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Studies on Apurinic and Reduced Apurinic Acids — the Hydrogen Ion Dissociation Curves

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A method for the reduction of the aldehyde groups in apurinic acid derived from sodium deoxyribonucleate and the chemical and dissociation properties of the reduced apurinic acid are described. Comparison of the titration curves of this material with that of the sodium deoxyribonucleate from which it was derived has enabled the dissociation ranges of the titratable groups in the deoxyribonucleate to be estimated.

Apurinic acid has been obtained from deoxyribonucleic acid by removal of its purine bases but with retention of an unchanged proportion of pyrimidines and has proved to be a useful substance for the study of the chemical structure of the original deoxyribonucleate. When apurinic acid was degraded by alkali [1] a decrease was observed in the intensity of the colour produced in the Dische test, probably because the sugar moieties containing free aldehyde groups were partly destroyed by the alkali. Hence it seemed likely that if the aldehyde groups in apurinic acid could be reduced, a more stable product would result. A suitable reducing agent would have to leave the pyrimidine bases unchanged and preliminary experiments with sodium borohydride showed that it did not reduce thymidine. In the following the reduction of the free aldehyde groups of apurinic acid to primary alcoholic groups by sodium borohydride is described together with the chemical and dissociation properties of the reduced apurinic acid so obtained. The greater stability of this reduced form made it particularly useful for titration purposes. Comparison of the titration curves of the reduced apurinic acid, which contained only thymine and cytosine bases, with those of the parent deoxyribonucleate, which in addition contained adenine and guanine, has enabled the dissociation ranges of the various groups in the deoxyribonucleate to be elucidated.

EXPERIMENTAL

Materials and preparations. Sodium deoxyribonucleate was isolated both from herring and cod roes. The herring sample used in this investigation has been described elsewhere [2] and has also been employed in concurrent titration studies [3,11]. Two samples of apurinic acid were prepared from the herring deoxyribonucleate by treatment with an acidic ion exchange resin, as previously described [4,11]. The first sample (APA—H1) of apurinic acid was isolated as the free acid by drying in the frozen state. A second sample (APA—H2) was first neutralized and isolated as the sodium salt, again by drying in the frozen state. Reduced apurinic acid (RAPA—H2) was then prepared in the form of its sodium salt from APA—H2 and was isolated after dialysis and drying in the frozen state. A second reduced apurinic acid sample (RAPA—C) was similarly obtained from apurinic acid (APA—C) which had been prepared from cod roe deoxyribonucleate by the resin method. Ribonucleoprotein was removed from the cod roes by extraction with 0.14 M sodium chloride solution. Deoxyribonucleoprotein was obtained by extraction with a stronger salt solution (1 M sodium chloride) followed by precipitation with 96 % ethanol. The protein was then removed by the use of chloral hydrate [5]. The sodium deoxyribonucleate was precipitated with ethanol, dissolved in water, dialysed against water and finally dried in the frozen state.

Reduction of apurinic acid. The course of the reduction of apurinic acid by the borohydride was studied in the following way. Apurinic acid (0.1 g of APA—C) was dissolved in distilled water (5 ml) and small amounts (0.05 ml and 0.025 ml) of this solution were withdrawn for use in the Schiff's and Dische test, respectively. Weighed amounts of solid sodium borohydride were then added successively. After each addition the mixture was shaken and half an hour was allowed to elapse before further samples were withdrawn for Dische and Schiff's tests. From these experiments (reported in Fig. 1) the following procedure for reducing apurinic acid was deduced. Apurinic acid (0.052 g of APA—H2) was dissolved in water (2 ml) and sodium borohydride (0.01 g) was added. The mixture was left at room temperature for nearly 4 hours. The absence of aldehyde groups in the product was always confirmed by Schiff's test, since control experiments showed that there was a linear relation between the amount of apurinic acid and the intensity of Schiff's colour. After reduction, the mixture which had a pH of 9 was neutralized with dilute hydrochloric acid and dried in the frozen state. Ethanol (10 ml, 96 %) was added to the white residue and the mixture was left to stand at room temperature for several days. The solid was separated by centrifugation, dried with ether, dissolved in distilled water (20 ml, pH 6.8) and dialysed at 2 °C against distilled water (300 ml). The dialysed solution was dried in the frozen state to yield the reduced apurinic acid (0.038 g). The ultraviolet absorption at 260 mμ of the dialysate indicated that negligible amounts of apurinic acid had passed through the dialysis tubing.

Chromatographic analysis. The purine and pyrimidine contents of the materials under examination were determined by hydrolysis of the samples (5 mg), previously dried at 110° C, with anhydrous trifluoroacetic acid (0.2 ml) [6] at 155° C for 1 hour, separation of the bases on paper chromatograms using *iso*propanol-hydrochloric acid [7] as the solvent system, elution of the spots and corresponding blanks with 0.1 N hydrochloric acid (5 ml) at 37° C for 12 hours, followed by examination of their ultraviolet absorption *. To obtain a more correct ultraviolet absorption of the bases at peak absorption the following formula was used [8]

$$\varepsilon_{net} = \text{net reading of base extinction} = \varepsilon_{\text{Spot at peak of base}} - \left[\varepsilon_{\text{Blank at peak}} \times \frac{\varepsilon_{\text{Base Spot at 320 m}\mu}}{\varepsilon_{\text{Blank at 320 m}\mu}} \right]$$

ε_{net} was used for the calculation of the amounts of bases present in the eluates.

Schiff's test. Schiff's reagent was prepared according to Tobie [8]. To a sample (0.05 ml) of the reduction mixture were added 0.1 N hydrochloric acid (0.05 ml), to destroy

* Eluates of spots and blanks were examined against 0.1 N hydrochloric acid in a Beckman Spectrophotometer DU.

excess borohydride, water (3 ml) and Schiff's reagent (1 ml). The mixture was left at room temperature for 135 minutes and its extinction at 560 mμ was measured in a Coleman spectrophotometer.

Dische test. The reagent was prepared according to Dische [9]. Water (2 ml) containing the material to be tested was heated with Dische reagent (5 ml) in a boiling water bath for 15 minutes. After cooling its extinction at 600 mμ was measured in a Coleman spectrophotometer.

Ultraviolet absorption measurements. The ultraviolet spectra of apurinic and reduced apurinic acid were determined at pH 7.0 in phosphate buffer and at pH 1.0 in 0.1 N hydrochloric acid. The phosphorus contents of the solutions were obtained by analysis according to the method of Jones *et al.*[10]

Titration methods. The procedures used were the same as those employed in parallel studies on the nucleic acids [11]. Solutions of the apurinic or reduced apurinic acids were prepared by dissolving the air-dry samples in sodium chloride (usually 0.05 M) and were titrated at 25 °C with acid and alkali using a cell consisting of a "Doran" Alkacid glass electrode and a saturated calomel electrode connected by a saturated potassium chloride bridge. During the titration hydrogen was passed through the cell in order both to stir the solution and to keep out carbon dioxide. The pH was measured with a Cambridge pH meter, previously standardized with 0.05 M potassium hydrogen phthalate (pH = 4.008) and 0.05 M sodium borate decahydrate (pH = 9.18) to give readings on the "saturated calomel scale" of Hitchcock and Taylor [12]. The determination of pH and of the number of equivalents of acid bound per 4 g-atoms of phosphorus had maximum errors of ± 0.01 and ± 0.03, respectively. "Water corrections" were made by the usual blank titrations.

RESULTS AND DISCUSSION

A. The preparation and properties of reduced apurinic acid

The decrease in Schiff's colour on the addition of increasing amounts of sodium borohydride continued until a total of about 20 mg of this reagent had been added to every 100 mg of apurinic acid (APA—C, Fig. 1). After this no Schiff's colour could be obtained so the aldehyde groups of the apurinic acid must have been completely reduced *. It is noteworthy that addition of 5 % of the total apurinic acid to an acidified reaction mixture could be detected by the Schiff's test. The completion of the reduction was further confirmed by the following tests. Spraying of a solution of *m*-phenylene diamine dihydrochloride on to a spot of the reaction product (400 μg) on filter paper failed to produce any fluorescence [13] in ultraviolet light as it did with apurinic acid, 20 μg of which could be readily detected by this method. Furthermore, Tollen's reagent [14], which could detect 20 μg of apurinic acid, produced a negative result with 500 μg of the reaction product, which must therefore be regarded as reduced apurinic acid. Apurinic acid in amounts of 1 mg is strongly reduced upon heating with Fehling solution. Under corresponding conditions, reduced apurinic gave only a very slight reduction. In quantities of 3 mg there was somewhat more reduction but considerably less than that given by 1 mg apurinic acid. The absence of reducing aldehyde groups is amply demonstrated by the other chemical tests and this latter observation is probably explained by the breakdown at 100° C of the 2-deoxy-D-ribitol moieties to reducing fragments by the very strong alkali in the Fehling solution. This latter view

* We have also reduced apurinic acid prepared by Chargaff's method.

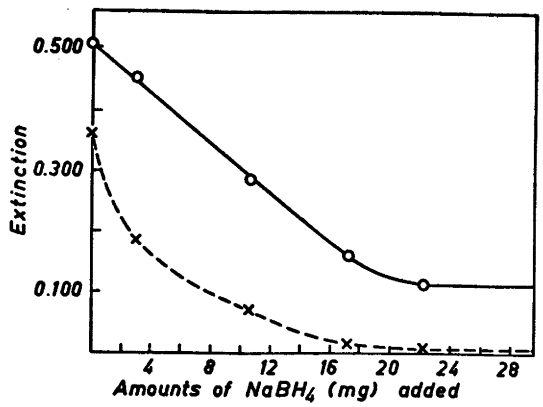

Fig. 1. Reduction of cod roe DNA (APA—C) with $NaBH_4$. The dotted line shows the decrease in the intensity of the colour produced by Schiff's reagent when added after various amounts of $NaBH_4$. The continuous line shows the decrease in the colour produced in the Dische test.

was confirmed by the fact that in a more concentrated Fehlings solution the reduction by reduced apurinic acid was greater.

On reducing apurinic acid (APA—C) there is a drop in the intensity of the colour developed with Dische's reagent. This is seen from Fig. 1 which gives the Dische colour of the reaction mixture following the addition of increasing amounts of reducing agent. It is noticeable that a constant Dische colour is obtained at a point where a negative Schiff's test indicates a complete reduction. The absorption spectrum of the Dische colour produced with reduced apurinic acid is identical with that given by apurinic acid. Comparative values (based on equal amounts of phosphorus) with cod roe sodium deoxyribonucleate, apurinic acid (APA—C) and reduced apurinic acid (RAPA—C) were 100, 83 and 15, respectively.

Chargaff [1] reported that the free aldehydo sugar in apurinic acid was unstable towards alkali, the Dische colour, for example, falling to 7 % of its original value after treatment with 2 N sodium hydroxide at 37° C for 24 hours. The reduction of the aldehydo group renders the sugar moiety stable to alkali for the Dische colour of reduced apurinic acid was almost unchanged after treatment with 2 N sodium hydroxide at 37° C for 24 hours.

Since the reduction of apurinic acid takes place in slightly alkaline solution (pH not above 9) a control experiment was carried out by keeping apurinic acid at room temperature in 0.01 M NaOH. The Dische colour of the solution was tested before and after three days and found to be the same. Thus the very slight alkalinity of the reduction mixture could not have affected the aldehydo sugar in apurinic acid.

The ultraviolet absorption spectra of apurinic and reduced apurinic acid from cod roe deoxyribonucleate are given in Fig. 2. The minima and maxima of absorption occurred at the same wavelengths. In Table 1 are given ε_{268} (P) for the different preparations. These data suggest that the reduction of apurinic acid with sodium borohydride does not affect the pyrimidines. This

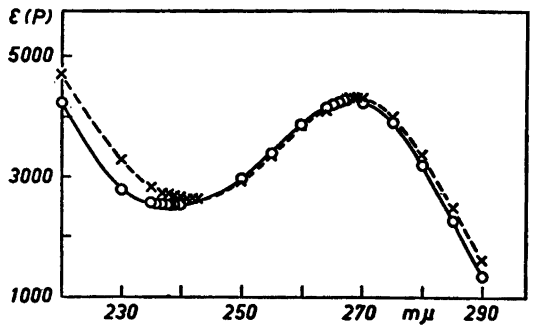

Fig. 2. The ultraviolet absorption spectra of apurinic acid (dotted line) and reduced apurinic acid (drawn line) from cod roe DNA at pH 7.

is further confirmed by the chromatographic analyses, which show no significant difference in cytosine and thymine content between a reduced apurinic acid and the apurinic acid from which it was obtained (*e. g.*, Table 2, 2nd and 4th columns).

B. The dissociation of apurinic and reduced apurinic acids

Typical titration curves in 0.05 M sodium chloride of herring apurinic and reduced apurinic acid are shown in Fig. 3 together with the reversible back titration of the herring sperm deoxyribonucleate from which they were derived [11]. The ordinate represents the acid or alkali bound by a unit amount of these substances containing 4 g-atoms of phosphorus so that, in spite of varying molecular weights, direct comparison between the curves is possible. No titration "anomaly" of the type found with sodium deoxyribonucleate was observed with either the apurinic or reduced apurinic acid, the forward and back titration curves being identical. All hydrogen bonds involving titratable groups must therefore have been ruptured in the course of the preparations.

Comparison of the apurinic and reduced apurinic acids. Comparison of the titration curves of these two substances is best effected by subtracting the alkali bound along the reduced apurinic acid curve (II) from that bound at the same pH's along the apurinic acid curve (I). The resulting comparison curve

Table 1. Ultraviolet absorption of apurinic and reduced apurinic acids. Extinctions (ε_{268}(P)) per g-atom of phosphorus per litre at λ = 268 mμ.

Source	Apurinic acid	Reduced apurinic acid
Cod roes	5 000 (APA—C)	5 100 (RAPA—C)
Herring roes	4 270 (APA—H 2)	4 290 (RAPA—H 2)

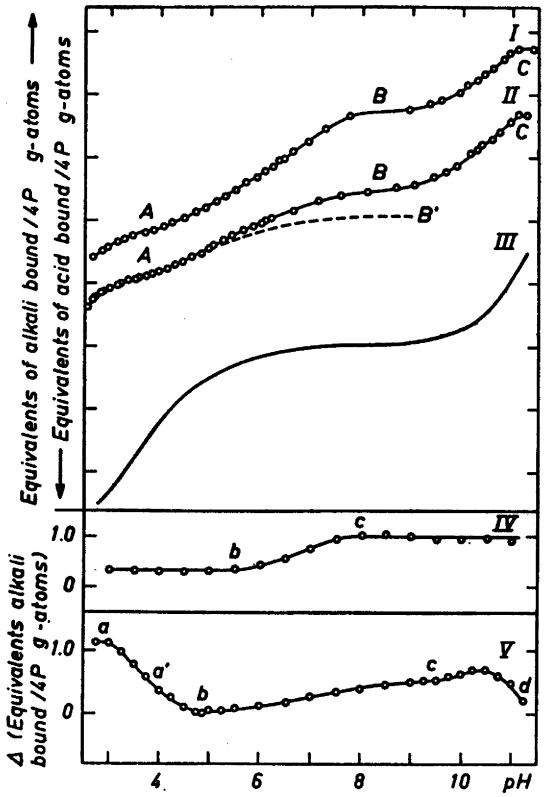

Fig. 3. Titration curves of herring apurinic, reduced apurinic and deoxyribonucleic acids. At 25° C in 0.05 M sodium chloride.

I. Herring apurinic acid (APA—H 2). Circles-experimental points.
II. Herring reduced apurinic acid (RAPA—H 2). Circles-experimental points.
III. Back titration curve of herring sodium deoxyribonucleate (from Ref. 11). Divisions on ordinate scale for curves I, II, III represent one equivalent of acid or alkali bound per 4 P g-atoms. Curves I, II, III separated vertically for clarity. Comparison curves:
IV. Alkali bound by apurinic acid, APA—H 2, along I, minus alkali bound by reduced apurinic acid, RAPA—H 2, along II.
V. Alkali bound by reduced apurinic acid, RAPA—H 2, along II, minus alkali bound by sodium deoxyribonucleate along III.

(IV) shows that only over the pH range 5.5—8.5 (*bc*) is there any significant difference in the number of titratable groups in the two derivatives. The groups titrating in this pH range are secondary phosphoryl end groups ($pK_a' = 6$—7) [15] and cytosine 6-amino groups ($pK_a' = 4.6$ according to Hurst *et al.*[15], but see below where $pK_a' = 5.0$ in 0.05 M salt is suggested). Since the analytical evidence (Table 2) indicates that the cytosine contents (in moles/4 P g-atoms) of apurinic and reduced apurinic acids are almost identical, and

equal to that of the original deoxyribonucleate, the shape of curve IV must be attributed to a larger proportion of titratable end groups in the apurinic acid than in the reduced form. This may well arise because smaller fragments, which would contain a higher proportion of end groups, were removed from the reduced apurinic acid preparation by dialysis but not from the apurinic acid preparation. In confirmation of this some of the APA—H 2 sample did appear to be dialysable after long storage.

The curves I, II and IV therefore show that in the reduction of apurinic acid there has been no structural modification of the molecule which affects the cytosine and thymine components.

Comparison of titration and analytical results. The chromatographic analytical evidence (Table 2) shows that the method of preparation of apurinic acid has entirely eliminated the purine bases, guanine and adenine, from the original nucleate structure, leaving only cytosine and thymine in the same ratio as before. Hence the titration curves I and II of apurinic and reduced apurinic acids must be interpreted in terms of the dissociations of the following groups only: primary phosphoryl groups ($pK_a' = 1—2$) [15]; cytosine 6-amino groups ($pK_a' = 4.6—5.0$, see above); secondary phosphoryl end groups ($pK_a' = 6—7$); and the —NH—CO-systems of thymine ($pK_a' \sim 10$)[15].

Curves I and II (Fig. 3) exhibit inflexion points at A (pH = 3.7), B (pH = 8.5) and an incipient one at C (pH = 11). In view of the evidence given above, the groups titrating in the various regions must be: below pH 3.7, the primary phosphoryl groups only; between pH 3.7 and 8.5 (AB), the cytosine amino groups and secondary phosphoryl groups; and between pH 8.5 and 11 (BC), the —NH—CO-systems of thymine. The difference between the alkali bound at the inflexion points B and C should be equal to the number of thymine

Table 2. Comparison of analytical and titration data. The numbers are the composition in moles or equivs/4 P—g atoms.

Preparation	Herring DNA	RAPA—H 2 [c]		APA—H 2	APA—H 1	
Method	Analysis [b]	Analysis	Titration [d]	Titration[d,e]	Analysis [f]	Titration [g]
Adenine	1.09	0	0	0	0	
Guanine	0.88	0	0	0	0	
Cytosine [a]	0.88	0.88	0.90	0.92	0.85	?
Thymine	1.08	1.07	1.14	1.02	1.06	1.1
Sec. phosphoryl groups			0.40	0.96		0.88

(a) Including 5-methylcytosine
(b) Analysis of Dr. G. E. Marsh (*Thesis*, University of Birmingham 1954)
(c) Prepared from APA—H 2
(d) In 0.05 M NaCl solution
(e) Analysis not available
(f) Analysis by Dr. S. Letham, Chemistry Department, University of Birmingham
(g) In 0.15 M NaCl solution

residues per 4 P g-atoms since there is only one —NH—CO-dissociation in each thymine molecule. The proportion of thymine residues so deduced from the titration curves is in good agreement with that obtained from chromatographic analysis (Table 2) both for the samples whose titration curves are given in Fig. 3 and for APA—Hl, which was titrated in 0.15 M sodium chloride solution.

It has recently been concluded [11,16] that the modern analytical and titration data show evidence for the presence in the original herring sodium deoxyribonucleate of no more than a negligible proportion of secondary phosphoryl end groups. Hence the difference curve (V) between the titration curve of reduced apurinic acid and of the original nucleate in the region of pH 5—8.5 (*bc*) must be entirely due to the (extra) secondary phosphoryl groups in the former. The estimates in Table 2 of the proportion of these end groups were obtained in this way.

If these arguments are valid, the curve (dotted line AB′, Fig. 3) obtained by subtraction of the alkali bound along *bc* of curve V from AB of curve II should be the dissociation curve of the cytosine amino groups in the reduced apurinic acid. The difference in the alkali bound at the inflexion points A and B′ should then be equal to the number of cytosine residues per 4 P g-atoms. The agreement between the amount of cytosine so determined from titration and that given by direct analysis is very satisfactory (Table 2, 2nd and 3rd columns of numbers) and demonstrates the validity of this method of deduction from the titration data.

Comparison of reduced apurinic acid and sodium deoxyribonucleate and the pK_a' *values of the dissociable groups.* Since there is no titration anomaly with the reduced apurinic acid, comparison must be made with the titration curve of non-hydrogen bonded deoxyribonucleate, *i. e.*, with its back titration curve (III). It has already been concluded that in the difference curve V (obtained by subtracting the alkali bound along III from that bound along II) the section *bc* represents the dissociation of the (extra) secondary phosphoryl groups in the reduced apurinic acid. No cytosine amino groups are involved in *bc* since analysis shows the same number of these groups in the two materials (Table 2) and therefore they cancel out in the subtraction which gives V. The section *ab* of V represents extra groups titrating in the deoxyribonucleate which are not present in the reduced apurinic acid and must be attributed to the amino groups of guanine and adenine in the former. The part *a′b* of V must be predominantly the dissociation of the adenine group and, knowing the amount of adenine present the deoxyribonucleate (1.09 moles/4 P g atoms) an estimate of the pK_a' of this group can be made. Similarly, the pK_a' of cytosine 6-amino groups in a polynucleotide can be obtained from AB′ of II, that of secondary phosphoryl groups from bc of IV and V, and that of the thymine —NH—CO-system from BC of II. Reliable values for the pK_a' of the guanine —NH—CO-group cannot be obtained from cd of V since its shape suggests some difference in pK_a' of this group as between curves II and III. The pK_a' values derived for the other groups in 0.05 M sodium chloride are given in Table 3 together with the values of some other authors [15–17]. The pK_a' for the cytosine amino group appears to increase slightly with its degree of dissociation. This may be due to the cumulative errors of the above

Table 3. pK_a' in 0.05 M sodium chloride of the dissociable groups of herring roe deoxyribonucleate. Obtained by comparison with the corresponding reduced apurinic acid.

Degree of acid dissociation of the group in 0.05 M NaCl	pK_a' values			
	Thymine —NH—CO—	Cytosine Amino	Adenine Amino	Secondary Phosphoryl
0.20	10.1	4.95	3.65 (approx.)	6.75 (approx.)
0.30	10.1	4.98		
0.50	10.1	5.10		
0.75	10.1	5.19		
0.80	10.1	5.20		
In deoxyribonucleotides, absence of salt (Ref. 15)	10.0	4.6	4.4	6.5
From analysis of DNA titration curves, at zero ionic strength.[a] (Table 1, Ref. 16)		4.85	3.65	6.0 [b]
From spectrophotometric back titration curves at ionic strength of 0.008 (Table 1, Ref. 17) [c]		5.3		

(a) DNA concentration of 0.139 mgm P/ml = 0.001125 equiv. 4P/l.
(b) By interpolation.
(c) The other figures quoted in Table 1, Ref. 17, refer to the forward titration curve of DNA, which corresponds not to a reversible dissociation but to a simultaneous ionisation and irreversible denaturation.

procedure but, on the other hand, it may also be a genuine decrease of acidity consequent upon the increasing negative charge of the deoxyribonucleate anion. However the change of pK_a' is much less than that observed with synthetic polyacids (*e. g.*, changes [18] of pK_a' of the order of 0.8 units over the degree of dissociation range of Table 3; this relative suppression of the polyelectrolyte effect by the sodium gegenions has been discussed elsewhere [11]). The mean value for the pK_a' of the cytosine amino group (*viz.*, 5.0 in 0.05 M salt) is higher than would have been expected from earlier data [15] and this has important consequences [11, 19] for the interpretation of the deoxyribonucleate titration curve over pH 5.5—9. It accords with the value of 5.3 in 0.008 M salt obtained by spectrophotometric titration of thymus DNA [17]. The values obtained by Jordan *et al.*[16] (and quoted in Table 3 for comparable conditions) are more open to doubt since they depend on an analysis of the back titration curves of thymus DNA where the dissociation ranges of various groups overlap and the allocation of pK_a' values is more ambiguous [3] than in the spectrophotometric method [17], and the method reported here.

C. Conclusion

The titration results indicate that the same proportion of pyrimidine dissociable groups are present in the apurinic and reduced apurinic acid. The reduction of the free aldehyde groups in apurinic acid by sodium borohydride has therefore been effected with no major structural change and has increased the stability of the sugar ring to alkali. The proportion of end groups is relatively high in both derivatives and suggests that considerable degradation has occurred relative to the original deoxyribonucleic acid. The apurinic acid (APA—H2, undialysed) contained 1 secondary phosphoryl group for every 4 phosphorus atoms, which implies that it was on the average about 4 nucleotides long, assuming every fragment carried one secondary phosphoryl group. The reduced apurinic (RAPA—H2, dialysed) contained 1 secondary phosphoryl group every 10 phosphorus atoms, implying an average chain length of 10 nucleotides. These are lower in molecular weight than the apurinic acid obtained by Tamm *et al.* which were thought to contain about 45 nucleotides but whose titration behaviour and end-group content were not obtained *. Any difference may arise not only because different preparative reactions were employed but also because the preparation of Tamm *et al.* was carried out under conditions of continuous dialysis during the acid hydrolysis, thereby concurrently removing all except the larger fragments.

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* *Note added in proof.* We have now titrated APA prepared from herring roe DNA using the method of Tamm *et al.*[1] The titration curve was identical with that of RAPA-H2 between pH 2.6-9. Hence the number of secondary phosphoryl groups in RAPA-H2 and the APA prepared by the method of Tamm *et al.*[1] must be identical, which means the number average chain length must be the same.