368. *The Nucleotide Sequence in Deoxyribonucleic Acids. Part V.* The Alkaline Degradation of Apurinic Acids.*

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Degradation of apurinic acids in N-potassium hydroxide at 37° or at pH 12 at 56° gave, in addition to oligonucleotides, an αβ-unsaturated aldehyde phosphate (probably II), the formation of which can be explained if the degradation proceeded by a β-elimination. The amount of this product indicated that about 45% of the degradation proceeded by this mechanism. Over 20% of the apurinic acid phosphorus was converted into inorganic phosphate, probably by simultaneous or consecutive cyclisation and β-elimination.

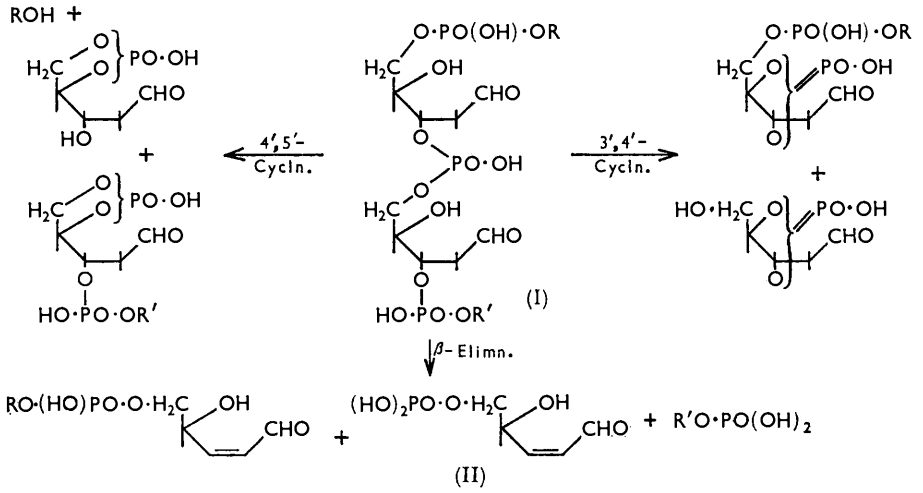
Apurinic acids (I), the products of the mild acid hydrolysis of deoxyribonucleic acids,[1] are degraded by alkali.[2] As all the interpyrimidine nucleotide linkages remain intact, examination of the degradation products should give information on the distribution of pyrimidines in deoxyribonucleic acids.[2] Two types of mechanism have been suggested for this alkaline degradation, (*a*) cyclisation,[2] which could be either at positions 3′, 4′ or at positions 4′, 5′, and (*b*) β-elimination.[3]

We have now obtained definite evidence that part of the degradation takes place by β-elimination. Thus apurinic acid was much more unstable than ribonucleic acid at pH 10 and 37°, so that they are probably degraded by different mechanisms, and ribonucleic acid is known to break down by cyclisation.[4] Paper chromatography of the products formed by degradation of apurinic acid with N-potassium hydroxide at 37° revealed, in addition to oligonucleotides, a component which appeared as a red spot with aniline hydrogen

* Part IV, *J.*, 1957, 2454.

[1] Tamm, Hodes, and Chargaff, *J. Biol. Chem.*, 1952, **195**, 49.
[2] Tamm, Shapiro, Lipshitz, and Chargaff, *J. Biol. Chem.*, 1953, **203**, 673.
[3] Brown and Todd, "Nucleic Acids," Vol. I, Academic Press, New York, 1955, p. 444.
[4] Brown and Todd, *J.*, 1952, 52.

phthalate and gave a positive reaction for phosphate.[5] In distilled water the product had $\lambda_{max.}$ 249 mμ and gave an immediate red colour with Schiff's reagent.[6] The ultraviolet absorption spectrum differed from those of the purines, pyrimidines, and their nucleosides and nucleotides, found in deoxyribonucleic acids, particularly by the absence of the peak or shoulder at 270—280 mμ which is present in the spectra of guanine derivatives. These properties indicated that the component was probably the αβ-unsaturated aldehyde



R and R′ = chain of pyrimidine nucleotide units.

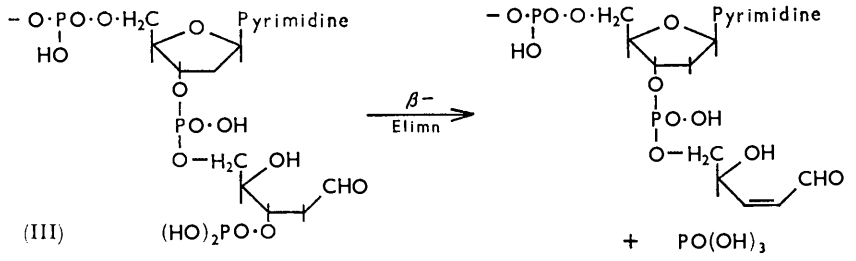
phosphate (II) which would be expected to arise by β-elimination. On the assumption that the substance contained 1 atom of phosphorus per molecule, $\varepsilon_{max.}$ was 8000, which is within the range quoted for αβ-unsaturated carbonyl compounds.[7] Woodward [8] found that αβ-unsaturated ketones usually have $\lambda_{max.}$ 225 ± 5 mμ; Evans and Gillam [7] showed that αβ-unsaturated aldehydes have $\lambda_{max.}$ 208—245 mμ, depending on the degree of substitution, increasing substitution generally increasing $\lambda_{max.}$. The even higher $\lambda_{max.}$ of this component can be attributed to substitution by both a hydroxyl and a $CH_2{\cdot}O{\cdot}PO(OH)_2$ group. Further evidence was that the substance formed a 2,4-dinitrophenylhydrazone whose $\lambda_{max.}$ (384 mμ) was characteristic of 2,4-dinitrophenylhydrazones of αβ-unsaturated aldehydes.[9]

The amount of αβ-unsaturated aldehyde phosphate (II) (measured by means of its phosphorus content [1]) produced in N-potassium hydroxide at 37° rose to a maximum of 6·3% after 14 hr. and then slowly decreased (to 1·1% after 356 hr.). The decrease may have been due to oxidation, hydrolysis, elimination of phosphate, reaction with other compounds in the alkaline solution, or tautomeric change. (αβ-Unsaturated carbonyl compounds in some cases, readily isomerise to βγ-tautomers in alkali.[10]) The present appears to be a particularly favourable case as further tautomerism to 5-hydroxylævulaldehyde 5-phosphate would ensue: the latter has, however, not been detected. Extrapolation of the results to zero decomposition showed that 6·5—7·5% of the apurinic acid phosphorus had been converted into " αβ-unsaturated aldehyde phosphorus." Similar

[5] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[6] Tobie, *Ind. Eng. Chem. Analyt.*, 1942, **14**, 405.
[7] Evans and Gillam, *J.*, 1943, 565.
[8] Woodward, *J. Amer. Chem. Soc.*, 1941, **63**, 1123.
[9] Roberts and Green, *J. Amer. Chem. Soc.*, 1946, **68**, 218.
[10] Ingold, " Structure and Mechanism in Organic Chemistry," Bell, London, 1953, p. 562.

results were obtained when apurinic acid was treated at pH 12 at 56°, but decomposition of the αβ-unsaturated aldehyde phosphate was very slight.

Compound (II) would arise by β-elimination only from those sites in the original deoxyribonucleic acid where at least two purine nucleotides were adjacent, so that if all the degradation proceeded by this mechanism the amount of compound (II) should be the same as the amount of inorganic phosphorus produced by the degradation of apurinic acid or deoxyribonucleic acid with diphenylamine and formic acid as described by Burton.[11] The apurinic acid used in this work gave 16·5% of inorganic phosphorus when degraded under these conditions. This was much lower than that obtained by Burton (25%) for



(III)

the degradation of herring testes deoxyribonucleic acid,[12] the difference arising because our apurinic acid was prepared by prolonged acid-hydrolysis of commercial herring-sperm deoxyribonucleic acid and was therefore considerably degraded. It contained 0·31 mol. of purines, 2·79 mol. of pyrimidines, and only 0·90 mol. of non-glycosidically bound 2-deoxy-D-ribose per 4 g.-atom of phosphorus. It can be concluded, therefore, that a maximum of 45% of the alkaline degradation of apurinic acid proceeds by a β-elimination.

During the alkaline degradation, 20—26% of the apurinic acid phosphorus was converted into inorganic phosphate. A small proportion of this could have arisen from the hydrolysis or elimination of phosphate from the αβ-unsaturated aldehyde phosphate (II). The remainder could arise by almost simultaneous rupture of both 3′- and 5′-phosphate linkages, as may occur during the Burton degradation;[11] however, this cannot account for all of the inorganic phosphate produced, and it seems more probable that compounds such as (III) are formed by a cyclisation degradation mechanism and that these then eliminate phosphate as shown.

The occurrence of two types of degradation also presents the possibility that the αβ-unsaturated aldehyde phosphate (II), or an isomer of it, arises as the result of a secondary reaction by elimination of phosphate from a compound formed by a cyclisation mechanism.

The alkaline degradation of apurinic acid is obviously very complex, so it is to be expected that for a given pyrimidine sequence many oligonucleotide fragments would result. The complexity of the decomposition has been somewhat reduced by the formation of dithioacetals of apurinic acid,[13] but even in this case it has been found[14] that calf-thymus apurinic acid di(carboxymethyl) dithioacetal gives over 70 components on alkaline degradation owing to the formation of several products for a given nucleotide sequence. The degradation of apurinic acid and deoxyribonucleic acid by Burton's method,[11] in which diphenylamine and formic acid are used, appears to give only one product for each sequence of pyrimidine nucleotides and as yet appears to cause little fission of inter-pyrimidine nucleotide linkages.

[11] Burton, *Biochem. J.*, 1956, **62**, 315; Burton and Peterson, *ibid.*, 1960, **75**, 17.
[12] Burton, *Biochem. J.*, 1960, **75**, 35P.
[13] Jones and Letham, *J.*, 1956, 2573; Jones, Letham, and Stacey, *J.*, 1956, 2579, 2584; Jones, Stacey, and Watson, *J.*, 1957, 2454; Kent, Lucy, and Ward, *Biochem. J.*, 1955, **61**, 529.
[14] Cunningham and Jones, unpublished results.

Experimental

Analytical Methods.—Nitrogen was determined by Ma and Zuazaga's method,[15] total phosphorus as described by Jones, Lee, and Peacocke,[16] and inorganic phosphorus by Berenblum and Chain's procedure.[17] Base analyses were carried out by Wyatt's method.[18]

Apurinic Acids.—(a) *Calf thymus.* This was prepared from highly polymerised calf-thymus deoxyribonucleic acid by the method described by Tamm *et al.*[1] The product contained 0·13 mole of guanine, 0·07 mole of adenine, 0·87 mole of cytosine, and 1·14 moles of thymine per 4 g.-atoms of phosphorus. (b) *Herring sperm.* Commercial herring-sperm deoxyribonucleic acid (3 g.; Isaac Spencer & Co.; more than half of the cytosine in this material had been degraded to uracil) was dissolved in 0·025N-hydrochloric acid (800 ml.) and set aside at 37° for 48 hr. The solution was then dialysed against running tap-water at room temperature for 24 hr. and then against frequent changes of distilled water at 0° for 48 hr. The dialysed solution was freeze-dried, to give apurinic acid (1·8 g.; N, 7·4%; P, 8·35%). Analysis of this material for purines and pyrimidines (by Mr. T. W. Thompson, B.Sc.) showed that it contained 0·16 mole of guanine, 0·15 mole of adenine, 0·61 mole of cytosine, 0·73 mole of uracil, and 1·45 moles of thymine per 4 g.-atoms of phosphorus.

Degradation of Apurinic Acid at pH 10.—Calf-thymus apurinic acid (4 mg.) and yeast ribonucleic acid (4 mg.) were separately dissolved in borate buffer of pH 10 (2 ml.) and dialysed at 37° for 36 hr. against the same buffer (20 ml.). The optical density of the diffusate at 260 mμ was 0·84 in the case of the apurinic acid and 0·50 in the case of the ribonucleic acid. This showed that 40% of the apurinic acid hydrolysate and 12% of the ribonucleic acid hydrolysate had diffused through the membrane. A similar experiment was carried out on herring-sperm apurinic acid in carbonate buffer of pH 10 for 3 days at 37°. 43% of the material (as measured by phosphorus analysis) diffused through the membrane. When herring-sperm nucleic acid was dissolved in distilled water and dialysed against distilled water at 37°, 7% of the phosphorus diffused through the dialysis membrane.

Degradation of Apurinic Acid with N-*Potassium Hydroxide.*—Herring-sperm apurinic acid (500 mg.) was dissolved in N-potassium hydroxide (90 ml.) and set aside at 37° for 18 hr. The solution was neutralised with perchloric acid, potassium perchlorate filtered off, and the filtrate concentrated *in vacuo* to 30 ml. Chromatography of this solution (5 ml.) on Whatman No. 3 paper in propan-2-ol–ammonia (*d* 0·880)–water (70 : 6 : 30) gave a component of R_F 0·10 which absorbed ultraviolet light ($\lambda_{max.}$ 249 mμ), gave a brown spot with a silver nitrate spray,[19] a red spot with aniline hydrogen phthalate,[20] and a blue spot with the Hanes and Isherwood [5] phosphate-detecting spray. Several other components were present. These had lower R_F values, had $\lambda_{max.}$ at about 265 mμ and gave positive reactions with silver nitrate and phosphate-detecting sprays, but gave no reaction with aniline hydrogen phthalate.

The component of R_F 0·10 was eluted from several sheets of paper, and the eluate (200 ml.) concentrated *in vacuo* to 20 ml. The solution gave an immediate colour with sensitive Schiff's reagent.[6] Phosphorus analyses showed that the eluate contained about 4% of the phosphorus of the apurinic acid. On the assumption that the compound contained 1 atom of phosphorus per mol. the $\varepsilon_{max.}$ was 8000. Appropriate paper controls were taken for all these determinations.

A portion of the eluate (3 ml.) and a similar eluate of blank paper were concentrated *in vacuo* to 0·5 ml. and added to a boiling methanol solution (5 ml.) of 2,4-dinitrophenylhydrazine (15 mg.) containing 1 drop of 10N-hydrochloric acid. The solutions were cooled and then examined by paper chromatography on Whatman No. 1 paper. With butan-1-ol–ethanol–ammonia (*d* 0·880)–water (40 : 10 : 1 : 49) as solvent, three components of R_F 0·00, 0·76, and 0·86 were obtained in the test solution. The component of R_F 0·86 was 2,4-dinitrophenylhydrazine and that of R_F 0·76 was also present in the solution from the eluate of blank paper. The component remaining on the origin when eluted with water had $\lambda_{max.}$ 384 mμ. The reaction mixture was also chromatographed in water saturated in butan-1-ol, a component of R_F 0·75

[15] Ma and Zuazaga, *Ind. Eng. Chem. Analyt.*, 1942, **14**, 280.
[16] Jones, Lee, and Peacocke, *J.*, 1951, 623.
[17] Berenblum and Chain, *Biochem. J.*, 1938, **32**, 295.
[18] Wyatt, *Biochem. J.*, 1951, **48**, 584.
[19] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[20] Partridge, *Nature*, 1949, **164**, 443.

and a streak extending from the origin to R_F 0·5 being obtained. The former had $\lambda_{max.}$ 384 mμ and the latter was mainly due to 2,4-dinitrophenylhydrazine.

Rate of Formation of the αβ-Unsaturated Aldehyde Phosphate (II).—(*a*) Herring-sperm apurinic acid (500 mg.) was dissolved in N-potassium hydroxide (100 ml.) and set aside at 37°. At intervals, samples (5 ml.) were removed and neutralised with perchloric acid, potassium perchlorate was filtered off, and the filtrate concentrated *in vacuo* and submitted to paper chromatography for 2 days on Whatman No. 3 paper with propan-2-ol–ammonia (*d* 0·880)–water (70 : 1 : 30). The αβ-unsaturated aldehyde phosphate was located by spraying with aniline hydrogen phthalate a strip cut from the chromatogram, and the required zone was cut out and eluted with distilled water at 37° for 18 hr. The ultraviolet absorption spectra and phosphorus contents were measured. Control determinations were carried out on strips cut from blank areas of the paper. The ultraviolet absorption spectra of all the samples had $\lambda_{max.}$ 249 mμ. The results of the phosphorus analyses were as tabulated.

Amount (%) *of apurinic acid* P *converted into* αβ-*unsaturated aldehyde* P.

Time (hr.)	1·5	4	6·5	11	12	13	14	15	16	17	18	37·5	44·5	61·5	86	133	158	188	230	356
Amount (%)	3·0	4·1	5·1	5·9	5·9	6·0	6·3	6·3	6·2	6·1	6·3	4·3	4·3	3·4	2·9	2·0	2·0	1·6	1·4	1·1

After 356 hr. at 37°, 21·4% of the apurinic acid phosphorus had been converted into inorganic phosphate.

(*b*) Herring-sperm apurinic acid (250 mg.) was dissolved in 0·01N-potassium hydroxide (200 ml.) and set aside at 56°. During the first 24 hr., N-potassium hydroxide was added at intervals to maintain the pH at 12. Samples (5 ml.) were withdrawn and the " αβ unsaturated aldehyde phosphorus " determined as described above. The results were as follows:

Time (hr.)	3·5	23	46·5	95	125	168	264
Amount (%) converted	4·1	6·4	6·4	5·7	5·7	6·0	6·0

After 23 and 168 hr., 9·7% and 25·8% respectively of the apurinic acid phosphorus had been converted into inorganic phosphate.

Treatment of Apurinic Acid with Diphenylamine and Formic Acid.[11]—Herring-sperm apurinic acid (10 mg.) and highly polymerised herring-sperm deoxyribonucleic acid (10 mg.) were separately dissolved in 66·7% formic acid (6 ml.) containing 2% of diphenylamine and set aside at 29° for 19 hr. The inorganic and total phosphorus contents of the solutions were then determined. 26·3% of phosphorus of the deoxyribonucleic acid and 16·5% of that of the apurinic acid were converted into inorganic phosphate.

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