Repair of DNA Treated with γ-Irradiation and Chemical Carcinogens

Progress Report

for period of February 1, 1976-February 28, 1977

David A. Goldthwait

Department of Biochemistry

Case Western Reserve University

Cleveland, Ohio 44106

Prepared For

The U. S. Energy Research and Development Administration

Under Contract No. EY-76-S-02-2725. #2

DISCLAIMER

ABSTRACT

1. Enzymes from E. coli which recognize damage in DNA due to chemical carcinogens and γ-irradiation are under study. Endonuclease II, which recognizes DNA treated with methylmethane sulfonate, methylnitrosourea, 7-bromomethyl-12-methylbenz[a]anthracene and γ-irradiation. Apurinic endonuclease (endonuclease VI) recognizes apurinic and apyrimidinic acid sites. These enzymes have now been purified to homogeneity and separated from exonuclease III which other groups have thought to be identical with one or both of the endonucleases.

A series of mutants of E. coli were found to lack one, two, or all of these enzymes. The genetic loci for the two endonucleases are very close to the Exonuclease III locus at 38.2 minutes. An attempt to relate in vivo sensitivity to MMS to lack of specific enzymes has been made.

2. Substrates for endonuclease II have been examined. β-propiolactone treated DNA has been found to be a substrate and details of this are being examined. The nature of the lesion in γ-irradiated DNA is under investigation.

3. A search for mammalian enzymes which are N-glycosidases is underway.

Progress Report for ERDA

In last year's proposal for renewal of contract No. E(11-1)2725, three areas of research for the ensuing year were outlined:

1. Purification and study of the mechanism of action of an E. coli enzyme which recognizes DNA treated with methylnitrosourea, 7-bromomethyl-12-methylbenz[a]anthracene and γ-irradiation.

2. Exploration of further substrates for (1) including the chemical nature of the enzyme sensitive sites in γ-irradiated DNA.

3. Purification of similar mammalian enzymes.

A discussion of progress in the past year will follow this outline.

1. Endonuclease II and Apurinic Acid Endonuclease of E. coli

a. Enzyme purification and separation from exonuclease III

Endonuclease II is an enzyme which to date has two activities--a phosphodiesterase and an N-glycosidase. The enzyme recognizes DNA treated with methylmethane sulfonate (MMS), methylnitrosourea (MNU), 7-bromomethyl-12-methylbenz[a]anthracene, and γ-irradiation. This enzyme was first isolated and described in this laboratory. A second enzyme, apurinic acid endonuclease, is also present in E. coli and this enzyme recognizes apurinic sites in DNA. In another laboratory it has been claimed that endonuclease II, was the same enzyme as exonuclease III, a enzyme which degrades in a 3' → 5' direction and also is a 3' phosphatase.

A large percentage of our time in the past year has been spent on purification of these three enzymes to demonstrate that they are separate entities. This work with all the appropriate references is publication number 1 listed at the end of the report, and a summary of this work follows.

The apurinic acid endonuclease can be separated from endonuclease II by DEAE chromatography (Fig. 1). When Fraction III, a 45-70% ammonium sulfate precipitate, was applied to a DEAE column, the apurinic acid endonuclease (Peak I) was eluted with 0.1 M NaCl prior to the gradient. This activity was assayed by the gel method using DNA with very few depurinated-reduced sites. This enzyme has been purified 11,000 fold in this laboratory, to a single band on SDS gel electrophoresis. It has a molecular weight by gel filtration of 31,500 (Table I).

Endonuclease II is eluted with 0.25 M NaCl as Peak III (Fig. 1). Endonuclease II has been purified in this laboratory over 12,500 fold to a single band on SDS gel electrophoresis. Its molecular weight on gel filtration is 33,000 and by SDS gel is 34,500 (Table I).

Exonuclease III is an enzyme which can be separated from the apurinic acid endonuclease and from endonuclease II. Therefore, it is not, the same enzyme as the apurinic acid endonuclease or endonuclease II. On DEAE chromatography, exonuclease III elutes in an area overlapping the apurinic acid endonuclease in Peak I (Fig. 1). A purified preparation of exonuclease III provided by Dr. Richardson was eluted in the same position. It is apparent that the exonuclease III activity does not coincide exactly with the apurinic acid endonuclease.

Exonuclease III could be partially separated from the apurinic acid endonuclease in early stages of purification because of a difference in molecular weights. When a 500 fold purified fraction of the apurinic acid endonuclease was passed through a Sephadex G-100 column, the two activities overlapped, but did not coincide. In this experiment, the molecular weight of the exonuclease III was 25,500 as opposed to that of the apurinic acid endonuclease of 31,500.

Exonuclease III was purified 2300 fold. The evidence that this was exonuclease III was as follows: (1) the major purification steps of the published procedure were followed. (2) the enzyme preparation possessed a 3'-phosphatase as well as the ability to release acid soluble fragments from labeled DNA. (3) the P_i released by this exonuclease III preparation was comparable to the P_i released from the same substrate by alkaline phosphatase. (4) the enzyme activity was inhibited 95% by 3×10^{-5} M $ZnCl_2$. (5) Dr. C. C. Richardson very kindly provided us with a sample of exonuclease III for comparison with our preparation. The ratio of P_i release to acid soluble nucleotide release was the same for the two preparations. (6) the chromatographic behavior of both preparations of the DEAE column was similar. (7) the molecular weight by gel filtration of the exonuclease III purified in this laboratory was 25,500 to 26,000 while the molecular weight of Dr. Richardson's preparation was 26,000. Therefore, although the preparation purified in this laboratory was not homogeneous, we can conclude that it is exonuclease III by the above criteria.

Exonuclease III purified in this laboratory had no endonucleolytic activity on either apurinic acid sites or alkylated sites in DNA (Fig. 2A, 2B). T4 DNA was depurinated and then reduced with $NaBH_4$ to prevent alkali catalyzed phosphodiester bond hydrolysis (4). After incubation with or without exonuclease III or the apurinic acid endonuclease, samples were examined in the alkaline sucrose gradients. Fig. 2A shows that exonuclease III was unable to recognize depurinated sites which were recognized by the apurinic acid endonuclease. Likewise, exonuclease III was unable to recognize sites in the DNA due to alkylation with MMS (Fig. 2B). These sites were recognized by endonuclease II. The 3' phosphatase and exonuclease activity of exonuclease III with the conditions noted above was comparable to that observed with Mg^{++} at pH 7.0.

The apurinic acid endonuclease purified 11,00 fold has negligible exonuclease III activity as measured by P_i release (Table I). Thus the apurinic acid endonuclease activity can be shown not to coincide with the exonuclease III acitivity on DEAE and G-100 Sephadex columns and the purified endonuclease does not have significant levels of exonuclease III. The low activity observed with alkylated DNA (Table 1) may be due to depurinated sites in this substrate. Endonuclease II activity, isolated from the DEAE column did not have any contaminating exonuclease III activity (Fig. 1, Peak III). Also in the 12,500 fold purified preparation, there was no significant exonuclease III 3'-phosphatase activity (Table 1). The low activity observed with depurinated reduced DNA seems to be an intrinsic property of the enzyme.

Exonuclease III, therefore, has no endonucleolytic activity directed against depurinated or alkylated sites, and as noted above, the purified apurinic acid endonuclease and the endonuclease II preparations have no significant exonuclease III activity.

One basis of the claim by other workers that exonuclease III and endonuclease II were the same protein was the use of the DNA-gel assay. The conclusion was that a homogeneous protein with a molecular weight of 28,000 had exonuclease, 3'-phosphatase, and endonuclease activity. If DNA is released from the gel by exonucleolytic activity, then an erroneous conclusion could be drawn. To measure the "endonuclease II" activity of the purified protein the other workers used a method, which was originally described in our laboratory, in which DNA is entrapped in a polyacrylamide gel and then alkylated heavily with MMS. We show here that exonuclease III can make double strand breaks in heavily alkylated DNA (Fig. 2C) under conditions where there is no endonucleolytic activity (Fig. 2A, 2B). T4 DNA was treated at the high MMS to nucleotide ratio and then used as a substrate. After incubation with the enzyme, the DNA was examined in neutral sucrose gradients to look for double strand breaks (Fig. 2C). It is apparent that double strand breaks occur with the exonuclease III preparation which does not make single strand breaks at either apurinic (Fig. 2A) or alkylated (Fig. 2B) sites. We suspect that the heavily alkylated DNA can undergo chemical depurination and at some of these depurinated sites, β-elimination with phosphodiester bond hydrolysis occurs. This creates sites for exonucleolytic action. If two sites are near but on opposite strands, exonucleolytic action will result in a double strand break. Double strand breaks are required to release DNA from a polyacrylamide gel. Therefore, we feel that the endonucleolytic activity observed to be associated with the exonucleolytic activity was actually due to the exonucleolytic action of the purified exonuclease III, which was able to produce double strand breaks without making single strand breaks.

A false assay was also observed in this laboratory with depurinated DNA. Exonuclease III, lacking the apurinic acid endonuclease activity (Fig. 2A) was chromatographed on Sephadex G-100 and the fractions examined both by the P_i release assay for exonuclease III, and by the gel assay using DNA with a small number of depurinated sites. Both activities were observed, but the endonucleolytic activity on the gel was very low compared to the P_i release and very low compared to the usual release by the apurinic acid endonuclease (see also Table I). The experiments described indicate that exonuclease III does not recognize apurinic or alkylated sites in DNA, but does give false positive reactions especially when heavily alkylated DNA is used.

b. Mutations in E. coli Altering Apurinic Acid endonuclease, Endonuclease II, and Exonuclease III.

An analysis of the levels of these enzymes is a series of mutants of E. coli was done to then relate the sensitivity in vivo to the presence or absence of any of these specific enzymes.

Assay for enzymes required the long laborious procedure of growing 50 gms of cells, treating the sonicated extract with streptomycin sulfate and ammonium sulfate and then after dialysis chromatographing the extract on DEAE and analyzing all the fractions for the three enzyme activities. The results with the wild type strain, AB 1157, are shown in Fig. 3A and appear similar to those shown in Fig. 1.

The strain AB 3027 was isolated by Dr. Paul Howard-Flanders. This was derived by treatment of AB 1157 with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and selected by its inability to grow in the presence of 1.2 mM MMS. When this strain

was examined by DEAE cellulose chromatography, the results, shown in Fig. 3B, were obtained. AB 3027 lacks both exonuclease III and endonuclease II.

This strain, AB 3027, has also been shown by others to lack DNA polymerase I. In order to obtain a strain with a normal DNA polymerase I, met E (very close to pol I) was transduced by others into AB 3027 and transductants were selected for their ability to grown on 1.2 mM MMS. The resulting strain, NH 5016, had normal levels of DNA polymerase I. The DEAE elution profile of NH 5016 is shown in Fig. 3C. Exonuclease III and endonuclease II were both absent as in AB 3027.

Strain BW 2001 was isolated by Yajko and Weiss by treating AB 1157 with MMNG and selecting a clone, the extract of which had a decreased ability to release heavily alkylated DNA from a gel. It was found to be deficient in exonuclease III and in an endonucleolytic activity on heavily alkylated DNA. The DEAE elution profile of this mutant is shown in Fig. 4A. Exonuclease III is missing as well as the apurinic endonuclease, but endonuclease II is present in normal levels.

The strain BW 2001 grows less rapidly than the parent strain. A spontaneous revertant, BW 2007, was selected by Yajko and Weiss because of its more rapid growth and therefore larger colony size. The DEAE elution profile, (Fig. 4B), shows that this mutant is lacking exonuclease III but has now a normal level of the apurinic endonuclease.

Another derivative of BW 2001 was constructed by the transduction of pheS and pps markers into BW 2001, followed by the transduction of these markers plus xthA1 into AB 1157. The DEAE elution profile of this mutant BW 2030, is shown in Fig. 4C. It is apparent that the apurinic endonuclease has returned to normal but exonuclease III is still absent. Peaks II and III are close together because of malfunction of the collector as explained in the legend.

Another revertant of BW 2001 was isolated by Yajko and Weiss because of its MMS resistance and was called BW 2021. The profile of this mutant is shown in Fig. 4D where the level of the apurinic endonuclease is normal and the exonuclease III level approaches normal.

A deletion mutant BW 9101 was defined by Yajko and Weiss in which the deletion included the pncA gene and extended into or through the exonuclease III (xth gene). It was of interest to note that in the DEAE elution profile (Fig. 5A) all three enzymes, apurinic endonuclease, endonuclease II and exonuclease III were absent. This suggests that the genes for all of these enzymes lie in the region of the xth gene located by White et al. (1976) at 38.2 minutes on the revised map.

The deletion mutant BW 9109 was obtained by Dr. Weiss by transduction of the deletion mutant, BW 9101, with the pncA marker into AB 1157. The DEAE profile of this mutant (Fig. 5B) is similar to that of BW 9109.

Milcarek and Weiss (1972) isolated a series of mutants of AB 1157 produced by N-methyl-N'-nitro-N-nitrosoguanidine which showed altered levels of exonuclease III. One of the mutants, BW 9093, had a thermosensitive exonuclease III which after a purification of 710 fold, showed an activity at 25° which approached that of the parent strain, but at 42°, was more rapidly inactivated than the wild type enzyme. This strain, grown at 25° in our laboratory, was used to prepare fraction

III for DEAE column chromatography. The profile, shown in Fig. 8A, has no detectable exonuclease III, but normal levels of apurinic endonuclease and endonuclease II.

Another mutant, BW 9059, isolated by Milcarek and Weiss (1972), was obtained by transduction of the pheS and pps markers into BW 9099. The latter was an exonuclease III mutant which was obtained in a fashion similar to BW 9093 and which had a low level of enzyme even at 25°. The DEAE profile of BW 9059, grown at 42° C is shown in Fig. 6B. Again, as in the other mutant, exonuclease III is absent while the endonuclease levels are normal.

A summary of the specific activities of peaks I and III of the DEAE elution patterns is presented in Table II. Fractions through each entire peak of activity were pooled and concentrated and this material was then tested with the different substrates: depurinated-reduced DNA in the gel, MMS treated DNA in the gel, and [^{32}P] DNA with 3' phosphomonoesters. Small differences in the numbers are not significant since the number of fractions pooled varied from column to column. Where no figures are given, there was no activity in the column fractions and therefore no fractions were pooled. The level of exonuclease III was measured only in peak I since it was present only there. Both endonuclease substrates were used. The activity observed in peak I with the MMS-DNA is approximately 1-2% of that observed with depurinated-reduced DNA and may be due to depurinated sites in the MMS DNA. In peak III, the level of activity found with depurinated reduced DNA is approximately 5-10% of that observed with MMS DNA. This ability to recognize depurinated DNA is a property of endonuclease II which has been observed even after purification of the enzyme to homogeneity.

Experiments were then done to determine the degree of sensitivity in vivo of the parent and mutant strains to methylmethane sulfonate. The wild type and mutant strains of E. coli were exposed to 0.05 M methylmethane sulfonate for varying times after which they were plated. Survival curves, determined for the wild type, for AB 3027 and for NH 5016 are shown in Fig. 7. As noted previously by others, AB 3027 was extremely sensitive to MMS. This mutant lacks polymerase I, exonuclease III and endonuclease II. The mutant NH 5016 with a normal polymerase but deficient in exonuclease III and endonuclease II was slightly more sensitive to MMS than wild type in their experiments, but in our laboratory had approximately the same sensitivity as the wild type strain.

The mutant strain, BW 2001, which lacked the apurinic endonuclease as well as exonuclease III, was also extremely sensitive to MMS both in the experiments of Yajko and Weiss (1975) and as shown in Fig. 8. This mutant was not as sensitive as AB 3027. The revertant BW 2007 which had regained the apurinic endonuclease but not the exonuclease III, had approximately the same sensitvity as BW 2001 (Fig. 8) while the other revertant BW 2021, with normal levels of both endonucleases and exonuclease III, was slightly more resistant than wild type. The mutant BW 2030, constructed from BW 2001, and lacking only exonuclease III had a sensitivity which was intermediate between BW 2001 and BW 2007 (Fig. 8).

The deletion mutants BW 9101 andBW 9109 were unusual in that in spite of a lack of apurinic endonuclease, endonuclease II and exonuclease III, they were slightly more resistant to MMS than the wild type strain (Fig. 9).

The mutants in which there is a defective exonuclease III were then examined (Fig. 10). BW 9093 was tested at 25° C and found to be less sensitive than the wild type, but at 42° it was considerably more sensitive. This mutant was shown by Milcarek and Weiss (1972) to have a thermolabile exonuclease III. BW 9059 which at 42° did not have exonuclease III was considerably more sensitive than wild type at 42° (Fig. 10) but at 37° it was only slightly more sensitive.

Thus, from these results the following conclusions can be drawn:

1. Evidence for separate genetic sites for endonuclease II, apurinic acid endonuclease, and exonuclease III has been provided.

2. The loss of all three enzyme activities in the deletion mutants localizes the part of the E. coli chromosome where both endonuclease genes reside to approximately 38.2 minutes.

3. Sensitivity in vivo to MMS when present is associated with an absence of exonuclease III. However, exonuclease III can be absent in cells such as the deletion mutants where no MMS sensitivity exists. The reasons for this are not clear.

2. Exploration of further substrates for Endonuclease II.

A series of experiments have been done with β-propiolactone treated DNA which has been found to be a substrate for endonuclease II. Varying concentratins of β-propiolactone have been tested to determine the optimum amount of alkylation for in vitro experiments. The pH optimum for the reaction has been determined. Experiments to determine the stability of the substrate have been done. Enzyme concentration curves for activity vs. DNA treated with β-propiolactone or MMS, or depurinated DNA have been determined. All of this work has been done utilizing the sucrose gradient technique. Preliminary experiments utilizing both thin layer chromatography and the high pressure liquid chromatography system for isolation of alkylated bases have been done.

Experiments have been started to determine the nature of the site in γ-irradiated DNA recognized by Endonuclease II. Because many of the experiments for this work require sucrose gradients, we are in the process of designing equipment which will collect gradients simultaneously and drip fractions onto filter paper. We have had many problems with the pump system and as yet do not have this resolved. We have also worked out a new computer program for analysis of gradients. Preliminary experiments have been done with several scavengers -- the objective being to determine the nature of the radical which produces the sites in DNA sensitive to endonuclease II. No definitive data has been obtained yet.

More work is continuing with MNU treated DNA and because of the cost, we have been synthesizing our own [^{3}H] MNU from [^{3}H] methylamine. A cold and a hot synthesis have been done, the latter with 25 mc and in both cases the expected 60% yield was obtained.

All of the work with carcinogenic substances -- the synthesis of MNU and the reaction of MNU and β-propiolactone has been done in a biohazard hood in a special laboratory established for this purpose. This hood, as well as other equipment such as the refrigerator, and pH meter were provided by this ERDA Contract. The work would not have been possible without this.

3. Mammalian enzymes

A search has started for an N-glycosidase activity in mammalian cells comparable to that of Endonuclease II. This has taken several tacks:

a. Assay methods

1. Rapid assay for analysis of column fractions. This has involved working out conditions for passage of the reaction mixture over mini-DEAE columns to collect the bases liberated in a volume small enough for assay by liquid scintillation counting.

2. High pressure liquid chromatography analysis of methylated bases. We have finally developed a system for separation of the alkylated purines which gives no cross contamination between the peaks and which can be done in twenty minutes.

b. Examination of tissues

Ehrlich ascites cell extracts have been chromatographed on DEAE columns and fractions have been analyzed. A number of peaks have been obtained which on further analysis appear to be due to exonuclease activities.

Human placenta is now under investigation. Again DEAE column fractions have been examined and one area looks like it might contain an N-glycosidase. Work is progressing on this.

Publications

1. Kirtikar, D.M., Cathcart, G.R. and Goldthwait D.A. Endonuclease II, Apurinic Acid Endonuclease and Exonuclease III. Proc. Nat. Acad. Sci. 73, 4324-4328, 1976.

2. Kirtikar, D.M., Cathcart, G.R., White, J.G., Ukstins, I., and Goldthwait, D.A. Mutations in E. coli altering apurinic Endonuclease, Endonuclease II and Exonuclease III and their Effect on in vivo Sensitivity to Methylmethane Sulfonate (Submitted to Biochemistry).

FIGURE LEGENDS

Figure 1. Chromatographic Behavior of Apurinic Acid Endonuclease, Endonuclease II and Exonuclease III on DEAE Cellulose. Fraction III (2), a 40-75% ammonium sulfate precipitate, (396) mg) was dialyzed against buffer C (0.05 M Tris HCl pH 8.0, 0.1 mM DTT and 20% glycerol), and then applied to a DE-52 column 2.5 x 48 cm. Elution was with one column volume of buffer C plus 0.03 M NaCl and then as indicated in Fig. 1. Ten ml fractions were collected and 50 µl of every fourth fraction was assayed for apurinic acid endonuclease, endonuclease II and exonuclease III.

For the endonuclease assays, labeled DNA was entrapped in a polyacrylamide gel which was forced through a screen to produce gel particles (14). Depurinated reduced DNA gel was prepared by suspending the gel in 4 vols of 0.1 M sodium citrate buffer, pH 3.5 plus 0.1 mM EDTA and heating at 45° for 30 minutes. The gel was then cooled, the pH was adjusted to 6.5 with NaOH, and then potassium phosphate buffer pH 6.5, was added to a final concentration of 0.5 M. Aldehyde groups at depurinated sites were reduced with $NaBH_4$. to prevent spontaneous β-elimination with phosphodiester bond hydrolysis. A final concentration of 0.25 M $NaBH_4$ was attained by three additions at 15 minute intervals at room temperature. After an incubation of 60 more minutes, the DNA gel was washed in 0.05 M Tris HCl pH 8.0 and resuspended in the same buffer. These conditions produce approximately one depurinated site per 1550 nucleotides[3]. The MMS-treated DNA gel was prepared by incubation of the DNA gel for 120 minutes at room temperature in 0.05 M Tris-HCl, pH 8.0 with MMS at a molar ratio of MMS to DNA nucleotide of 500 to 1. Gels were washed extensively with 0.05 M Tris-HCl pH 8.0, and resuspended in the same buffer. The packed DNA gels contained 60 to 80 nmoles of DNA nucleotide per ml of packed volume. Both types of gels were used on the day of their preparation. Incubation mixtures contained 12 to 16 nmoles of DNA substrate, 0.05 M Tris HCl pH 8.0, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M DTT and 1.5 mg of bovine serum albumin in a volume of 1.5 ml. Incubations were at 37° for 30 min and were stopped with 0.1 ml of 1% SDS. After dilution with water to 2.0 ml and centrifugation, a 1.0 ml aliquot was counted in a liquid scintillation counter. One unit represents 1 µmole DNA nucleotide released per hour.

Exonuclease III was assayed by its 3' phosphatase activity. E. coli [^{32}P]-DNA (2 x 10^4 cpm/nmole) was digested with micrococcal nuclease until 30% was acid soluble; the higher molecular weight material remaining after dialysis was used (15). Incubation mixtures contained 50 nmoles of DNA, 10^{-3} M β-mercaptoethanol, 10^{-2} M $MgCl_2$, and potassium phosphate 0.066 M, pH 7.0 in a volume of 0.3 ml. After incubation for 30 minutes at 37°, 0.5 mg of calf thymus DNA was added followed by 0.5 ml of 10% TCA. The radioactivity which did not absorb to Norit charcoal was determined. One unit of 3' phosphatase activity is defined as the amount of enzyme able to release 1 nmole of [^{32}P] per 30 minutes at 37°. Exonuclease III activity was also measured by counting the 5% TCA soluble material from the [^{32}P] DNA used for the 3' phosphatase assay after a 30 minute incubation at 37°.

Figure 2. Sucrose Gradient Analysis of Exonuclease III Action on Depurinated-Reduced DNA, Lightly Alkylated DNA and Heavily Alkylated DNA. The reaction mixtures contained 0.05 M Tris-HCl pH 8.0, 10^{-4} M β-mercaptoethanol, 10^{-4} M 8-hydroxyquinoline, plus treated DNA and enzyme units as indicated in Fig. 2 in a volume of 0.25 ml. Depurinated [^{3}H] T4 DNA was prepared by heating the DNA at pH 3.5 at 37° for 60 minutes, conditions which produced approximately 1 depurinated site per 1150 nucleotides. Incubations were for 1 hr at 37° and were stopped by the addition of SDS and EDTA at final concentrations of 0.05% and 0.02 M respectively. Centrifugation

was through 3.6 ml of 5-20% alkaline or neutral sucrose gradients in an SW 56 Spinco rotor.

a. Alkaline sucrose gradient fractionation of depurinated reduced DNA. The reaction mixture was as described in Methods and contained 10 nmoles depurinated reduced DNA (2.4×10^3 cpm/nmole), plus the indicated units of either exonuclease II or apurinic acid endonuclease purified approximately 2000 fold. Centrifugation was at 35,000 rpm for 3 hours at 20° C.

b. Alkaline sucrose gradient fractionation of lightly alkylated DNA. T4 DNA was alkylated at a molar ratio of MMS to DNA nucleotide of 10:1 as described in Methods. The reaction mixture contained 7 nmoles of alkylated DNA (1.3×10^3 cpm/nmole) and the indicated units of 1500 fold purified endonuclease II. Centrifugation was at 30,000 rpm for 3 hours at 20° C.

c. Neutral sucrose gradient fractionation of heavily alkylated DNA. The DNA was alkylated at a molar ratio of 6000:1, MMS to DNA nucleotide, as described. The reaction mixture contained 23.6 nmoles DNA (4.7×10^3 cpm/nmole) and enzyme as indicated. The sedimentation in the neutral gradient was at 28,00 rpm for 3 hours.

Figure 3. DEAE-Cellulose Column Profile of AB 1157, AB 3027 and NH 5016. In all profiles, endonuclease levels were tested in every fourth fraction. Exonuclease III was tested in every fourth fraction between 1 and 100. Where no values are shown, the level of enzyme activity is insignificant. Fractions 1-100 were eluted with 0.- N NaCl and fractions 100-260 were eluted with a linear 0.1-015 N NaCl gradient (arrow) as described in Methods. AB 1157 was grown at 42° C, AB 3027 at 37° C, and NH 5016 at 37° C. The activities are presented in nmoles per ml of fraction in Figure 3-6.

Figure 4. DEAE-Cellulose Column Profile of BW 2001, BW 2007, BW 2021, and BW 2030. BW 2001, BW 2007 and BW 2021 were grown at 42° C; BW 2030 was grown at 37° C. The profile for strain BW 2030 shows peak II and III close together. Between fractions 135 and 150 the collector malfunctioned and tubes overflowed. Sodium chloride molarities for fractions were as follows: 120, 0.1; 130, 0.115; 140, 0.16; 150, 0.23; 160, 0.27; 170, 0.295, 180, 0.35. If the gradient was linear, peak III would be displaced to the right by at least 8 fractions.

Figure 5. DEAE-Cellulose Column Profile of BW 9101 and BW 9109. Both strains were grown at 37° C.

Figure 6. DEAE-Cellulose Column Profile of BW 9093 and BW 9059. BW 9093 was grown at 25° C and BW 9059 at 42° C.

Figure 7. In Vivo Sensitivity to MMS of AB 1157, AB 3027 and NH 5016. Strains were tested at 37° C.

Figure 8. In Vivo Sensitivity to MMS of AB 1157, BW 2001, BW 2007, BW 2021, and BW 2030. Strains were tested at 37° C.

Figure 9. In Vivo Sensitivity to MMS of AB 1157, BW 9101 and BW 9109. Strains were tested at 37° C.

Figure 10. *In Vivo* Sensitivity to MMS of AB 1157, BW 9093 and BW 9059. AB 1157 was tested at 37° C and 42°; BW 9093 was tested at 25° and 42° and BW 9059 was tested at 37° and 42° C.

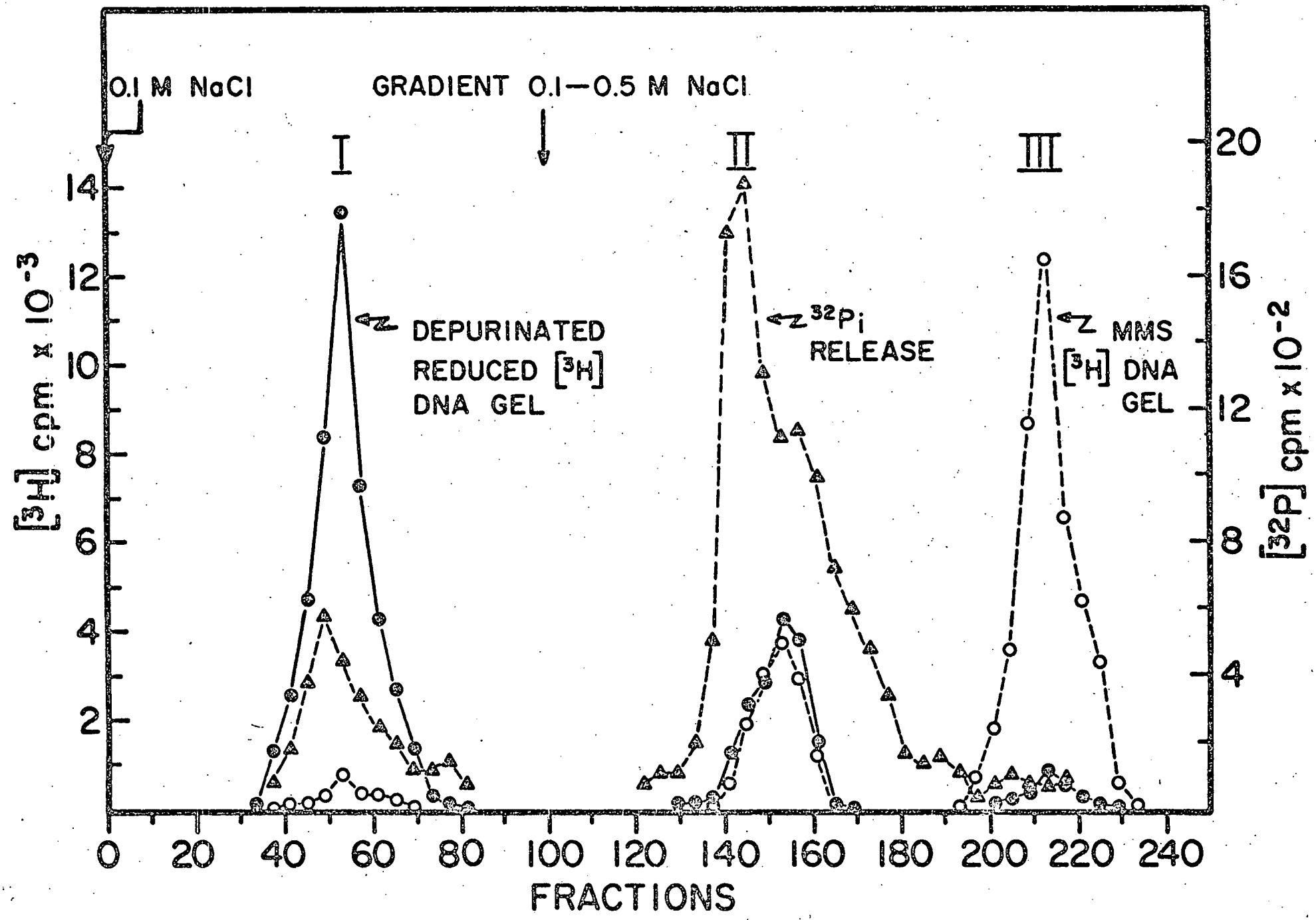

0.1 M NaCl
GRADIENT 0.1—0.5 M NaCl
I
II
III
DEPURINATED REDUCED [3H] DNA GEL
32Pi RELEASE
MMS [3H] DNA GEL
[3H] cpm x 10-3
[32P] cpm x 10-2
FRACTIONS
0
20
40
60
80
100
120
140
160
180
200
220
240
2
4
6
8
10
12
14
4
8
12
16
20

Figure 1

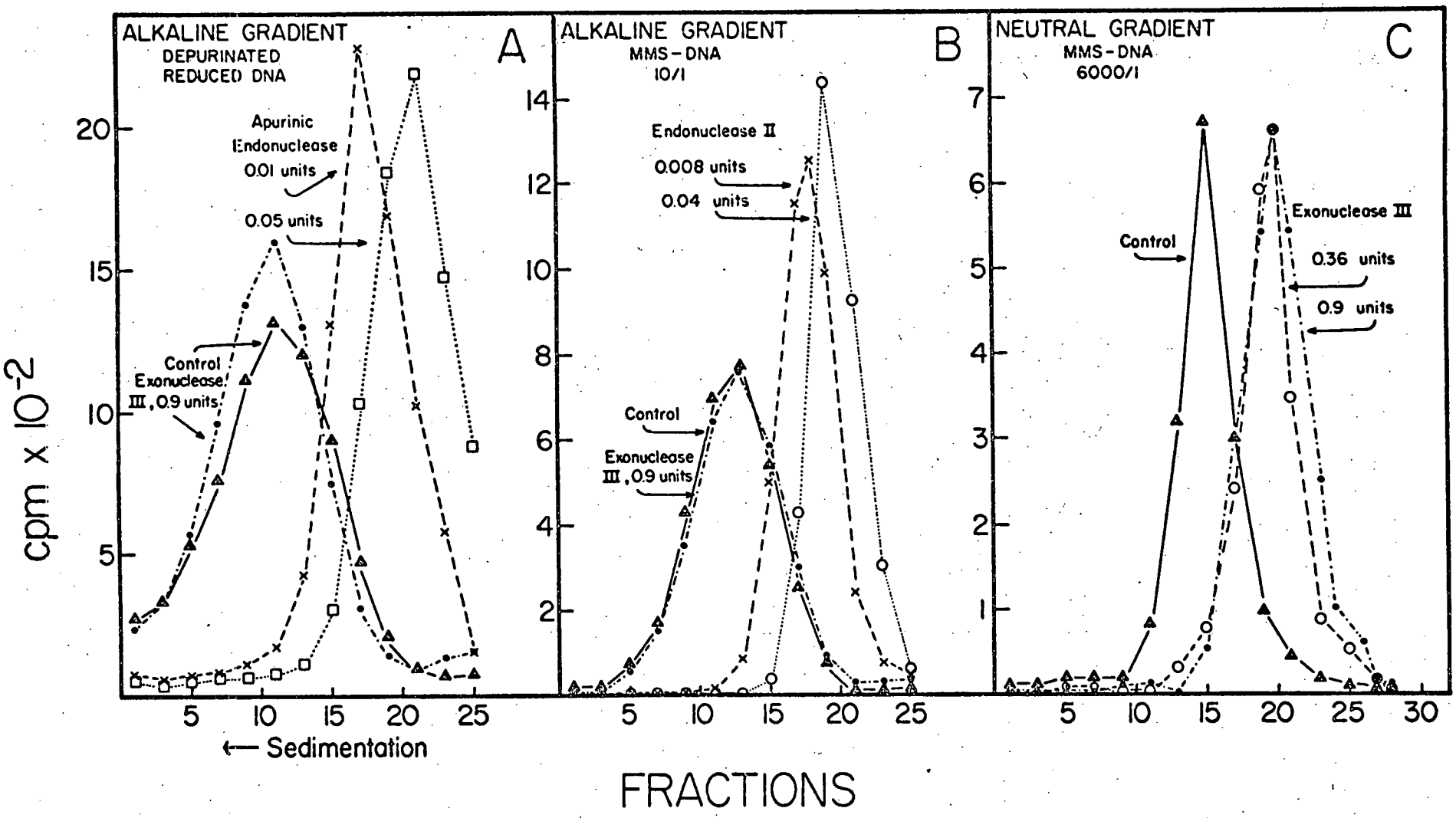

ALKALINE GRADIENT
DEPURINATED
REDUCED DNA
A
Apurinic
Endonuclease
0.01 units
0.05 units
Control
Exonuclease
III, 0.9 units
ALKALINE GRADIENT
MMS-DNA
10/1
B
Endonuclease II
0.008 units
0.04 units
Control
Exonuclease
III, 0.9 units
NEUTRAL GRADIENT
MMS-DNA
6000/1
C
Control
Exonuclease III
0.36 units
0.9 units
cpm x 10-2
← Sedimentation
FRACTIONS

Figure 2

Figure 3

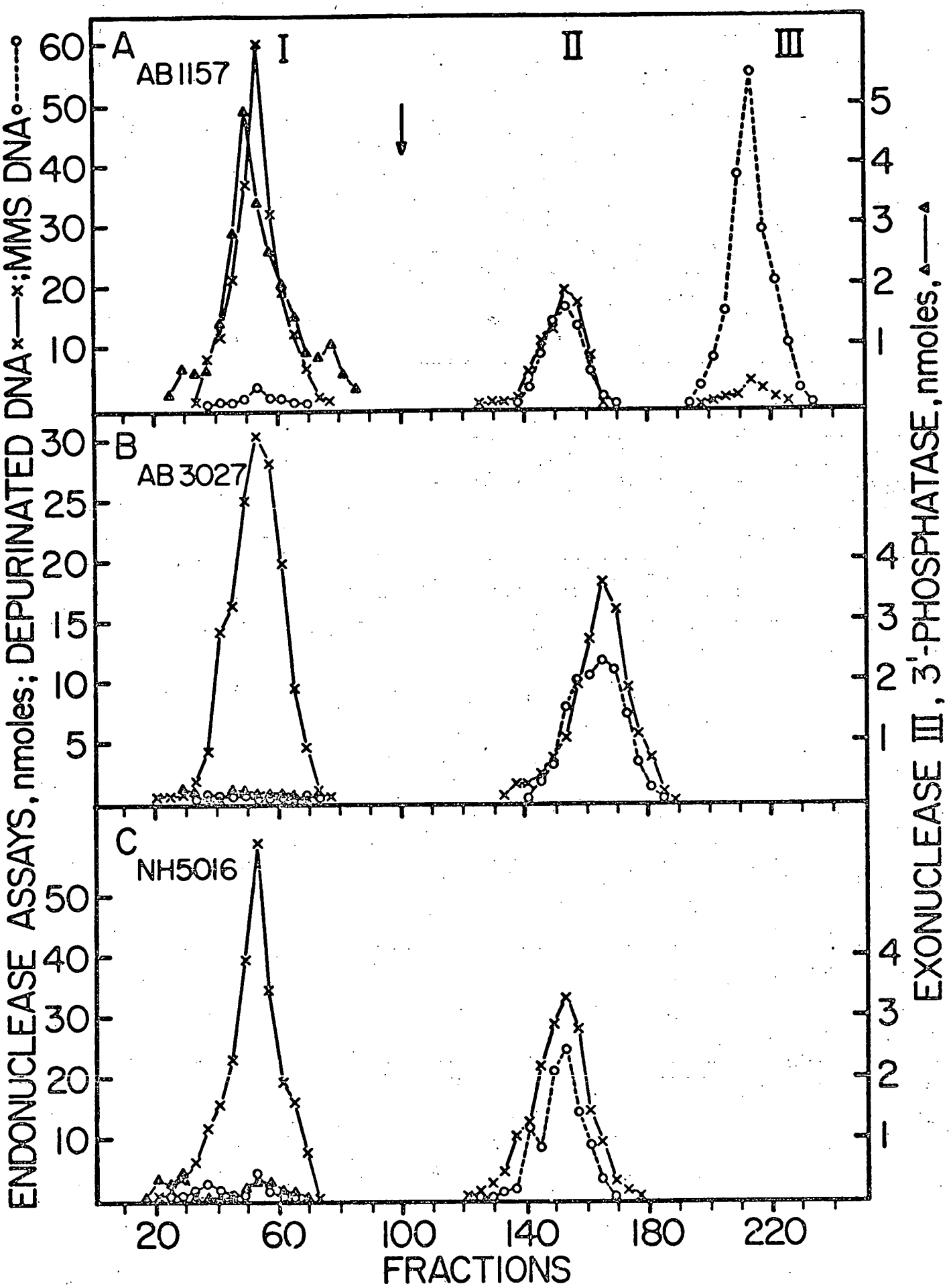
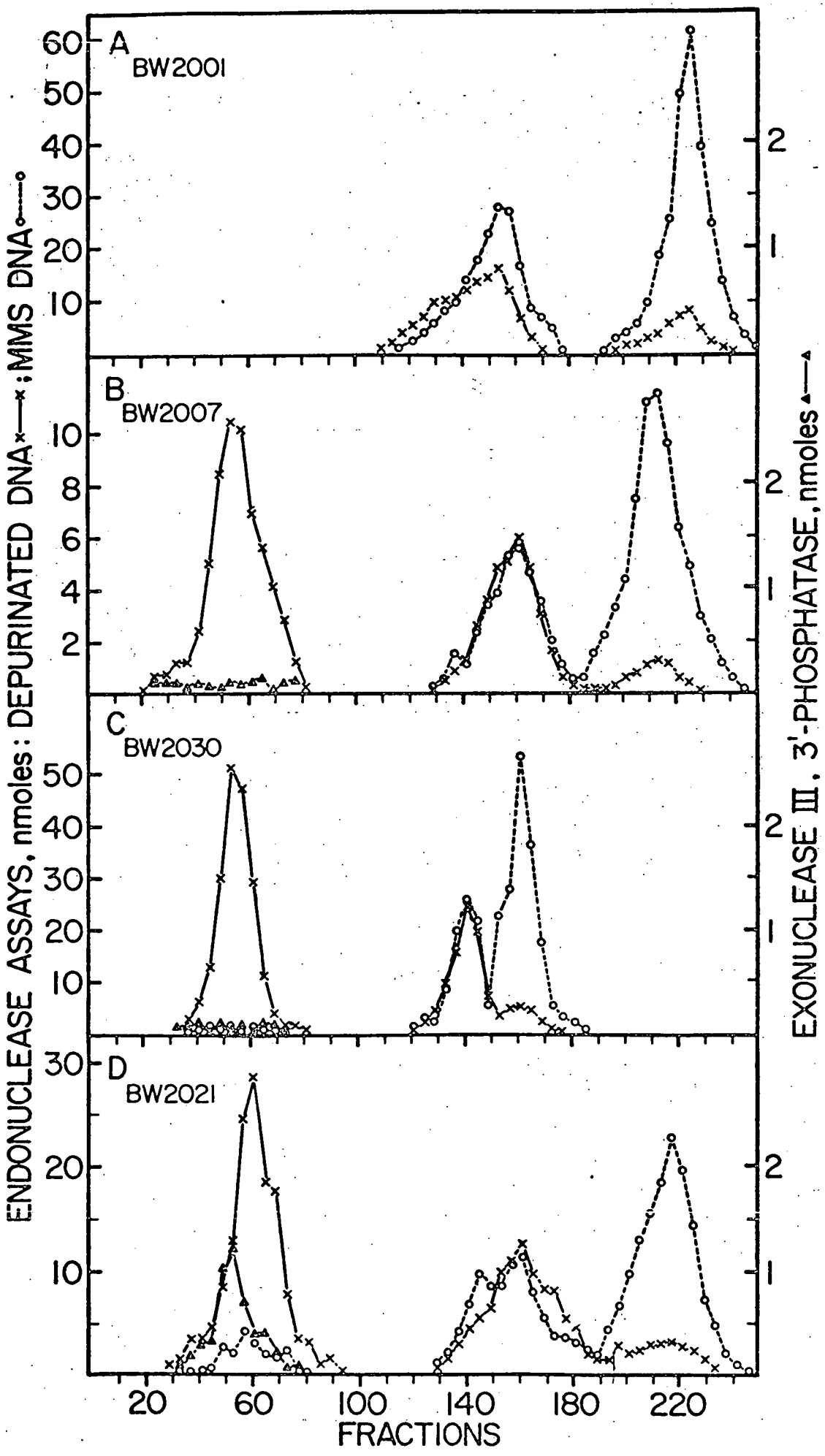

A
AB1157
I
II
III
B
AB3027
C
NH5016
ENDONUCLEASE ASSAYS, nmoles; DEPURINATED DNA×—×; MMS DNA○----○
EXONUCLEASE III, 3'-PHOSPHATASE, nmoles, ▲—▲
FRACTIONS
20
60
100
140
180
220

Figure 4


A
BW2001
B
BW2007
C
BW2030
D
BW2021
ENDONUCLEASE ASSAYS, nmoles: DEPURINATED DNA×—×; MMS DNA○—○
EXONUCLEASE III, 3'-PHOSPHATASE, nmoles ▲—▲
FRACTIONS
20
60
100
140
180
220

A
BW9101
B
BW9109
ENDONUCLEASE ASSAYS, nmoles, DEPURINATED
DNA ×—×; MMS DNA °----°
EXONUCLEASE III 3'-PHOSPHATASE, nmoles
FRACTIONS
20
60
100
140
180
220
4
8
12
16
20
2
4
6
8
10
1
2
3
4

Figure 5

A
BW9093
B
BW9059
ENDONUCLEASE ASSAYS, nmoles:
DEPURINATED DNA ×—×; MMS DNA °—°
EXONUCLEASE III, 3'-PHOSPHATASE, nmoles, ▲—▲
FRACTIONS
2
4
6
8
10
12
14
16
5
10
15
20
25
30
35
20
60
100
140
180
220

Figure 6

Figure 7

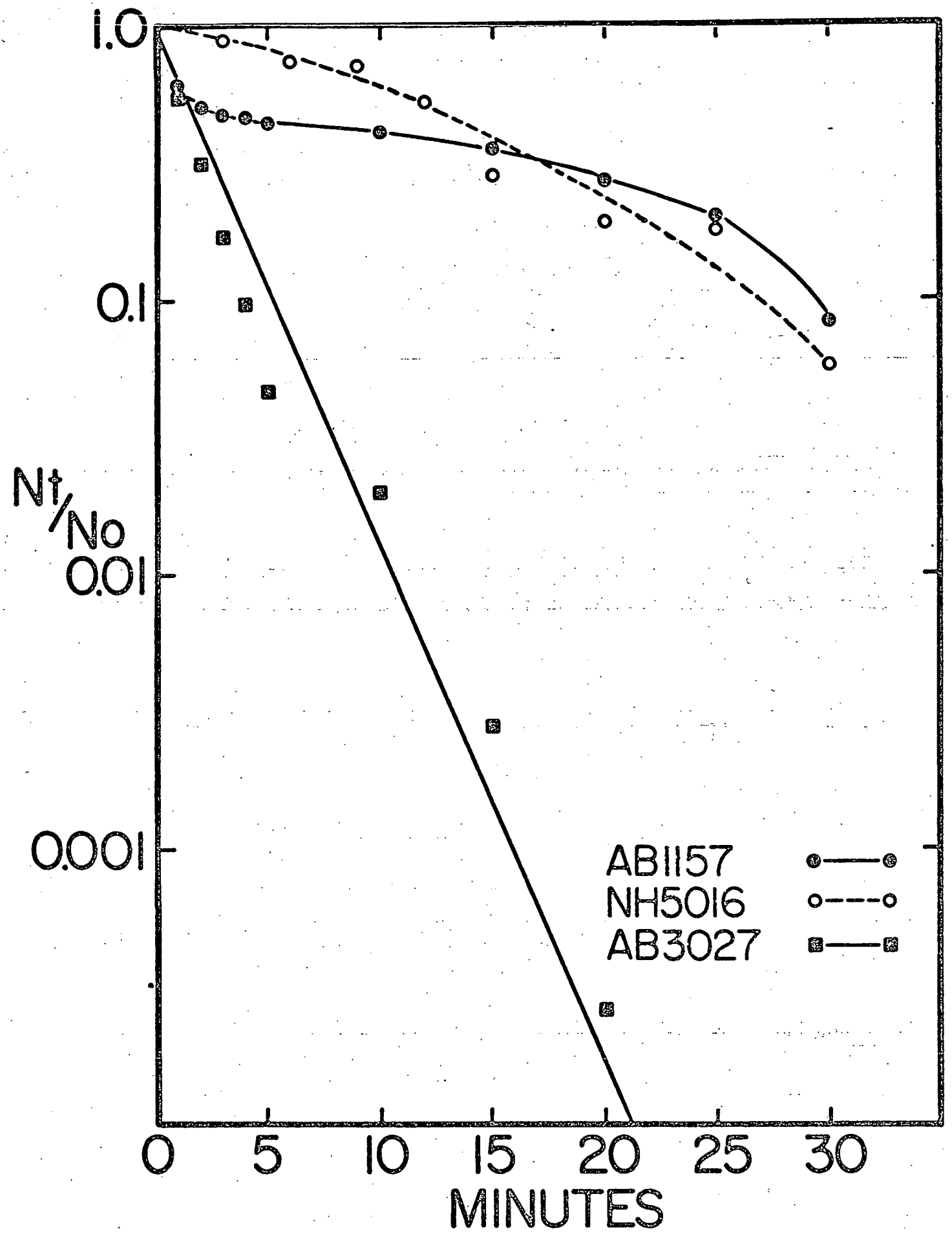
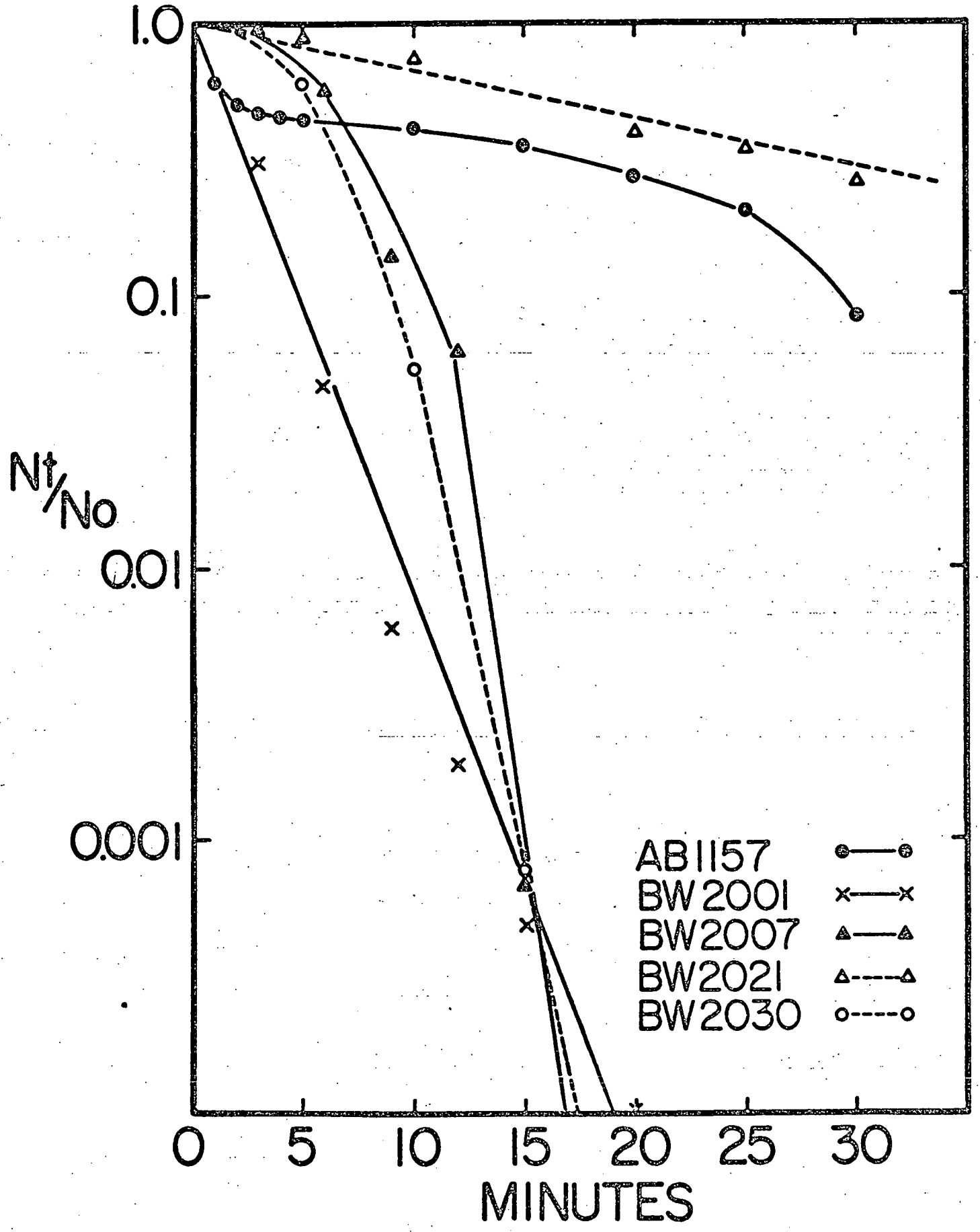

1.0
0.1
0.01
0.001
Nt/No
0
5
10
15
20
25
30
MINUTES
AB1157
NH5016
AB3027

Figure 8


1.0
0.1
0.01
0.001
Nt/No
0
5
10
15
20
25
30
MINUTES
AB1157
BW2001
BW2007
BW2021
BW2030

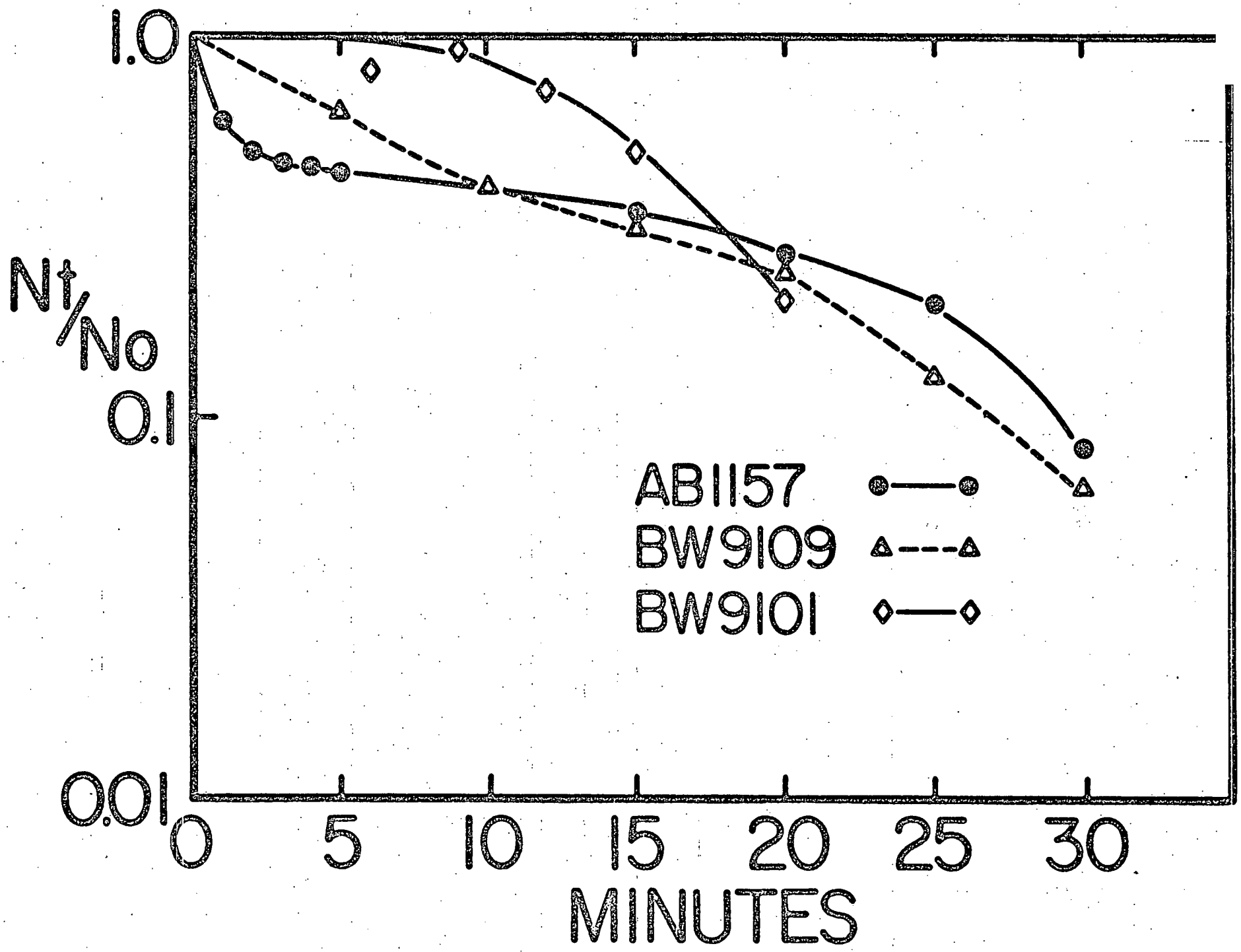


Figure 9

Figure 10

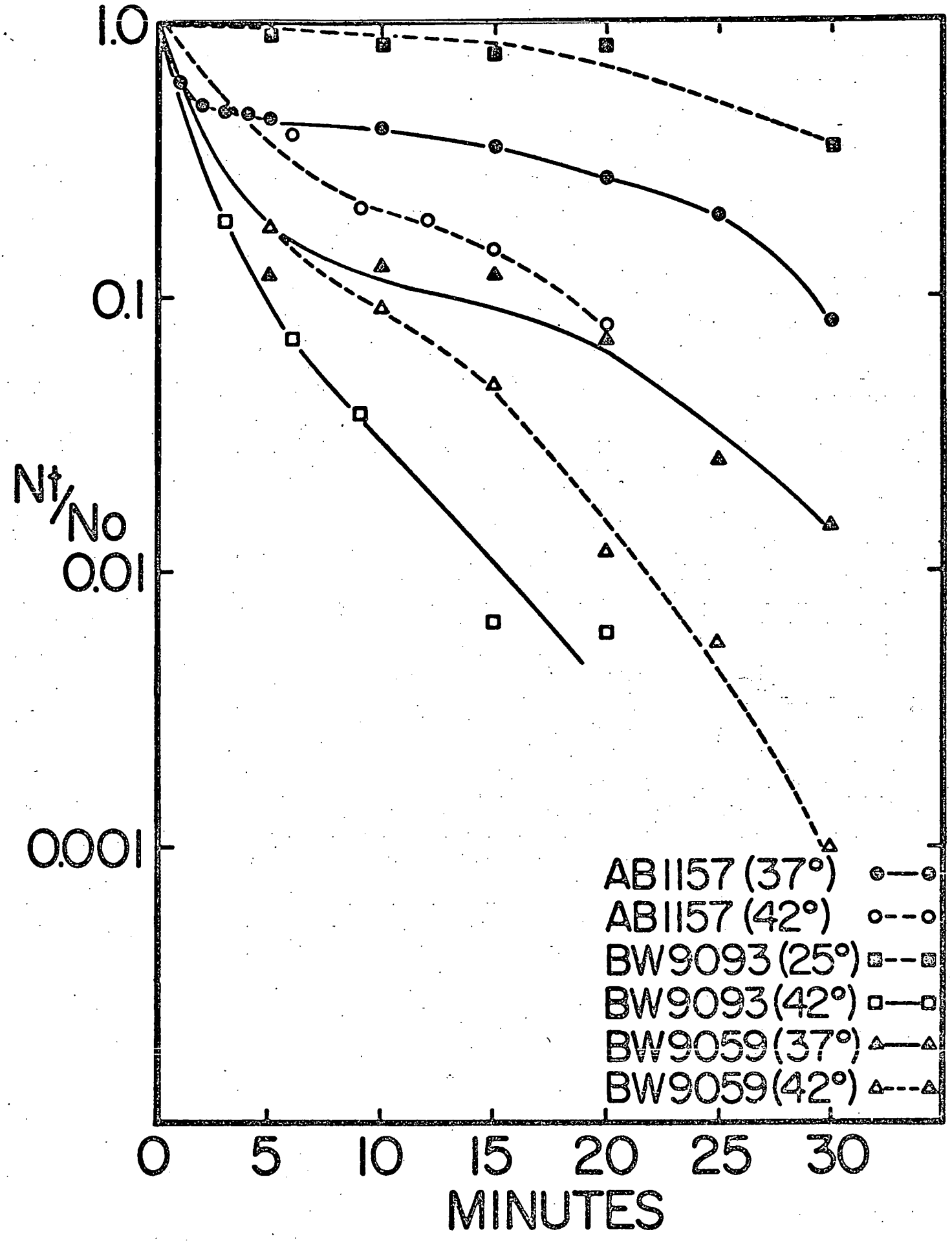

1.0
0.1
0.01
0.001
Nt/No
0
5
10
15
20
25
30
MINUTES
AB1157 (37°)
AB1157 (42°)
BW9093 (25°)
BW9093 (42°)
BW9059 (37°)
BW9059 (42°)

Table I

Activities of Purified Enzymes on Various Substrates

ENZYME	FOLD PURIFICATION	UNITS per mg	Percent units per unit of enzyme tested		
			MMS treated DNA gel	Depurinated reduced DNA gel	[^{32}P] release 3'-phosphate
Endonuclease II	12,500	295	(100)	7.1	0
Apurinic Acid Endonuclease	11,000	365	12.4	(100)	0
Exonuclease III	2,300	2300	0.046*	0.044*	(100)

*Determined by the gel assay; no activity when determined by the gradient technique (Fig. 2).

TABLE II

ENZYME ACTIVITIES IN DEAE PEAKS I AND III

Strain	Temp. of Growth	Peak I Depurinated Reduced DNA µm/mg/hr	Peak I MMS DNA µm/mg/hr	Peak I 3' Phosphatase nm/mg/30 min	Peak III Depurinated Reduced DNA µm/mg/hr	Peak III MMS DNA µm/mg/hr
AB 1157	37°	0.69	0.006	32.2	0.059	0.68
AB 1157	42°	0.40	0.009	4.5	0.064	0.44
AB 3027	37°	0.54	0.007	0.44	-	-
NH 5016	37°	0.57	0.009	0.11	-	-
BW 2001	42°	-	-	-	0.047	0.51
BW 2007	42°	0.54	0.011	0.37	0.036	0.55
BW 2030	37°	0.42	0.008	0.23	0.039	0.49
BW 2021	42°	0.48	0.008	13.1	0.021	0.54
BW 9093	25°	0.40	0.009	1.3	0.030	0.52
BW 9059	42°	0.51	0.008	0.44	0.031	0.53

BW 9101 and 9109 had no apurinic endonuclease or exonuclease III activity in peak I and no endonuclease II activity in peak III. The fractions in each peak were pooled and concentrated prior to measurement of specific activity. Where a blank (-) appears, no enzyme activity could be detected in the column fractions and therefore they were not pooled.

TABLE III

THE RELATIONSHIP OF APURINIC ENDONUCLEASE, ENDONUCLEASE II AND EXONUCLEASE III TO *IN VIVO* SENSITIVITY TO MMS IN THE PARENT AND MUTANT STRAINS

Strain		Apurinic Endonuclease	Endonuclease II	Exonuclease III	MMS Sensitivity
AB 1157		+	+	+	1
AB 3027		+	-	-	4
NH 5016		+	-	-	1
BW 2001		-	+	-	3-4
BW 2007		+	+	-	3
BW 2030		+	+	-	3
BW 2021		+	+	+	0
BW 9101		-	-	-	0
BW 9109		-	-	-	0
BW 9093	25°	+	+	±	0
	42°	N.D.*	N.D.	N.D.	3
BW 9059	37°	N.D.	N.D.	N.D.	1
	42°	+	+	-	2

MMS sensitivity is graded on a scale of 0-4 with 1 representing the parent strain, 4 the most sensitive strain and 0 the least sensitive strains.

*N.D. means not done.

Progress Report (continued)

The principal investigator has complied with the contract. He has devoted 20 percent of his effort in the past and will continue to do so.