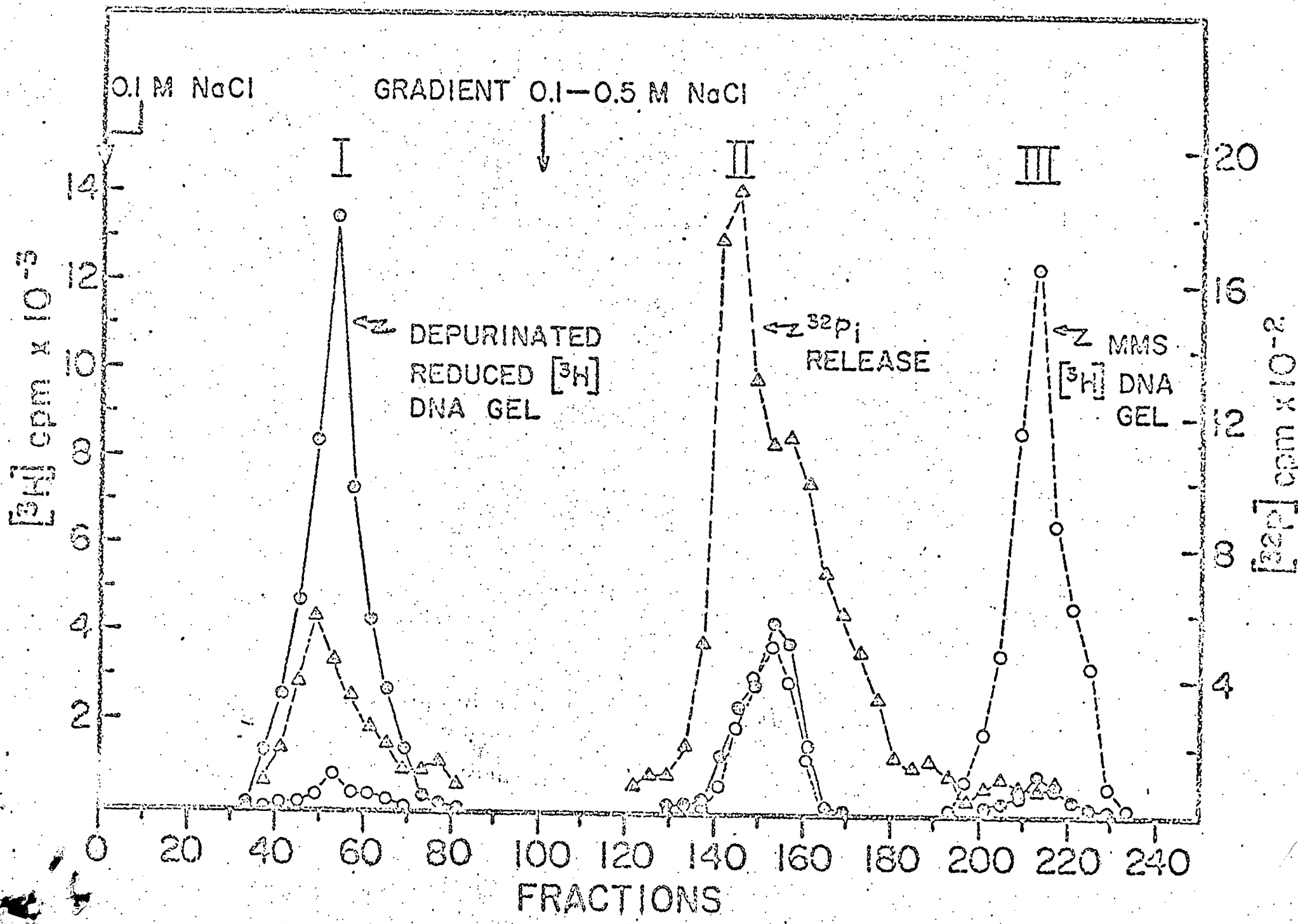
0.1 M NaCl
GRADIENT 0.1—0.5 M NaCl
I
II
III
DEPURINATED REDUCED [3H] DNA GEL
32Pi RELEASE
MMS [3H] DNA GEL
[3H] CPM x 10-3
[32P] CPM x 10-2
FRACTIONS
0
20
40
60
80
100
120
140
160
180
200
220
240

Figure 1