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Repair of DNA Treated with γ-Irradiation and Chemical Carcinogens

Progress Report

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Progress Report

Abstract: Enzymatic mechanisms of DNA repair are under investigation. From E. coli an endonuclease active on apurinic acid has been separated from endonuclease II active on DNA treated with methylmethane sulfonate, methylnitrosourea, 7-bromomethyl-12-methylbenz[a]anthracene and γ-irradiation. Mutants have been identified for each enzyme. The purification of both enzymes is proceeding and the mechanism of action of endonuclease II which has both an N-glycosidase and a phosphodiester hydrolase activity is under investigation. Evidence demonstrating exonuclease III is an enzyme separate from the apurinic acid endonuclease and endonuclease II has been accumulated but this is still under investigation. Work has been completed on 7-bromomethyl-12-methylbenz[a]anthracene treated DNA as a substrate and is being continued on the inhibitory effects of phorbol ester on endonuclease II. Finally, the properties of an enzyme from calf liver active on depurinated DNA have been compared with those of a comparable enzyme from calf thymus. Evidence that they are isozymes has been found.

Progress Report for ERDA

Repair of DNA Treated with γ-Irradiation and Chemical Carcinogens

Endonuclease II of E. coli has, because of recent work, been defined as the enzyme activity which recognizes DNA which has been reacted with methyl-methane sulfonate (MMS), methylnitrosourea (MNU), 7-bromomethyl-12-methyl-benz[a]-anthracene and γ-irradiation. This enzyme has now been separated from an enzyme active on depurinated DNA which will be referred to as the apurinic acid endonuclease.

Endonuclease II and the Apurinic Acid Endonuclease of E. coli-Separation and Control by Genes

Endonuclease II was separated from the apurinic acid endonuclease by chromatography on DEAE. The E. coli cells were disrupted with glass beads, and after a high speed centrifugation, treatment with 0.8% streptomycin and ammonium sulfate precipitation between 45% and 80%, the fraction was applied to a DEAE column and eluted as shown in Fig. 1. Peak I is active on depurinated DNA or depurinated-reduced DNA. This peak, now purified over 3000 fold has very slight activity on MMS treated DNA which may be due to minimal depurination, although this is difficult to prove. The molecular weight of this fraction after further purification was 33,000 as determined by gel filtration. Peak III is active on DNA treated with MMS but is able to degrade depurinated DNA at a rate which is less than 10% of the rate of degradation of MMS treated DNA. This activity on depurinated DNA compared to MMS DNA remained constant through various steps of purification and evidence will be presented in another section to support the concept that this is an intrinsic activity of endonuclease II and not a contamination with the apurinic acid enzyme. In previous publications it was concluded erroneously that the activities on depurinated DNA and on MMS DNA were due to the same enzymes. Peak II contains both activities and on gel filtration has a molecular weight of 58,000. On rechromatography the same peak is recovered and does not split into Peaks I and III. This suggests that it is a stable dimer.

Using the chromatographic procedure shown above, we have examined two mutants and found that these mutations correspond to Peaks I and III. A mutant of E. coli originally isolated by Dr. Paul Howard-Flanders, AB 3027, on the basis of its sensitivity to MMS, has been shown to lack endonuclease II, Peak III, as illustrated in Fig. 2. Of note is the activity in Peak II on MMS-DNA which is decreased to approximately one half that on the depurinated DNA. A second mutant, BW 2001, was isolated in Dr. Bernard Weiss' laboratory on the basis of a decreased endonucleolytic activity on DNA treated with MMS. The ammonium sulfate fraction of this mutant when examined on a DEAE column produced the pattern shown in Fig. 3. Peak I, the apurinic acid activity, is absent and this activity is decreased to approximately one half in Peak II. Our interpretation of Peak II is that monomers of either enzyme can combine to form, for reasons unknown, a stable dimer. If one polypeptide has a missense mutation it is unstable as a monomer but is partially stabilized by the normal polypeptide in the dimer.

Endonuclease II Properties

The molecular weight by gel filtration of the material in Peak III is 33,000. When MMS treated DNA is used as the substrate, there is no requirement for divalent cation, although the activity is increased approximately two fold by Mg^{++} (1). The enzyme is active in the presence of various chelating agents such as 8-hydroxyquinoline of 6×10^{-4} M, but EDTA does inhibit the enzyme 71%

at 10^{-4} M and 90% at 10^{-3} M. t-RNA does not inhibit the enzyme. The sulfhydryl reagent, p-choromercurisulfonate, does inhibit the enzyme activity. By examining the DNA in neutral and alkaline gradients it has been noted that the enzyme makes one double strand break for every four single strand breaks. Since the enzyme had very limited activity on untreated single-stranded DNA, these results suggest that the DNA may be alkylated in a non-random fashion.

The endonuclease II (Peak III) has now been purified over 3000 fold and with this preparation the nature of the phosphodiester bond break has been studied using snake venom and bovine spleen exonucleases, with and without alkaline phosphatase. Results are presented in Fig. 4 and from this it can be concluded that the enzyme produces 3'-hydroxyl and 5'-phosphate residues. Similar experiments have been done with the apurinic acid endonuclease and similar results have been obtained.

The Mechanism of Action of Endonuclease II

The problem still remains whether endonuclease II is a single enzyme with both the N-glycosidase and the phosphodiester hydrolase activities. It has been proposed that there is an N-glycosidase active on purine base derivatives in DNA and that repair involves this enzyme followed by the apurinic acid endonuclease. Several lines of evidence are against this interpretation: (1) It has been shown as noted above that the apurinic acid activity can be separated from the endonuclease II (MMS) activity. (2) A mutation which eliminates the apurinic acid activity does not affect the MMS activity and vice-versa. (3) When endonuclease II is purified over 3000 fold the base releasing activity and the phosphodiester bond hydrolysis are still associated. (4) If two enzymes were required, and they were separate and worked sequentially then the rate of the overall reaction should be determined by the rate limiting enzyme. Table I shows an experiment in which varying concentrations of the apurinic acid endonuclease and of endonuclease II were tested on three different substrates--depurinated reduced DNA, MMS treated DNA, and the same MMS treated DNA which had been then treated to depurinate the alkylated bases. It is clear that there is a high degree of specificity of the two enzymes for their respective substrates. The endonuclease II when acting on depurinated DNA has less than 10% of the activity it has on MMS treated DNA, while the apurinic acid endonuclease when acting on MMS-treated DNA has also less than 10% of the activity it has on depurinated DNA. Finally, endonuclease II which was able to recognize 75 sites in the MMS treated DNA was only able to recognize four sites when this DNA was depurinated. Therefore, action of endonuclease II on depurinated DNA would be limited to four breaks and this rules out the possibility that two separate enzymes, an N-glycosidase and a phosphodiester hydrolase, are acting sequentially. (5) If a complex of the apurinic acid enzyme and an N-glycosidase were responsible for the activity on MMS DNA then the molecular weight of the complex should be the sum of the apurinic acid endonuclease, which is 33,000 plus the N-glycosidase. Instead the molecular weight of the endonuclease II is also 33,000. (6) Phorbol ester inhibits the activity of endonuclease II on MMS treated DNA, but has no inhibitory effect on the apurinic acid endonuclease. However, phorbol ester does inhibit the activity of endonuclease II on apurinic acid (Table II). This should not be the case if sequential action of an N-glycosidase followed by the apurinic acid endonuclease was the mechanism.

The Endonucleases and Exonuclease III

Other investigators have indicated that endonuclease II (or the apurinic acid endonuclease) and exonuclease III are the same enzyme on the basis of results with mutants. In our laboratory extensive purification has shown that

these activities although they purify together can be separated. With a preparation of the apurinic acid endonuclease purified over 3000 fold the exonuclease III activity, as measured by P_i release from ^{32}P labeled DNA digested with micrococcal nuclease, can be separated from the apurinic acid endonuclease activity on a Sephadex G-100 column. The apurinic acid endonuclease has a molecular weight of 33,000 while the exonuclease III has a molecular weight of 26,000. A similar separation of endonuclease II from exonuclease III has been observed. In these cases the purification of the endonucleases has been pursued. When the exonuclease III was purified by ammonium sulfate, DEAE, phosphocellulose Sephadex G-100, hydroxyapatite and DEAE Sephadex, the activities were not separated, but experiments are continuing to resolve this problem.

7-Bromomethyl-12-Methylbenz[a]anthracene Treated DNA, A Substrate for Endonuclease II

7-Bromomethyl-12-methylbenz[a]anthracene was synthesized originally by Dipple and his colleagues and found to be a potent carcinogen in several animal test systems. It reacts with the amino groups of adenine, guanine and cytosine of DNA both *in vitro* and *in vivo*. We have shown that the enzyme endonuclease II makes phosphodiester bond breaks and also releases the hydrocarbon-purine base derivatives of DNA treated with the brominated hydrocarbon. The relationship of phosphodiester bond breaks to enzyme concentration is shown in Table III. The enzyme catalyzed release of the purine base derivatives is shown in Fig. 5 and in Table IV. After an incubation period with or without enzymes, alcohol was added to the reaction mixture to precipitate the DNA. The supernatant or alcohol soluble fraction which contained bases liberated by the enzyme was then chromatographed on Sephadex LH-20. The alcohol precipitate was hydrolyzed enzymatically to yield nucleosides and then hydrolyzed with acid to obtain the base derivatives which were also chromatographed. The chromatographic patterns of one experiment are shown in Fig. 5. By the use of marker derivatives and by subsequent examination of the peaks by thin layer chromatography it was demonstrated that the derivatives of adenine and guanine, the free bases and not the nucleosides, are liberated. Good stoichiometry of release of the purine derivatives is shown in Table IV. Further experiments showed that the rate of release of the adenine derivative was approximately four times that of the guanine derivative. From these experiments it is concluded that endonuclease II can recognize DNA treated with 7-bromomethyl-12-methylbenz[a]anthracene and break phosphodiester bonds as well as liberate N^6 [12-methylbenz[a]anthracenyl-7-methyl] adenine and N^2 [12-methylbenz[a]anthracenyl-7-methyl] guanine. No evidence for liberation of the cytosine derivative was obtained, and this suggests that the enzyme may be specific for purine derivatives.

Inhibition of Endonuclease II But Not the Apurinic Acid Endonuclease by Phorbol Ester

Phorbol myristate acetate, the active cocarcinogen in croton oil inhibits both the hydrolysis of phosphodiester bonds and the N-glycosidase activity of endonuclease II. As shown in Fig. 6, the phorbol ester inhibits phosphodiester bond hydrolysis of DNA treated with MMS or with 7-bromomethyl-12-methylbenz[a]anthracene. The hydrolysis of phosphodiester bonds in depurinated DNA by the apurinic acid endonuclease is not significantly inhibited by phorbol ester. The preparation used for these experiments contained both enzyme activities. The marked sensitivity of the hydrocarbon treated DNA (with 95% inhibition at 3.3×10^{-7} M) compared to the less sensitive and incomplete inhibition of the hydrolysis of MMS treated DNA is of note. The lack of complete inhibition could be due to some depurination, but the fact that higher concentrations of the phorbol ester

were required for 50% of the maximal inhibition suggests that the K_m for the two substrates may be different. Release of hydrocarbon-labeled bases from DNA reacted with 7-bromomethyl-12-methylbenz[a]anthracene was also inhibited by phorbol myristate acetate. The percent inhibition of release of the bases as a function of concentration of the ester is shown in Table V. Again the sensitivity to very low concentration is apparent. Careful studies at these low levels comparing inhibition of phosphodiester bond hydrolysis with inhibition of base release have not yet been done. Also the experiments described here have been done with a mixture of endonuclease II and the apurinic acid endonuclease although other experiments have shown that all the base release can be ascribed to endonuclease II and none to the apurinic acid endonuclease.

Mammalian Enzymes Active on Depurinated DNA

A search for a mammalian repair endonuclease using a somewhat heavily alkylated DNA containing depurinated sites led to the isolation and partial purification of such an enzyme from calf liver. By employing various ratios of alkylation, this activity was found to be specific for the depurinated sites present in methylmethane sulfonate-treated DNA. It has been purified some 600-1000 fold using the depurinated-reduced DNA-gel assay employed by our laboratory to isolate repair enzymes from E. coli. A summary of a typical purification sequence is presented in Table VI.

Some of the properties of this partially purified protein are similar to those reported by Ljungquist and Lindahl for an activity purified to about the same degree from calf thymus. However, the two endonucleases seem to differ markedly in many other aspects, as can be seen from Table VII. Although both enzymes are specific for depurinated DNA; introducing very few nicks into native DNA, the conditions for optimal activity vary widely.

To eliminate any false interpretations made on the basis of different assays, the enzyme from calf thymus was isolated and purified through the first few steps described by Ljungquist and Lindahl using the gel assay employed for the calf liver nuclease. This rather impure preparation was found to possess a Mg^{++} optimum and a salt sensitivity similar to that already reported by these workers, Table VII. It will be noted that the calf liver enzyme is stimulated only slightly if at all by Mg^{++}, and is much more sensitive to salt. Another interesting difference is observed when the inhibition of equal amounts of enzyme by various concentrations of transfer-RNA is compared. This substance markedly inhibits the calf liver endonuclease, but has little effect on the calf thymus enzyme.

These nucleases also appear to differ in their mode of action upon DNA. The calf thymus enzyme has been reported to introduce single-strand nicks into depurinated DNA, whereas the calf liver endonuclease seems to make both single and double-strand breaks. The different curves observed as a function of time for the production of these two types of cleavage, may indicate, however, that two separate activities are responsible for their appearance. Using exonucleases of known specificity, it was found that both the calf liver and impure calf thymus preparations introduced 3'-hydroxyl and 5'-phosphate end groups in nicked DNA. Using a different method, this result was earlier reported by Ljungquist and Lindahl for the purified calf thymus endonuclease. The position of the nick relative to the depurinated site is unknown for the calf liver enzyme. In addition to its activity on depurinated-reduced DNA, the liver endonuclease appears to attack single-stranded DNA at high enzyme concentrations, degrading it to a

certain limit, about 20 S for T4 single-stranded DNA, and then no further. A similar phenomenon has also been described for the E. coli preparation, but not for the enzyme from calf thymus, single stranded DNA apparently being resistant to all protein concentrations used. Although the activity observed with the partially purified calf liver enzyme may be due to a contaminant present in the preparation, heat inactivation studies employing both substrates yielded identical decay curves and would seem to make this explanation unlikely. That single-stranded DNA is not the primary substrate for the enzyme can be appreciated when the amount of nuclease needed to demonstrate this activity is compared to that necessary to show extensive nicking of depurinated DNA. One possible explanation for this action on single-stranded DNA is that it may be responsible for the secondary cleavage of enzyme-nicked depurinated sites, thereby introducing the double-strand breaks observed in endonuclease treated DNA. However, when DNase I-nicked DNA was treated with the calf liver preparation, no additional breaks were observed in the molecule.

A few preliminary experiments have indicated that RNA may be a substrate for the calf liver endonuclease, but it seems that this phenomenon also requires large amounts of enzyme and may be due to a contaminat. On the other hand, most DNA substrates not seen by this enzyme are also not effected by similarly active nucleases from different sources, including rat liver, calf thymus, and E. coli. Thus the endonuclease from calf liver possesses no observable activity on ultraviolet or x-irradiated DNA, the latter being tested after all the alkali-labile depurinated sites were removed by incubation with base. In addition, the preparation does not act exonucleolytically on either single or double-stranded DNAs from a number of sources. Nor does the enzyme introduce nicks, or release damaged bases from dimethylsulfate or 7-bromomethylbenz[a]anthracene treated DNA, an activity reported to be present in an E. coli preparation.

Finally, a rather vigorous search for enzymes which attack alkylated DNA has been conducted. To date, no activity on alkylated DNA-gel has been observed in various preparations and fractions from rat or calf liver, or calf thymus. The attempted isolation of base release activities from the latter two tissues has also been unsuccessful.

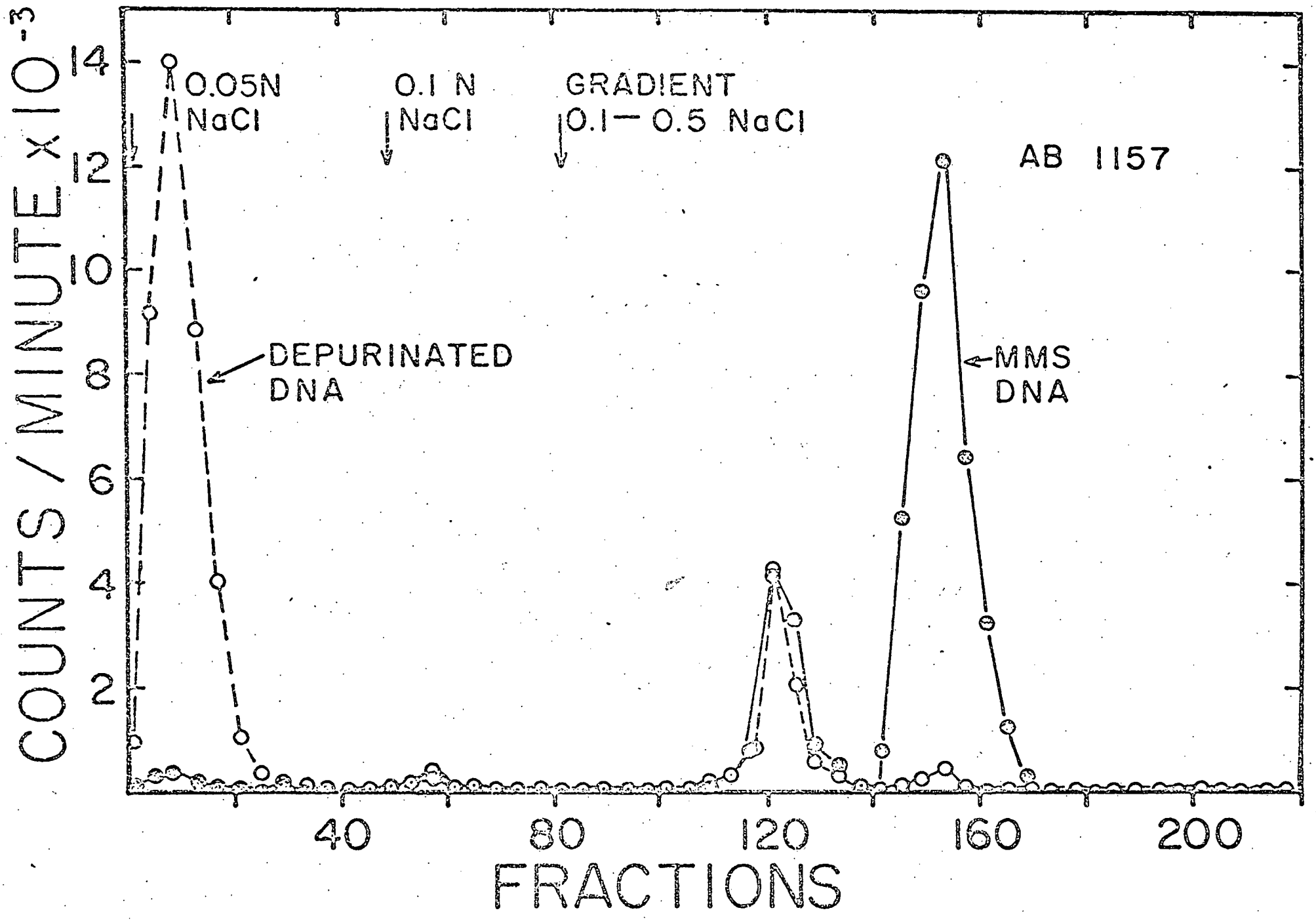

COUNTS / MINUTE x 10-3
14
12
10
8
6
4
2
0.05N NaCl
0.1 N NaCl
GRADIENT 0.1 — 0.5 NaCl
AB 1157
DEPURINATED DNA
MMS DNA
40
80
120
160
200
FRACTIONS

Figure 1

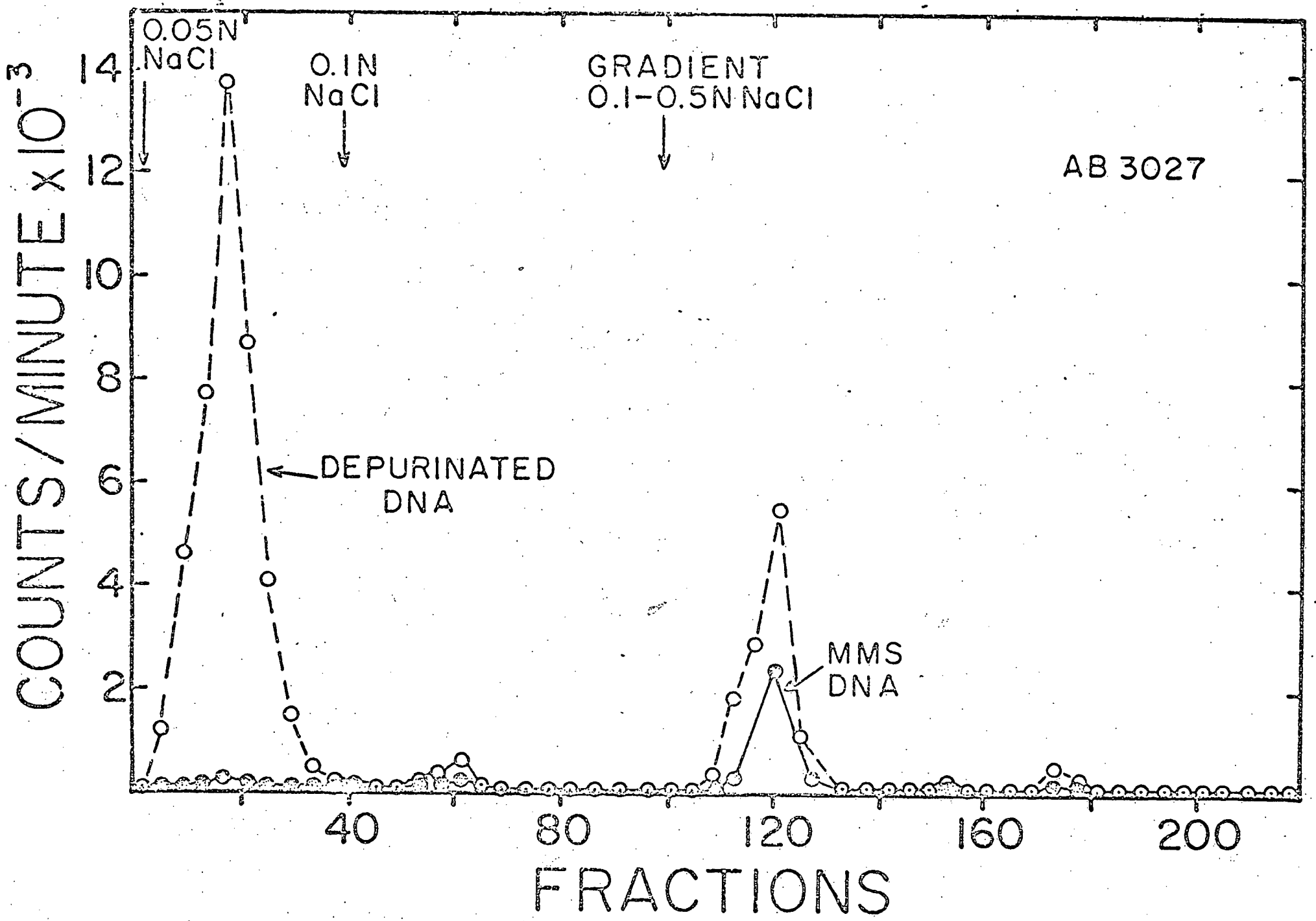

0.05N NaCl
0.1N NaCl
GRADIENT 0.1–0.5N NaCl
AB 3027
DEPURINATED DNA
MMS DNA
COUNTS/MINUTE x 10⁻³
FRACTIONS
2
4
6
8
10
12
14
40
80
120
160
200

Figure 2

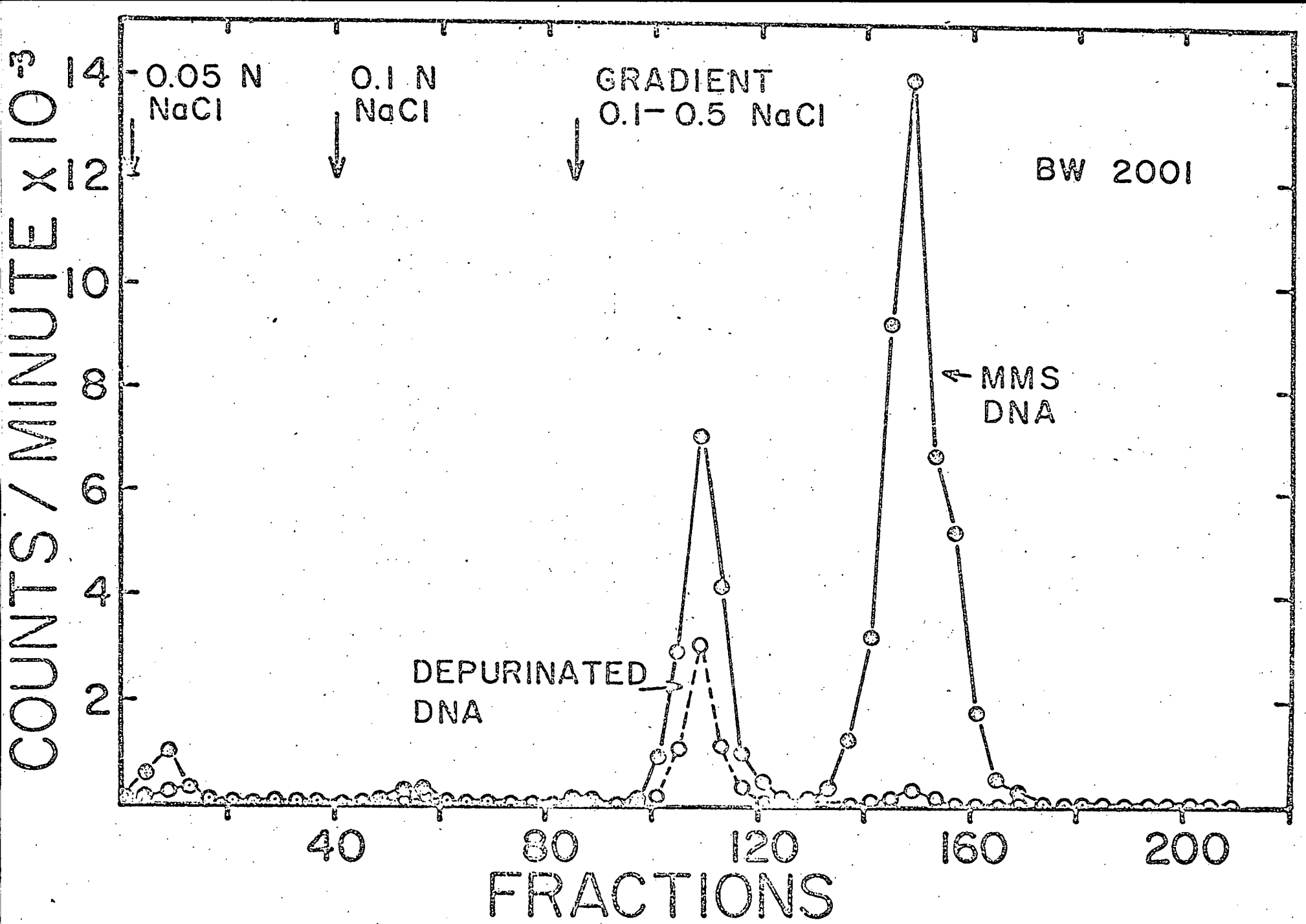
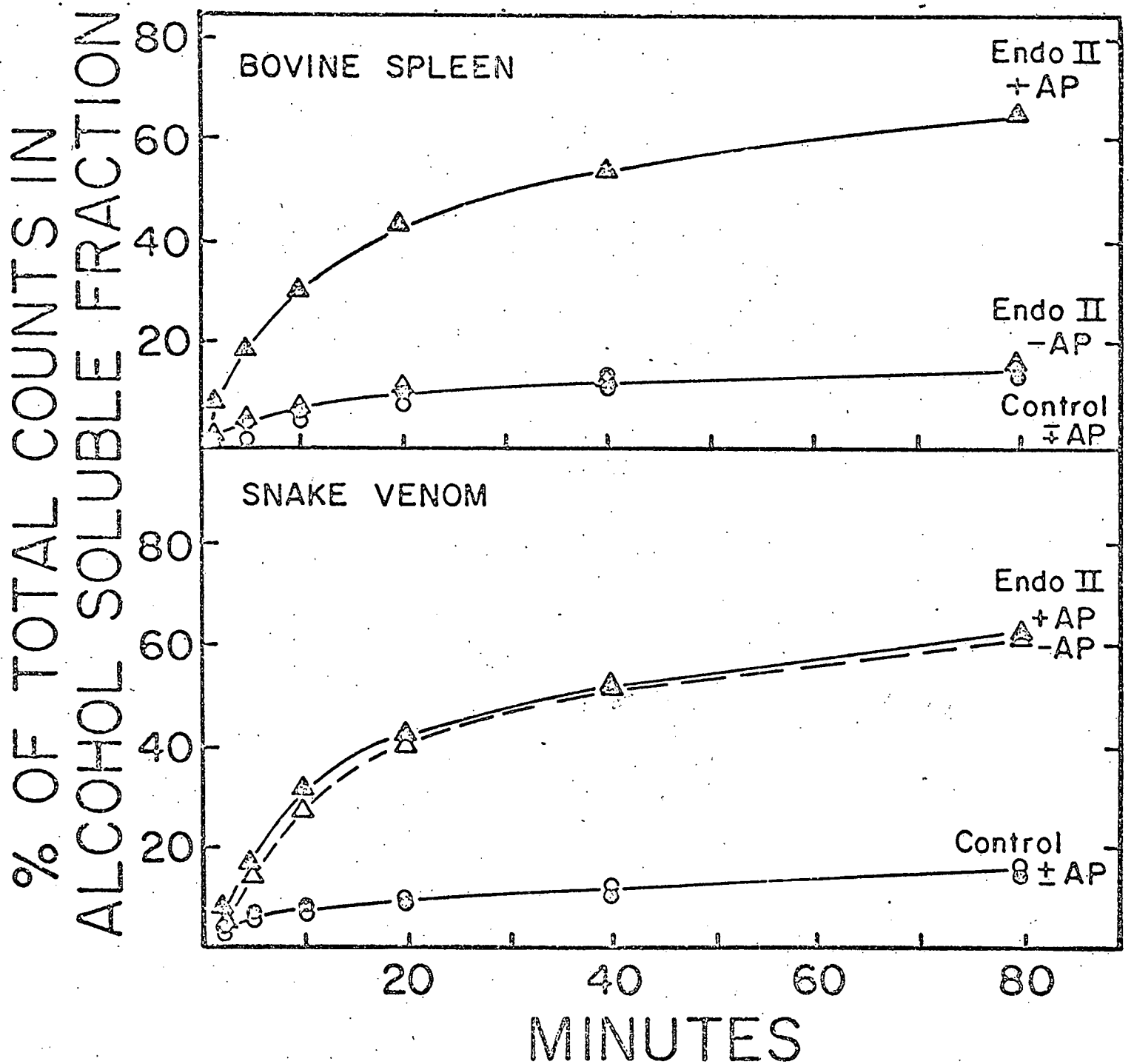

0.05 N NaCl
0.1 N NaCl
GRADIENT 0.1–0.5 NaCl
BW 2001
MMS DNA
DEPURINATED DNA
COUNTS / MINUTE x 10³
FRACTIONS
2
4
6
8
10
12
14
40
80
120
160
200

Figure 3


% OF TOTAL COUNTS IN ALCOHOL SOLUBLE FRACTION
BOVINE SPLEEN
Endo II +AP
Endo II -AP
Control ∓AP
SNAKE VENOM
Endo II +AP -AP
Control ± AP
80
60
40
20
20
40
60
80
MINUTES

Fi ;e 4

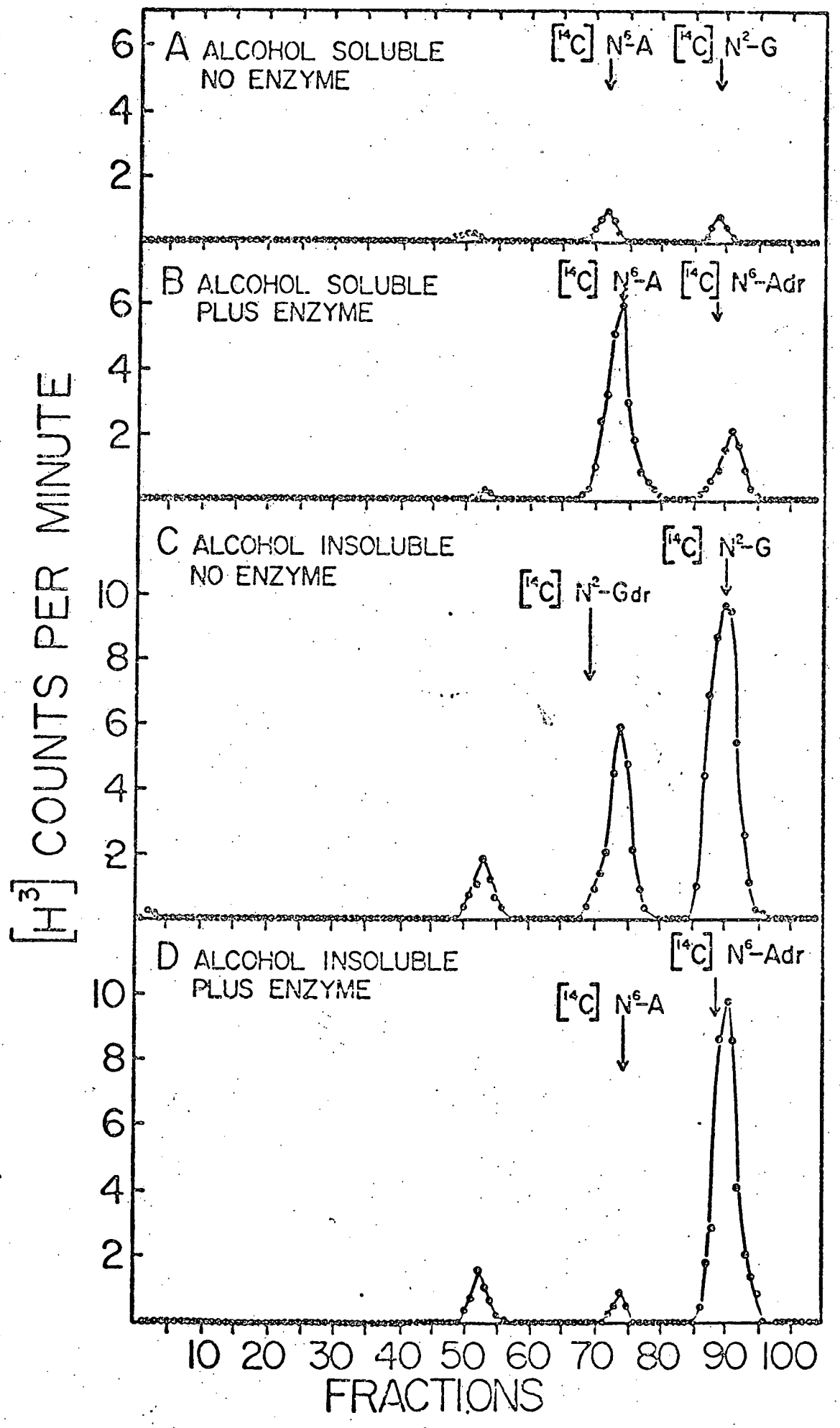

A ALCOHOL SOLUBLE
NO ENZYME
[14C] N6-A
[14C] N2-G
B ALCOHOL SOLUBLE
PLUS ENZYME
[14C] N6-A
[14C] N6-Adr
C ALCOHOL INSOLUBLE
NO ENZYME
[14C] N2-G
[14C] N2-Gdr
D ALCOHOL INSOLUBLE
PLUS ENZYME
[14C] N6-Adr
[14C] N6-A
[H3] COUNTS PER MINUTE
FRACTIONS
10 20 30 40 50 60 70 80 90 100

Fig. 5

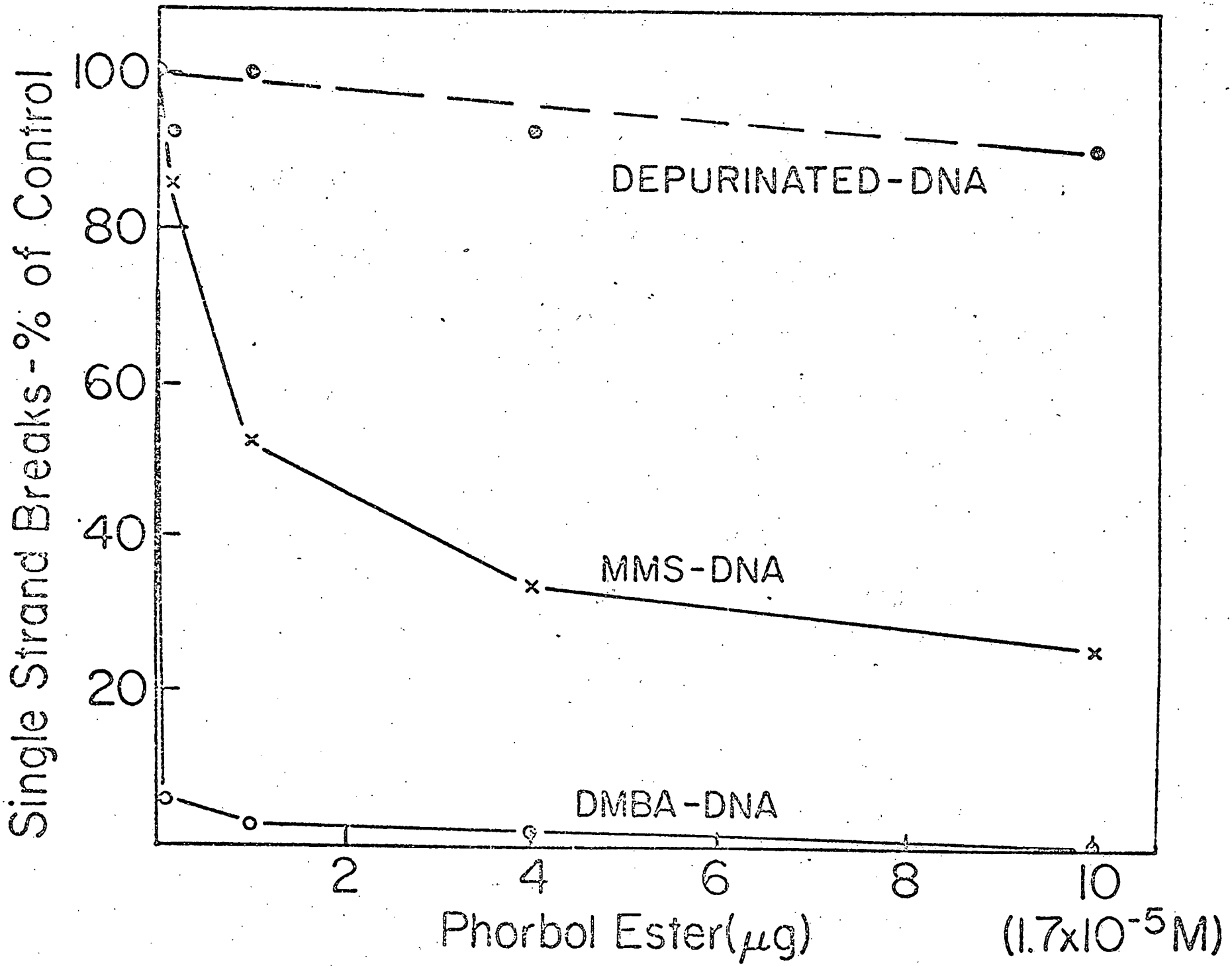

Single Strand Breaks - % of Control
100
80
60
40
20
DEPURINATED-DNA
MMS-DNA
DMBA-DNA
2
4
6
8
10
Phorbol Ester(μg)
(1.7x10⁻⁵M)

Fig. 6

Table I

The Activity of the Apurinic Acid Endonuclease and Endonuclease II on Depurinated-Reduced DNA and MMS-Treated DNA

Substrate / Enzyme	Depurinated Reduced DNA		MMS-DNA		MMS DNA→ Depurinated-Reduced	
	Endonuclease		Endonuclease		Endonuclease	
Units of Enzyme	Apurinic	II	Apurinic	II	Apurinic	II
1×10^{-3}	8	0.3	0.2	3.6	-	0.1
5×10^{-3}	21	0.4	1.4	15	20	0.4
2.5×10^{-2}	27	0.9	2.6	26	48	0.5
5×10^{-2}	69	3	3.4	50	97	2
1×10^{-1}	92	8	5.4	75	157	4

Table II

The Inhibition of Endonuclease II Activity on Depurinated Released DNA by Phorbol Ester

		Enzyme induced Breaks	
	Phorbol Ester	MMS-DNA	Depurinated-Reduced DNA
Endonuclease II	-	102	7
	+	2	4
Apurinic Acid Endonuclease	-	9	97
	+	9	97

Table III

Enzyme induced single strand breaks in DNA treated with 7-bromomethyl-12-methylbenz[a]anthracene.

Enzyme Units	Single Strand Breaks
--	3
0.01	3
0.02	6
0.04	11
0.08	16
0.16	25

[^{3}H] purine-labeled T7 DNA [sp. act. 1960 cpm per nmol DNA nucleotide] was reacted with unlabeled 7-bromomethyl-12-methylbenz[a]anthracene at a hydrocarbon to DNA nucleotide ration of 1:10 (13). Incubation mixtures (0.25 ml) contained 15 nmoles of hydrocarbon modified DNA nucleotides, 1×10^{-4}M β-mercaptoethanol, 1×10^{-4}M 8-hydroxyquinolene, 5×10^{-2}M Tris·HCl buffer, pH 8.0 and enzyme units as indicated. After 60 minutes at 37°, reactions were terminated by adding EDTA and sodium dodecylsulfate at final concentrations of 2×10^{-2}M and 0.25% respectively. The samples were then incubated in alkali [0.066M final concentration] at 37° for 20 minutes and aliquots were centrifuged through 5-20% alkaline sucrose density gradient solutions. Single strand breaks were calculated.

TABLE IV

ENZYMATIC RELEASE OF DMBA DERIVATIVES OF DNA BASES

Base Derivative	Alcohol Soluble Δ(+) vs (-) Enzyme CPM	Alcohol Insoluble Δ(+) vs (-) Enzyme CPM
N-6 DMBA Adenine	+22,423	-21,590
N-2 DMBA Guanine	+ 6,764	- 6,757
N-4 DMBA Cytosine	- 89	- 512

The incubation mixtures (1.0 ml) contained 85 nmoles DNA nucleotide [(^{3}H)-hydrocarbon-salmon sperm DNA, 0.82 mmoles carcinogen permole DNA-nucleotide, specific activity 500 cpm per nmole DNA nucleotide], $1x10^{-4}$ M β-mercaptoethanol, $5x10^{-2}$M Tris HCl, pH 8 buffer, $1x10^{-4}$M 8-hydroxyguinoline and 0.4 units of enzyme where indicated. After 60 minutes at 35° the reaction was terminated, and an alcohol soluble and an alcohol insoluble fraction were obtained. The latter was digested to nucleosides (17) and hydrolyzed to bases. The hydrolyzed sample as well as the alcohol soluble material were supplemented with [^{14}C]-labeled markers and chromatographed on Sephadex LH-20 eluted in spectroanalyzed methanol.

TABLE V

INHIBITION OF ENZYMATIC RELEASE OF DMBA-BASE BY PHORBOL ESTER

Phorbol Ester μg	% Inhibition
0	0
0.02	51
0.10	92
1.0	96
5.0	99
10.0	99

(1 μg = 1.66×10^{-6} M)

Incubation mixtures (0.25 ml) contained 60-75 nmoles [^{3}H] hydrocarbon-treated DNA, 5×10^{-2} M Tris-HCl, pH 8.0 buffer, 1×10^{-4} M β-mercaptoethanol, 1×10^{-4} M 8 hydroxyguinoline, phorbol ester as indicated. Enzyme (0.06 units for 0.02 and 0.1 μg of phorbol ester and 0.25 units for 1-10 μg) was reacted with phorbol ester 5 minutes at 0° prior to addition of substrate. After 45 and 60 minute incubation at 36° with the two enzyme concentrations, reactions were terminated by adding EDTA to final concentration of 2×10^{-2} M. The samples were supplemented with unlabeled T_4 DNA (50-100 μg per tube) and unhydrolyzed DNA was precipited out with alcohol. Alcohol soluble and alcohol insoluble radioactivity was determined.

Table VII

Calf Apurinic Acid Enzymes

Properties	Liver (Kuebler)	Thymus (Lindahl)	Thymus (Kuebler)
Purification	900	830	10-30
pH optimum	9.5	8.5	----
Max. Mg^{++} stimulation	1.3 x	1,000X	8X
Conc. Mg^{++} for max. stimulation	0.01 mM	3.0 mM	0.5-2.0 mM
50% inhibition by salt	0.023 M	0.2 M	0.12 M
Stimulation by low salt	none	----	> 4 fold at 0.01 M
DNA breaks	SS and ds	SS	----
M.W.	29,000	32,000	

The principal investigator has complied with the contract requirements to the best of his knowledge. He has devoted 20% of his time to this project since its start and he will continue to devote the same percentage.

Publications resulting from work supported by this contract:

1. Kirtikar, D. M., Kuebler, J. P., Dipple, A., and Goldthwait, D. A. (1976) Endonuclease II of E. coli and Related Enzymes 6th International Symposium of the Princess Takamatsu Cancer Research Fund (in press).

2. Kirtikar, D. M., Kuebler, J. P., Dipple, A. and Goldthwait, D. A. (1976) Enzymes Involved in Repair of DNA Damaged by Chemical Carcinogens and γ-Irradiation. Eighth Miami Winter Symposium. Academic Press (in press).