patible with the experimental system being studied and free from serious ambiguity. Some experimental results may be unsuitable for work with the present methods. The confidence to be held in the final results is dependent on the quality of the experimental data: perfect measurements will lead to essentially perfect results and error-containing data will produce approximations, good or poor, depending on the amount of error in the data.

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Endonuclease II of *Escherichia coli*. Degradation of Partially Depurinated Deoxyribonucleic Acid*

Sheikh-Mumtaz Hadi and David A. Goldthwait†

ABSTRACT: Endonuclease II of *Escherichia coli*, which was partially purified on the basis of its ability to catalyze single- and double-strand breaks in DNA alkylated with methylmethanesulfonate, has been shown to degrade partially depurinated DNA. DNA either entrapped in an acrylamide gel or free in solution was converted to a substrate by heating at various temperatures at pH 3.5. Depurination and molecular weight studies, a melting curve, viscosity, and density studies indicated that the heating at various temperatures produced either double-stranded or single-stranded partially depurinated DNA. The exact nature of the single- and double-stranded forms is not clear. The depurinated DNA was unstable in alkali, but could be stabilized by treatment with $NaBH_4$ or with NH_2OH. After either treatment, the DNA was still a substrate for the enzyme.

Endonuclease II of *Escherichia coli* hydrolyzes phosphodiester bonds of alkylated DNA. The enzyme makes both single- and double-strand breaks in DNA alkylated with the monofunctional alkylating agent methylmethanesulfonate. It also makes a limited number of single-strand breaks in native T-4 and T-7 DNA (Friedberg and Goldthwait, 1968, 1969; Friedberg *et al.*, 1969). Work in this laboratory, which has characterized this enzyme from *E. coli*, was started with the belief that distortions in DNA structure were responsible for the endonucleolytic scissions required for recombination (Friedberg and Goldthwait, 1968). Alkylation of DNA was considered to be a chemical means of producing distortions which might be analogous to those produced biologically. Previous work by others (Reiter *et al.*, 1967; Strauss and Robbins, 1968) aided in the initiation of our work.

Endonuclease II has been purified some 1600-fold on the basis of its ability to degrade alkylated DNA. Experiments have been designated to determine whether the enzyme will hydrolyze phosphodiester bonds of double-stranded DNA the structure of which is altered by other methods. In this paper, the activity of the enzyme on depurinated and depurinated reduced DNA is described.

Materials and Methods

Enzyme Preparation. The original purification procedure (Friedberg and Goldthwait, 1969) has been modified and the

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† National Institutes of Health Research Career Award Fellowship KG-gm-21444; to whom to address correspondence.

details will be published elsewhere. Preparation of the crude extract, fractionation with streptomycin and then with ammonium sulfate, the first three steps in the original purification, have been retained. Further purification involved fractionation on columns of phosphocellulose, Sephadex, and finally DNA-cellulose. The enzyme has been purified approximately 1600-fold by these procedures and the final fraction used for the experiments reported had a specific activity of 210 units/mg. One unit of activity is defined as the amount of enzyme capable of liberating 1 μmole of DNA nucleotide/hr from a standard amount of DNA gel alkylated with methylmethanesulfonate (Friedberg and Goldthwait, 1969). The purified enzyme can be stored at −15° in 0.05 M Tris-HCl (pH 8.0) with 20% glycerol for several months without loss of activity. Occasional thawing and refreezing does not appear to inactivate the enzyme.

DNA. [^{3}H]Thymine-labeled T-4 DNA was prepared by phenol extraction of T-4 phage grown on *E. coli* B-3 (thy$^-$) (Friedberg and Goldthwait, 1969). This DNA had a specific activity of 1–2 × 10^3 cpm/nmole of DNA nucleotide. [^{3}H]-Purine-labeled DNA was prepared in a similar manner from T-4 bacteriophage grown on the same strain with [^{3}H]hypoxanthine. The specific activity of this DNA was 130 cpm/nmole of DNA nucleotide.

Assay. The use of alkylated DNA entrapped in polyacrylamide gel as a substrate for the assay of endonuclease II of *E. coli* has been described (Friedberg and Goldthwait, 1969; Melgar and Goldthwait, 1968). Nonalkylated DNA gel, prepared in a similar fashion, was depurinated rather than alkylated and used for many of the experiments described in this paper. Depurinated DNA gel suspensions with 10–20 nmoles of DNA were added to incubation mixtures (1.5 ml) with a final concentration of 0.05 M Tris-HCl (pH 8.0), 0.006 M $MgCl_2$, and 10^{-4} M β-mercaptoethanol or dithiothreitol. Incubations were for 30 min at 37° unless otherwise noted and reactions were stopped by the addition of 0.5 ml of 0.2% sodium dodecyl sulfate. After centrifugation for 5 min at 3000 rpm, 1.0-ml aliquots of the supernatant fraction were used for the determination of radioactivity by scintillation counting (Friedberg and Goldthwait, 1969).

Depurinated DNA in Acrylamide Gel. Depurination of the DNA was done by first suspending one volume of the packed DNA gel in approximately nine volumes of 0.1 M sodium citrate (pH 3.5) plus 10^{-3} M EDTA. The DNA gel was then heated either in 3-ml aliquots in 12-ml centrifuge tubes or in a 25-ml aliquot in a 125-ml erlenmeyer flask. The rack of tubes or flask was placed in a 70° water bath for 6 min with occasional shaking, removed, and placed in an ice bath. In the centrifuge tubes the temperature reached 70° in 1 min and in the erlenmeyer flask in 2 min. After rapid cooling, the DNA gel was then centrifuged for 3 min at 2000 rpm and, resuspended in 0.05 M Tris-HCl (pH 8.0) plus 10^{-3} M EDTA, and recentrifuged at 4°. The gel was washed with Tris-EDTA three times and resuspended in Tris-HCl (pH 8.0) without EDTA at 0°. This DNA gel was used immediately in incubations. No temperature corrections of the pH values were made in any of the experiments reported in this paper.

Reduction of Depurinated DNA in the Gel. The DNA gel was depurinated in the erlenmeyer flask as described above and after rapid cooling, the pH was adjusted to 6.5 by the addition of 2 N NaOH. $NaBH_4$ (5 M) was added to the gel suspension to give a final concentration of 0.25 M, and the suspension was kept at room temperature for 1 hr. The DNA gel was then washed five times by centrifugation with 0.05 M Tris-HCl (pH 8.0) at room temperature and resuspended in 0.05 M Tris-HCl (pH 8.0). It was stored at 4° and generally used within 2 weeks. The gel was always centrifuged and resuspended in fresh buffer prior to use. When the DNA gel was stored at 4° for 6 weeks, and then washed, 66% of the DNA had escaped from the gel. The DNA remaining in the gel after the storage period was as good a substrate as the freshly depurinated, reduced material.

Depurination and Reduction of DNA in Solution. Double-stranded DNA was also depurinated in a manner similar to the DNA entrapped in the gel. To 1.85 ml of 0.1 M sodium citrate, pH 3.5, containing 10^{-3} M EDTA, 166 nmoles of [^{3}H]thymine-labeled T-4 DNA with a specific activity of 1500 cpm/nmole was added to make a final volume of 2.0 ml. The solution was heated for 6 min at the desired temperature, cooled rapidly and then dialyzed against 1 l. of 0.05 M Tris-HCl (pH 8.0) at 0° for 16 hr. If the DNA was to be reduced with $NaBH_4$, the pH was adjusted to 6.5 with NaOH immediately after the heating and cooling and then 2 M potassium phosphate buffer (pH 6.5) was added to a final concentration of 0.5 M. Sodium borohydride (5 M) was added to a final concentration of 0.25 M in three aliquots at 15-min intervals and the solution was allowed to stand at room temperature for 1 hr. It was then dialyzed against 1 l. of 0.05 M Tris-HCl (pH 8.0) for 16 hr at 0°.

Sedimentation Velocity Experiments. Enzyme reactions were run in a total volume of 0.2–0.25 ml containing 5–10 nmoles of native, depurinated, or depurinated reduced [^{3}H]thymine-labeled T-4 DNA, 5 × 10^{-3} M $MgCl_2$, 10^{-4} M mercaptoethanol, and endonuclease II. Incubations were at 37° for 1 hr. Control incubations of both native and depurinated DNA without enzyme were done. Reactions were terminated by the addition of 0.005 ml of 10% sodium dodecyl sulfate and 0.2 ml of 0.2 M EDTA. The total reaction mixture was layered on a gradient of 5–20% sucrose in 0.05 M Tris-HCl (pH 7.0), 10^{-3} M EDTA, and 1 M NaCl (neutral gradient) or 5–20% sucrose in 0.9 M NaCl, 10^{-3} M EDTA, and 0.1 N NaOH (alkaline gradient). All gradient centrifugation was done at 20°.

Viscosity. Viscosity measurements on depurinated DNA were made with a Beckman rotating cylinder viscometer. T-4 DNA (144 μg) was depurinated at various temperatures in a 4-ml solution containing 0.1 M sodium citrate buffer (pH 3.5) and 10^{-3} M EDTA. The solution was cooled rapidly and the viscosity was measured at 30° either directly or after adjustment of the pH to 6.5 with NaOH.

Results

Effect of Heating the DNA Gel at Varying Temperatures at pH 3.5 on the Nonenzymatic and Enzymatic Release of DNA. The heating of DNA, entrapped in polyacrylamide gel and suspended in citrate buffer at pH 3.5, converted it to an effective substrate for endonuclease II of *E. coli.* Figure 1 shows the effect of varying temperatures on the nonenzymatic and enzymatic release of DNA from the gel. The DNA gel was heated at the temperatures indicated for 6 min at pH 3.5, cooled, washed as described in Methods, and then resuspended for immediate incubation at pH 8.0 in Tris buffer with Mg^{2+} at 37° for 30 min without and with enzyme (Figure 1).

The nonenzymatic release of DNA from the gel increased with increasing temperature. The enzymatic release of DNA (which represents the total amount of DNA released during the incubation with enzyme minus the DNA released nonenzymatically) increased up to 70° and then decreased. The DNA liberated from the gel has a molecular weight of approximately 400,000 or less (Melgar and Goldthwait, 1968) and

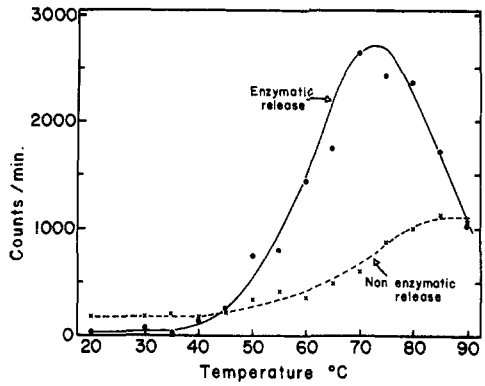

FIGURE 1: Effect of heating the DNA gel at pH 3.5 on the nonenzymatic and enzymatic release of DNA. Aliquots of [^{3}H]T4 DNA gel suspension containing 20 nmoles of DNA (1500 cpm/nmole of nucleotide) were equilibrated with 0.1 M sodium citrate buffer (pH 3.5) plus 10^{-3} M EDTA by repeated centrifugation and resuspension in 3 ml of this buffer. Tubes were heated for 6 min in water baths at the temperatures indicated, the gel was centrifuged and resuspended twice in 0.05 M Tris-HCl (pH 8.0). The final incubation mixture (2 ml) contained the buffer and gel, 6×10^{-3} M $MgCl_2$ 10^{-4} M dithiothreitol, and 0.22 μg of enzyme (specific activity 210) Incubation was for 30 min at 37°. Aliquots (1 ml) were counted.

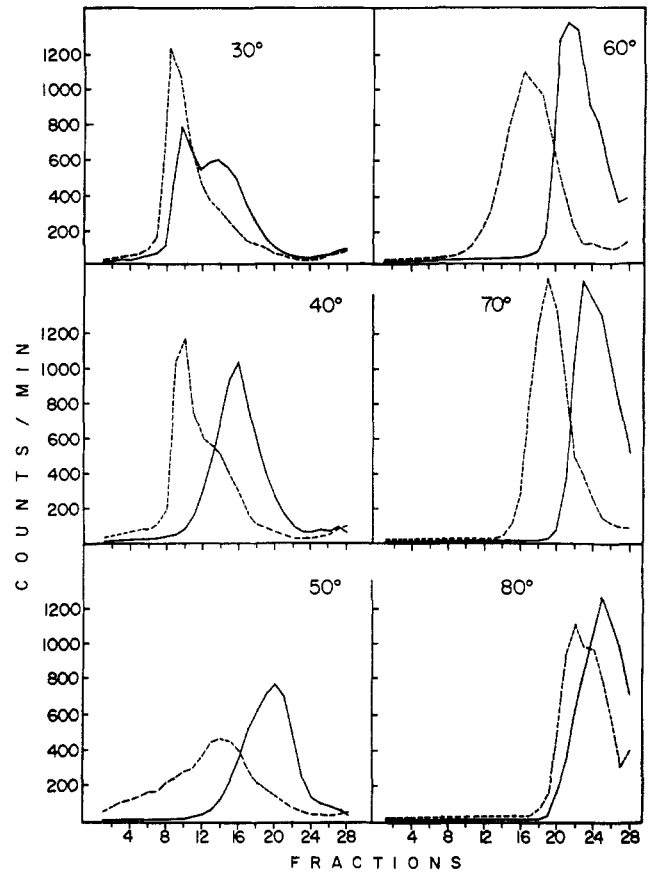

FIGURE 2: Degradation by endonuclease II of DNA heated at varying temperatures at pH 3.5 and examined in neutral sucrose gradients. [^{3}H]T4 DNA (135 nmoles; specific activity 1500 cpm/nmole) was depurinated at pH 3.5 for 6 min at various temperatures in a total volume of 2 ml as described in the Methods. The solution was cooled rapidly and dialyzed immediately against 500 ml of 0.05 M Tris-HCl (pH 8) for 16 hr at 4°. The reaction mixture contained 10 nmoles of DNA, 5×10^{-3} M $MgCl_2$, 10^{-4} M 2-mercaptoethanol, and 0.45 μg of enzyme (specific activity 210) in a total volume of 0.2 ml. The reaction was stopped after 1-hr incubation at 37° by the addition of 0.005 ml of 10% sodium dodecyl sulfate and 0.02 ml of 0.2 M EDTA. The total reaction mixture was layered on a 5–20% neutral sucrose gradient (see Methods) and centrifuged at room temperature in a Spinco SW 56 rotor at 28,000 rpm for 3 hr. No enzyme (- - - - -); plus enzyme (—).

the smaller amounts of DNA liberated from the gels heated at temperatures below 70° are most likely due to insufficient depurinated sites to allow the extent of degradation necessary for release. One reason for the decrease at temperatures above 70° was the decrease in total DNA in these gels at the start of the incubation. This occurred because after heating at pH 3.5 and prior to the start of the nonenzymatic or enzymatic incubation, the gels were washed, and DNA was lost in these washings, particularly in the gels heated at the higher temperatures. In a comparable experiment, the percentage of the original DNA which remained in the gel after heating at 30, 50, 70, and 85° and then washing, was 100, 80, 63, and 26%, respectively. It was shown previously that the amount of DNA released enzymatically from the gel is a function of the amount of DNA per unit volume of packed gel (Melgar and Goldthwait, 1968). Thus, the decreased amount of DNA in the gel heated at 85° compared to 70° could account for a drop of at least 50% in the observed enzymatic release. Evidence will be presented to show that DNA heated at pH 3.5 at 80° can be degraded enzymatically.

Other Variables Affecting the Production and Stability of Depurinated Substrate. The pH value of 3.5 is optimum for production of substrate under these conditions. At lower pH values the nonenzymatic release increases abruptly and at pH 4.0 the enzymatic release is only one-half that at pH 3.5. When the time of heating the gel at 70° and pH 3.5 is varied, the enzymatic release reaches a plateau value by 5 min. Therefore 6 min was chosen as an optimum time.

Since the pH optimum of endonuclease II is 8.0 (Friedberg *et al.*, 1969) and the β-elimination reaction, which produces phosphodiester bond cleavage, is base catalyzed (Tamm *et al.*, 1953; Brown and Todd, 1955; Shapiro, 1967), a study of the pH dependence of nonenzymatic release of depurinated DNA from the gel was done. Depurinated DNA gel was prepared by heating at 70°, pH 3.5, for 6 min. The gel was washed and then resuspended in phosphate buffer, in bicarbonate buffer, and in Tris buffer. All gels were incubated at 37° for 30 min to observe the nonenzymatic release of DNA. In phosphate buffer no significant increase in nonenzymatic release was observed, between pH 6 and 8. In Tris-HCl, the release at pH values from 7 to 8 was similar to the results in phosphate, but at 8.5 it was twice and at 9.0 almost four times this value. At pH 9 in bicarbonate buffer, the release was similar to that in phosphate buffer. The effect of amine buffers on the degradation of depurinated DNA has been noted (Strauss and Hill, 1970). All enzyme reactions were run in Tris buffer at pH 8.0.

Nonenzymatic and Enzymatic Degradation of Free DNA Heated at Varying Temperatures at pH 3.5. Seventy degrees, the optimum temperature for heating the DNA gel to make substrate, was considerably above the T_m for the DNA at pH 3.5 (see below). However, the trapping of DNA in the gel may have allowed renaturation. Therefore a series of experiments was done with DNA in solution in the absence of gel. The results of nonenzymatic and enzymatic degradation are shown in Figure 2. The DNA was treated at pH 3.5 for 6 min and rapidly cooled. The pH was readjusted, the DNA was then incubated without or with enzyme at pH 8.0 for 1 hr, and the reaction mixtures were layered on top of neutral

TABLE I: DNA Depurinated at Different Temperatures—a Comparison of the Size of Single Strands after Exposure to Alkali and the Per Cent of Purine Released.[a]

Condition	$s_{20,w}$ (S)	Mol Wt × 10^6	Depurinated Sites	Purines Released % of Total	Depurinated Sites
Unheated	45	22.4			
30°, pH 3.5	26	5.2	3.3	0.03	10
40°, pH 3.5	16	1.5	14	0.00	0
50°, pH 3.5	10	0.44	50	0.13	44
60°, pH 3.5	8	0.28	80	0.57	190
70°, pH 3.5	3.5	0.04	560	1.9	630

[a] [^{3}H]T4 DNA (55 nmoles) in 0.5 ml of 0.1 M sodium citrate buffer (pH 3.5) with 10^{-3} M EDTA was heated at the temperatures indicated for 6 minutes and cooled rapidly. Sufficient 2 M NaOH was added to bring the pH to 7.0. To 0.1 ml (10 nmoles) of this DNA was added water and 2 M NaOH to a final concentration of 0.1 M. This solution was incubated for 1 hr at 37° and layered on a 5–20 % alkaline sucrose gradient and centrifuged at room temperature in a Spinco SW 56 rotor at 28,000 rpm for 3 hr. Catalase was used as a marker. The molecular weight was calculated by the formula of Studier (1965). Total purines released were obtained by heating purine-labeled [^{3}H]T4 DNA at the various temperatures as follows: 0.1 ml of DNA (108 nmoles; specific activity 130 counts/nmole) in 0.05 M Tris-HCl (pH 8) was added to 0.4 ml of 0.1 M sodium citrate (pH 3.5) containing 10^{-3} M EDTA, heated for 6 min, and cooled rapidly. Unlabeled DNA (1 mg) and bovine serum albumin (1 mg) were added with 0.2 ml of 1 M Tris-HCl and the final volume was adjusted to 1.0 ml. The solution was chilled and 2 ml of cold absolute ethanol were added. The tube was kept at 0° for 30 min, centrifuged, and then 1 ml of the supernatant fraction was counted. Untreated controls were handled in the same manner.

sucrose gradients and centrifuged. No evidence for nonenzymatic degradation of the DNA was obtained at 30 or 40°, but at 50° and above, the size of the DNA decreased. The nature of the DNA at elevated temperatures will be discussed.

Enzymatic degradation did occur after the DNA was heated at all temperatures. At 30°, approximately half of the DNA was degraded from molecules with an estimated $s_{20,w}$ of 50 S to molecules with an $s_{20,w}$ of 38 S. The corresponding decrease in approximate molecular weight was from 89 × 10^6 to 40 × 10^6 (Studier, 1965). There was no double-strand cleavage of untreated native T-4 DNA incubated with enzyme under the same conditions, although the enzyme did make a limited number of single-strand breaks (Friedberg *et al.*, 1969). When the DNA was heated at elevated temperatures at pH 3.5, the nonenzymatic degradation was increased and this material was still a substrate for the enzyme. Since previous experiments had shown that the enzyme does not degrade single-stranded T-4 DNA produced in a specific manner, it was important to determine the nature of the substrate, produced by heating at pH 3.5.

Effect of Heating DNA at Varying Temperatures at pH 3.5 on the Amount of Depurination Measured by DNA Size after β Elimination and by Release of Labeled Purines. A series of experiments was then done to determine the nature of the DNA substrate produced by heating at the different temperatures at pH 3.5. When depurinated DNA is incubated in 0.1 N NaOH at room temperature, hydrolysis of the phosphodiester bonds at the sites of depurination occurs (Tamm *et al.*, 1953). Therefore one way to determine the number of depurinated sites in a DNA molecule is to determine the decrease in size of the single-stranded DNA after treatment with alkali. This has been done using alkaline sucrose gradient centrifugation and the data are shown in Table I. Exposure of DNA to pH 3.5 even at 30° produces depurination. When compared to the untreated DNA the estimated number of sites per single strand, which on exposure to alkali produced breaks, was 3.3. At 40° this number rose to 14, and at 50°, where double-strand breaks were also observed (Figure 2), the number was 50. The number of single-strand breaks produced by heating at 70°, was approximately 560.

With a T-4 DNA labeled with [^{3}H]hypoxanthine, the release of purines due to exposure of the DNA to pH 3.5 at varying temperatures was examined (Table I). At the lower temperatures, the release could not be estimated accurately because the specific activity of the DNA was too low. However, at higher temperatures, results were obtained which could be used to estimate the number of depurinated sites in a DNA molecule, the single strands of which originally had a molecular weight of 22.4 × 10^6 (Table I). The correspondence between these two approaches is reasonably good and indicates that at every depurinated site, alkali-catalyzed hydrolysis of a phosphodiester bond can occur. Therefore, the treatment at pH 3.5 produces depurinated DNA.

Evidence for the Double- or Single-Stranded State of DNA Heated at pH 3.5. The T_m of DNA heated in 0.1 M sodium citrate buffer at pH 3.5 was 46.5° and by 52° the DNA was completely melted. The T_m at pH 7.0 was 81°. When the specific viscosity of DNA heated in 0.1 M citrate buffer for 6 min at varying temperatures was measured the major decrease occurred between 48 and 60° with a midpoint around 50°.

The densities of DNA heated at temperatures from 30 to 90° are shown in Table II. After six minutes at 50° at pH 3.5, 71% of the double-stranded DNA was converted to single-stranded DNA, while at 60° 100% conversion occurred. It is of interest that the DNA heated at 80 and 90° had a density greater than single-stranded DNA, presumably due to the more extensive depurination (Kohn and Spears, 1967). In Figure 2, it is apparent that substrate for endonuclease II is produced by heating DNA at pH 3.5 at temperatures ranging from 30 and 80°. The data on T_m, viscosity and density indicate that between 30 and 60°, the predominant DNA changes from a double-stranded to a single-stranded form. No forms with intermediate density were observed. It appears

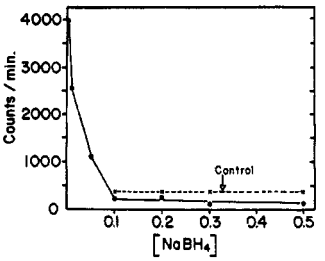

FIGURE 3: Effect of varying concentrations of $NaBH_4$ on the alkali-catalyzed release of partially depurinated DNA. [^{3}H]T4 DNA gel (1500 cpm/nmole of nucleotide) was equilibrated with 0.1 M citrate buffer (pH 3.5) and 10^{-3} M EDTA. The suspension was heated at 70° for 6 min in a water bath and cooled rapidly. The gel was washed twice with 0.05 M potassium phosphate buffer (pH 6.6) and finally resuspended in it. To aliquots of the gel each containing 9 nmoles of [^{3}H]DNA (1500 cpm/nmole) a concentrated solution of $NaBH_4$ was added to the final concentrations indicated in the figure and a final volume of 1 ml. The suspension was kept at room temperature for 60 min before washing extensively with 0.05 M Tris (pH 8) and resuspension. NaOH was now added to a final concentration of 0.1 M in a volume of 1 ml and the aliquots incubated for 30 min at room temperature. The suspension was centrifuged and 0.5-ml aliquots of the supernatant fraction were counted. The control was with native DNA treated in the same manner with $NaBH_4$.

TABLE III: Effect of Different Agents on the Activity of Endonuclease II on Depurinated Reduced DNA and on Alkylated DNA.[a]

Addition	Concn (M)	% of Control Activity: DR[b] DNA	% of Control Activity: Alkylated DNA
Control		100	100
8-Hydroxquinoline	1×10^{-4}	105	108
EDTA	1×10^{-4}	30	29
$MgCl_2$	6×10^{-3}	163	185
p-Chloromercuribenzoate	1×10^{-4}	38	23
tRNA	66 μg/ml	91	92

[a] Depurinated reduced DNA in the gel was prepared by heating at 70° as in Figure 1 and reduction with 0.25 M $NaBH_4$. The assay procedure was as described in Methods, and 0.046 unit of enzyme was used. The data for the alkylated DNA has been published (Friedberg *et al.*, 1969). [b] DR, depurinated reduced.

that the enzyme is active on both double- and single-stranded depurinated DNA. This point will be considered in the Discussion.

Stabilization of Depurinated DNA by Reduction. As has been noted, depurinated DNA is unstable at alkaline pH and phosphodiester bonds are cleaved. In the acid-catalyzed depurination reaction, the aldehyde group of the deoxyribose remains and a base-catalyzed β-elimination reaction can occur (Tamm *et al.*, 1953; Brown and Tood, 1955; Shapiro, 1967; Strauss and Hill, 1970). Therefore, stabilization to alkaline conditions was attempted by reduction of depurinated DNA with sodium borohydride. The relationship of the concentration of $NaBH_4$ to the amount of depurinated DNA liberated by 0.1 N NaOH from the gel is shown in Figure 3. Under the conditions utilized, the optimum concentration of the reducing agent was 0.1 M or above. There was no effect of $NaBH_4$ on the nonenzymatic release of native DNA from the gel.

TABLE II: Variation of Density of DNA Due to Heating at pH 3.5.[a]

Temp (°C)	pH	Double-Stranded DNA: %	Double-Stranded DNA: *p*	Single-Stranded DNA: %	Single-Stranded DNA: *p*
20	7.0	100	1.694		
100	7.0			100	1.709
30	3.5	93	1.693	7	1.708
40		85	1.693	15	1.708
50		29	1.693	71	1.708
60		0		100	1.708
70		0		100	1.708
80		0		100	1.713
90		0		100	1.724

[a] T4 DNA (73 μg) in 1 ml of 0.1 M sodium citrate (pH 3.5)–10^{-3} M EDTA was heated for 6 min at the temperatures indicated and cooled rapidly. Sufficient 2 M NaOH was added to bring the pH to 6.5. This depurinated DNA (2.3 μg) and poly [d(A-T)] (4 μg) as a reference were centrifuged in CsCl of density 1.70 at 44,000 rpm for 22 hr in a Model E Spinco ultracentrifuge. Tracings of films were made with the Joyce-Loebl densitometer and analyzed by the method of Szybalski (1968).

Degradation of Depurinated Reduced DNA in the Gel by Endonuclease II. To demonstrate that the reduction of depurinated DNA did not alter its effectiveness as a substrate, the enzymatic release of depurinated and depurinated reduced DNA from the gel was compared. Native DNA was incorporated in the gel, depurinated at pH 3.5 at 70°, and then a portion of this was reduced. The enzymatic release of the depurinated DNA was 47% of the total DNA in the gel and under the same conditions, the enzymatic release of depurinated reduced DNA was 56%. Thus, the depurinated and the depurinated reduced DNA are equally effective as substrates for endonuclease II.

The possibility existed that the preparation of endonuclease II, purified 1600-fold based on its activity on alkylated DNA, was contaminated with another nuclease responsible for the activity observed on depurinated reduced DNA. The effect of various agents on the enzyme activity with depurinated reduced DNA was compared with the activity on alkylated DNA and the results are presented in Table III. The enzyme was active on both substrates in the absence of Mg^{2+}, and also in the presence of 8-hydroxyquinoline. Mg^{2+} gave a similar stimulation and EDTA a similar inhibition. The sulfhydryl reagent, *p*-chloromercuribenzoate, inhibited to the same degree, and tRNA had no significant effect with either substrate. These data support the conclusion that the enzyme responsible for the degradation of alkylated DNA, is also the one active on depurinated reduced DNA.

Enzymatic Degradation of Depurinated Reduced Free DNA. Reduction of depurinated DNA was shown to increase its stability in alkali as measured by its release from the acryl-

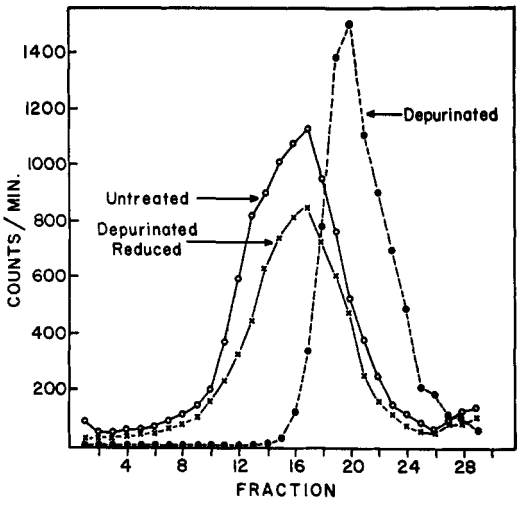

FIGURE 4: Effect of alkali on the size of untreated, depurinated, and depurinated reduced DNA. [^{3}H]T-4 DNA, 166 nmoles in 2.0 ml of 0.1 M sodium citrate (pH 3.5) containing 10^{-3} M EDTA, was heated at 40° for 6 min and was cooled rapidly. The pH was adjusted to 6.5 with NaOH and potassium phosphate buffer (pH 6.5) was added to a final concentration of 0.5 M and final volume of 3 ml. To a 1.5-ml sample, $NaBH_4$ was added at 0, 15, and 30 min to give a final concentration of 0.25 M, while water was added to the other 1.5-ml sample. The two solutions were kept at room temperature for 1 hr after which they were each dialyzed against 500 ml of 0.05 M Tris-HCl (pH 8.0) at 4° for 16 hr. Aliquots were diluted with an equal volume of water, 2 M NaOH was added to a final concentration of 0.1 N, and 0.2-ml volumes were layered on 5–20% alkaline sucrose gradients and centrifuged in an SW 56 rotor at 28,000 rpm for 3 hr at 20°.

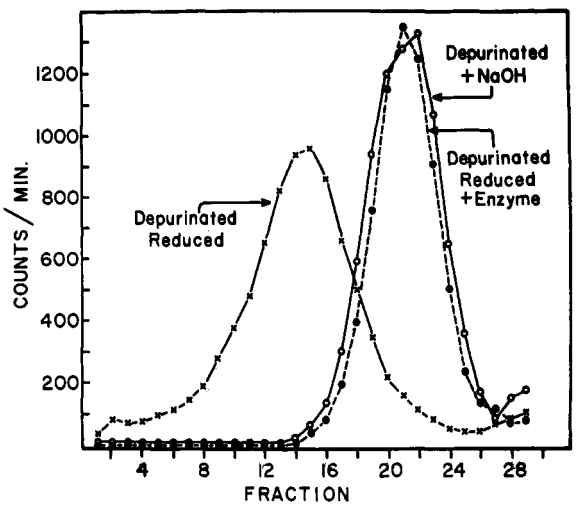

FIGURE 5: Base-catalyzed degradation of depurinated DNA and enzyme degradation of depurinated reduced RNA. The preparation of the DNA samples was described in Figure 4. The reaction mixture for endonuclease II contained in a total volume of 0.2 ml, 5 nmoles of depurinated reduced DNA, 3.3×10^{-3} M $MgCl_2$, 1×10^{-4} M 2-mercaptoethanol, and 0.44 μg of enzyme (specific activity 210). The additions to depurinated DNA were the same with the exception of the enzyme. Incubation was for 1 hr at 37° and the reaction was stopped by the addition of 0.005 ml of 10% soduim dodecyl sulfate and 0.02 ml of 0.2 M EDTA, NaOH (2 M) was added to a final concentration of 0.1 M, and the reaction mixture layered on a 5–20% alkaline sucrose gradient. Centrifugation was as described in Figure 4.

amide gel. When DNA free of the acrylamide gel was depurinated at 40° and reduced with $NaBH_4$, minimal if any alkaline instability was observed. This DNA after reduction was incubated in 0.1 N NaOH at 37° for 1 hr and then examined in an alkaline sucrose gradient along with native DNA treated similarly (Figure 4). The native DNA and the depurinated reduced DNA both have the same pattern which indicates that no alkali-catalyzed single-strand breaks occurred in the depurinated reduced DNA. The same DNA depurinated but not reduced, is shown as a control to indicate that there was depurination which, without reduction, produced alkali-labile phosphodiester bonds.

An experiment was then designed to show that the enzyme cleaved the same number of sites in the depurinated reduced DNA as were cleaved by alkali in the depurinated DNA. The results shown in Figure 5 indicate that the products of enzymatic and nonenzymatic cleavage when examined as single-stranded DNA, were similar in size. Excess enzyme was used under conditions which produced limit degradation. Similar results were obtained in two other experiments.

Single- and Double-Strand Breaks in Depurinated Reduced DNA Made by Endonuclease II. DNA was depurinated at 40° for 6 min and then reduced with $NaBH_4$. The material was incubated with 0.01 or 0.48 unit of enzyme and these samples plus the control were examined in neutral and alkaline sucrose gradients. The results are shown in Figure 6. The higher amount of enzyme was shown to produce limit degradation of the DNA. The *s* values relative to catalase are shown. When these *s* values are used to calculate molecular weights, using the constants of Studier (1965), it is apparent that there are approximately 1.8 single-strand nicks in the double-stranded material prior to incubation with enzyme. With 0.01 unit of enzyme the ratio of single- to double-strand breaks was 4.5,

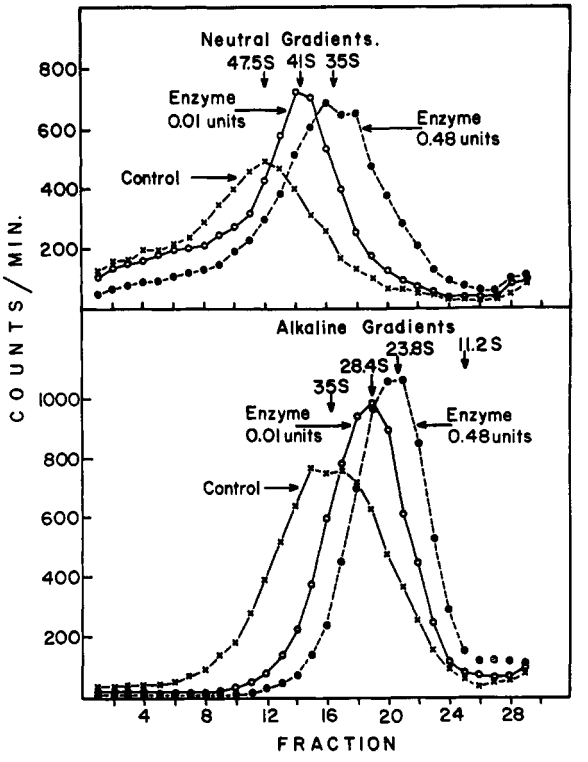

FIGURE 6: Enzymatic single- and double-strand breaks in depurinated reduced DNA. The DNA was depurinated and reduced as described in Figure 4. The reaction mixture contained in a total volume of 0.2 ml, 6.8 nmoles of depurinated reduced DNA, 5×10^{-3} M $MgCl_2$, and 10^{-4} M 2-mercaptoethanol and enzyme as indicated. After incubation at 37° for 1 hr the reaction was stopped by the addition of 0.005 ml of 10% sodium dodecyl sulfate and 0.02 ml of 0.2 M EDTA. Before layering on the alkaline gradient 0.01 ml of 2 M NaOH was added to each sample. Centrifugation was as described in Figure 4.

TABLE IV: Nonenzymatic and Enzymatic Release from the Gel of Depurinated DNA Treated with Hydroxylamine.[a]

Expt	Treatment of DNA Gel	Incubation	Time of Incubation (min)	Cpm released $\times 10^{-3}$
1	Depurinated	0.1 N NaOH	5	0.30
	Depurinated	0.1 N NaOH	10	0.53
	Depurinated	0.1 N NaOH	20	1.53
	Depurinated	0.1 N NaOH	40	2.10
	Depurinated	0.1 N NaOH	60	2.00
	Depurinated + NH_2OH	0.1 N NaOH	5	0.23
	Depurinated + NH_2OH	0.1 N NaOH	10	0.24
	Depurinated + NH_2OH	0.1 N NaOH	20	0.25
	Depurinated + NH_2OH	0.1 N NaOH	40	0.33
	Depurinated + NH_2OH	0.1 N NaOH	60	0.38
2	Depurinated + NH_2OH	Enzyme (0.05 unit)	0	0.6
	Depurinated + NH_2OH	Enzyme (0.05 unit)	10	4.4
	Depurinated + NH_2OH	Enzyme (0.05 unit)	20	6.1
	Depurinated + NH_2OH	Enzyme (0.05 unit)	30	10.5
	Depurinated + NH_2OH	Enzyme (0.05 unit)	60	9.9
	Depurinated	Enzyme (0.38 unit)	60	13.2
	Depurinated	0.1 N NaOH	60	17.7
		Enzyme (0.38 unit)	60	18.1
	Depurinated + NH_2OH	0.1 N NaOH	60	3.1

[a] The [^{3}H]T4 DNA gel (1500 cpm/nmole of nucleotide) was heated at 70° at pH 3.5 for 6 min in an erlenmeyer flask as described in the Methods. To one-half of the suspension a 10 M solution of NH_2OH–HCl was added to a final concentration of 0.2 M and incubated at 37° for 1 hr. At the end of this period the hydroxylamine-treated and untreated gels of expt 1 were washed three times and resuspended with 0.05 M potassium phosphate buffer (pH 6.5) in the original volume. In expt 2, the gel was washed with 0.05 M Tris-HCl. Aliquots (1 ml; containing 5 nmoles of [^{3}H]DNA in expt 1 and 20 nmoles of [^{3}H]DNA in expt 2) of the gel suspension were used for treatment with alkali or enzyme. The gel used in expt 1 had been stored for 4 weeks before use and had only 25% as much DNA as was present in the gel used in expt 2.

while with 0.48 unit the ratio was 4.0. It is recognized that the s values are not accurate and therefore these calculations can only give an approximation.

Nonenzymatic and Enzymatic Degradation of Depurinated DNA Treated with Hydroxylamine. Treatment of depurinated DNA with $NaBH_4$ prevented cleavage of the phosphodiester bonds in alkali due to the β-elimination reaction. The stabilization was considered to be due to the reduction of the aldehyde group of the deoxyribose to the alcohol (Wolfrom and Thompson, 1963). If this assumption was correct, then other reactions with the aldehyde group of depurinated DNA might also stabilize the DNA in alkali and furthermore this treated DNA might be a substrate for endonuclease II. Phenylhydrazine derivatives of depurinated DNA have been made, but these derivatives undergo chemical degradation of the sugar–phosphate backbone (Coombs and Livingston, 1969). Reaction of depurinated DNA with hydroxylamine (NH_2OH) to give the oxime derivative of the deoxyribose does not result in degradation of the backbone (Coombs and Livingstone, 1969). Table IV shows the results of an experiment in which the base-catalyzed release of depurinated and depurinated, hydroxylamine-treated DNA in the gel was compared. In expt 1, the same type of stabilization to alkali was observed with hydroxylamine as with $NaBH_4$ (Figure 3). In expt 2, depurinated hydroxylamine-treated DNA was incubated with endonuclease II for varying times. A maximum of 13,200 counts was released by the higher concentration of the enzyme. The base-catalyzed and enzyme-catalyzed release of the depurinated DNA (prior to treatment with NH_2OH) was similar. Finally, the amount of depurinated hydroxylamine-treated DNA liberated by alkali was about one-sixth the amount of depurinated DNA (not treated by NH_2OH) which was liberated in alkali.

Discussion

Previous studies in this laboratory indicated that DNA alkylated with methylmethanesulfonate was a substrate for endonuclease II (Friedberg and Goldthwait, 1969; Friedberg *et al.*, 1969). Friedberg and Goldthwait (1969) in Figure 6 of their paper demonstrated that the alkylated DNA was not degraded in the alkaline gradient, but was degraded by the enzyme. This evidence is the basis for concluding that endonuclease II can degrade alkylated DNA which has not been depurinated prior to incubation with the enzyme. A similar conclusion was reached earlier by Strauss and Robbins (1968) regarding the activity of extracts of *Bacillus subtilis* and *Micrococcus lysodeikticus* on alkylated DNA. Papirmeister *et al.* (1970) have concluded that the alkylated adenine rather than the alkylated guanine is the site of the enzymatic activity which inactivates alkylated bacteriophage, and they have supported this conclusion by experiments with extracts of *Escherichia coli* and differentially labeled polymers (Papirmeister *et al.*, 1970).

Data presented in this paper indicate that partially depurinated DNA, either as such or stabilized by reduction with $NaBH_4$ or by reaction with NH_2OH, also serves as a substrate for endonuclease II. Evidence has been presented to indicate

that excess enzyme hydrolyzes a phosphodiester bond at each site of depurination, and also that it is the same enzyme that acts on alkylated DNA and depurinated DNA.

The exact nature of the depurinated substrate is not known. Although evidence is presented in Table II and in the text, which indicates that heating at pH 3.5 produces DNA which is either double or single stranded, this does not determine whether there are microareas in the single-stranded DNA which are double stranded due to folding with base pairing (Studier, 1969). Also the possibility exists that the enzyme recognizes areas of DNA where the double helix opens into two single strands. When native T-4 DNA was denatured by heating at 100° for 5 min cooled rapidly, incubated with the enzyme (an amount which produces the maximum number of single-strand breaks in native T-4 DNA) and then examined in an alkaline sucrose gradient, no enzymatic degradation was observed. Thus, the enzyme preparation does not have any nonspecific single-strand endonuclease activity. The enzyme does make several breaks in T-4 DNA which has been denatured by alkali and is of a higher molecular weight than the material denatured by heat. Further work is being done on the problem of the limited enzymatic degradation of high molecular weight single-stranded DNA.

Since the same enzyme degrades alkylated DNA and depurinated DNA, the question arises whether depurination is an intermediate step in the degradation of alkylated DNA. To date we have no answer to this question.

A further puzzling feature of depurinated reduced DNA as a substrate for endonuclease II is the ratio of single- to double-strand breaks (approximately 4–4.5). As noted, this DNA was heated at 40° and had very few depurinated sites. In this experiment an excess of enzyme was used to ensure limited degradation. As observed in Figure 5 and in two similar experiments, the number of enzyme-induced breaks in the depurinated reduced DNA was similar to the number of base-catalyzed breaks in the depurinated DNA, and from this evidence it was concluded that the enzyme degraded a phosphodiester bond at each depurinated site. However, double-strand breaks were also observed (Figures 2 and 6). These two observations are not compatible with enzymatic cleavage on the opposite strand at a nondepurinated site. One possible explanation is that depurination does not occur initially at random sites but in specific areas on both chains, some of which are close enough to allow double-strand breaks. No experimental evidence is available to support this. The ratio of single- to double-strand breaks was approximately 4 to 1 in these experiments while the same ratio for random breaks introduced by DNase I was approximately 200 to 1 (Thomas, 1956). It should be reemphasized that it is the breaking of double strands that allows the use of the DNA gel as an enzymatic assay. Similar double-strand breaks were observed with the degradation of alkylated DNA (Friedberg *et al.*, 1969).

The function of endonuclease II of *E. coli* is still obscure. Some experiments suggest that the enzyme may not degrade alkylated DNA in the cell. When T-7 bacteriophage was alkylated *in vitro* with $MeSO_3Me$, the mean lethal dose required was 280 Me groups/DNA molecule (Lawley *et al.*, 1969). This represents approximately 1 nucleotide alkylated per 285 nucleotide residues. Furthermore inactivation of T-7 phage by MMS increased with prolonged incubation after the alkylation, and the degree of inactivation appeared to be directly related to the degree of depurination. With six to eight depurinations, the phage viability was reduced by 63%. This *in vitro* depurination was not necessarily associated with *in vitro* strand cleavage (Lawley *et al.*, 1969). These data suggest that for methylmethanesulfonate to be biologically active, it must not only alkylate the phage DNA, but this DNA must then be depurinated. Our studies indicate that endonuclease II is active on both alkylated *and* depurinated DNA and a very crude comparison suggests that both of these substrates are about equally as effective. If this is correct, it is difficult to see how endonuclease II could be responsible for the inactivation of depurinated T-7 DNA, but not of alkylated T-7 DNA.

Alkylation of the DNA of *B. subtilis* with 1 methyl group/400 nucleotide residues does not effect viability (Prakash and Strauss, 1970). Furthermore, the DNA replicates and the cells go through several generations without loss of the methyl groups. Also with this degree of methylation, there is no observable decrease in the size of the DNA when examined by the sucrose gradient technique (Prakash and Strauss, 1970). Strauss and Robbins (1968) have observed and we have confirmed that an enzyme similar to endonuclease II exists in *B. subtilis*. In this organism they have presented evidence that multiple alkylated residues can remain in the DNA without any effect on viability. This suggests that either the endonuclease makes phosphodiester bond breaks which are repaired rapidly without removal of the methyl groups, or that a mechanism is present which prevents chain scission by the endonuclease of the alkylated DNA. It is possible that the enzyme described in this paper has no role in the repair of alkylated DNA.

No explanation for the ability of the enzyme to make a limited number of single-strand breaks in native T-4 and T-7 DNA is available. Possibilities for enzyme action which still have not been ruled out are in the initiation of DNA synthesis, in the cleavage of the newly synthesized DNA at the replication fork, and in recombination.

Acknowledgment

The authors thank Mrs. Irene Ukstins for excellent technical assistance.

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CORRECTIONS

"IUPAC–IUB Revised Tentative Rules for Nomenclature of Steroids," Volume 8, Number 6, June 1969, page 2227. The following amendments have been made by IUPAC–IUB.

On page 2227: Under Contents, Rule 2S-10, for Hetero read Heterocyclic.

Add the following to the footnote: The commissions are greatly indebted to R. S. Cahn, formerly Titular Member and later Associate Member of the Commission on the Nomenclature of Organic Chemistry, who has taken a great part in the work on nomenclature of steroids.

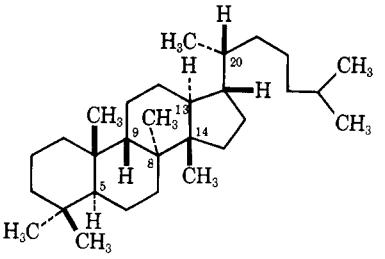
Column 2, paragraph 3: delete the last sentence (Decisions on . . . dealing with them).

Page 2228: Under 1.1, delete "or order of complexity." For Rule* C-15.11(e), read Nomenclature of Organic Chemistry, Section C (Butterworths, London, 1971). Delete footnote.

Under 1.4, last line, delete "is unspecified."

Under 1.5, Notes, add: If two carbon chains are attached at position 17, see notes 4 and 5 [found in these addenda] to Rule 2S-2.3.

Page 2230: The name under formula 31 should be 19(10→9β)*abeo*-5α,10α-lanostane. Added to these formulas should be

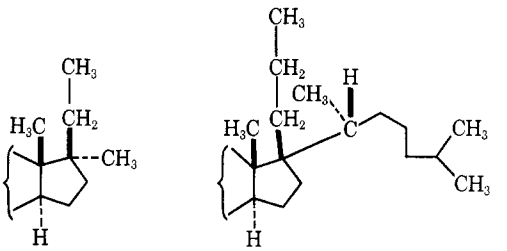


31A
5α-Protostane
4,4,8,14-Tetramethyl-18-nor-5α,8α,9β,13α,14β,17β,20*R*-cholestane (this is an important biogenetic precursor of tetracyclic triterpenoids and steroids)

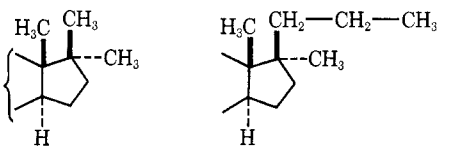
Under 2.3, Notes, should be added:

(4) If a steriod has two carbon chains attached at position 17 and one of them is included in Table I under Rule 2.3, the compound is named as a 17-alkyl derivative of the steroid in the table carrying that substituent [*e.g.*, 17-methyl-5α-pregnane (31B); 17-propyl-5α,17α-cholestane (31C)].

(5) If a steroid has two carbon chains attached at position 17, neither of which is included in Table I under Rule 2.3, the compound is named as a 17,17-disubstituted androstane [*e.g.*, 17,17-dimethyl-5α-androstane (31D); 17α-methyl-17β-propyl-5α-androstane (31E)].

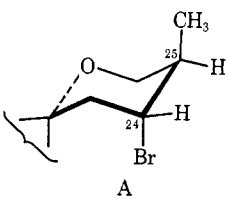


31B 31C



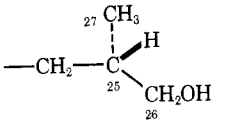
31D 31E

Page 2232: Column 2, line 28, delete unspecified or. Formula A should be amended at C-24 and its name corrected.



A
Name of A should be (24*R*,25*R*)-24-Bromo-5β-spirostan-3β-ol

Page 2233: In formula 55, for (20*R*,22*R*,25*R*), read (20*R*,-25*R*). In 56 and 59, the ends of the side chains should be redrawn, as shown. This makes the epimerism with 57 and 58 clearer.



56, 59

Page 2234: Table II, formula 55, for (20*R*,22*R*,25*R*) read