506. *The Nucleotide Sequence in Deoxypentosenucleic Acids. Part II.* The Alkaline Degradation of Calf-thymus* aldehydo*Apurinic Acid Di(carboxymethyl) Dithioacetal.*

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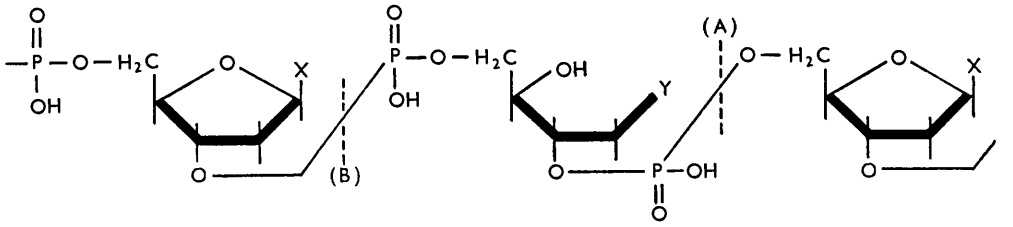
Degradation of calf-thymus *aldehydo*apurinic acid di(carboxymethyl) dithioacetal (I) with dilute alkali gave dialysable fragments. These have been separated by paper chromatography and paper electrophoresis into at least 20 components, some of which have been identified, including thymidine, deoxycytidine, nucleoside diphosphates, and derivatives of dinucleotides and of a trinucleotide. The results show that in calf-thymus deoxyribonucleic acid there are regions in which at least three pyrimidine nucleotides are linked together.

The preceding paper described the preparation of calf-thymus *aldehydo*apurinic acid di(carboxymethyl) dithioacetal (I) by the action of mercaptoacetic acid on calf-thymus deoxyribonucleic acid in the presence of zinc chloride and anhydrous sodium sulphate. On alkaline hydrolysis of the dithioacetal (I), fission could apparently take place in two ways represented by (A) and (B) in (I). If fission occurred at (A), 3′- and 4′-phosphates corresponding to the "a" and "b" nucleotides from pentosenucleic acids would be formed. The structure with the terminal 3′-phosphate could presumably undergo fission at (B), but the 4′-phosphate would be expected to be stable to alkali since cyclic phosphate formation could not take place. If fission occurred at (B), the 4′- and the 5′-phosphates would be formed, of which the 5′-phosphate would be expected to undergo further fission and the 4′-phosphate to be stable. Hence two series of products might arise; the first would consist of pyrimidine oligonucleotides carrying a terminal 2-deoxy-*aldehydo*-D-ribose phosphate di(carboxymethyl) dithioacetal residue, and the second of sulphur-free pyrimidine oligonucleotides without a terminal phosphate group.



(I) X = pyrimidine

Y = $CH(S{\cdot}CH_2{\cdot}CO_2H)_2$

Tamm *et al.*[1] found that apurinic acid was degraded by dilute alkali and put forward a mechanism similar to that proposed above for the degradation of the dithioacetal (I). Brown and Todd [2] suggested, however, that when apurinic acid is treated with dilute alkali, elimination of the phosphate groups occurs at position 3′ which is β with respect to the free aldehydo-groups. With the dithioacetal the latter reaction would not be expected.

Hydrolysis of the dithioacetal (I) at pH 12·4 at 37° for 15 hr. rendered 45% of the phosphorus dialysable against sodium hydroxide solution (pH 12·4) and a further 50% dialysable against 2M-sodium chloride. The hydrolysate was resolved into eight zones (numbered 1—8 in order of increasing R_F value) by paper chromatography with propan-2-ol–ammonia. These were eluted and analysed for phosphorus, sulphur, and pyrimidines.

* Part I, preceding paper.

[1] Tamm, Shapiro, Lipshitz, and Chargaff, *J. Biol. Chem.*, 1953, **203**, 673.
[2] Brown and Todd, "Nucleic Acids," Academic Press Inc., New York, 1955, Vol. I, p. 409.

All except zones 7 and 8 contained sulphur, and each zone which contained cytosine also contained 5-methylcytosine in a similar proportion to that in the intact deoxyribonucleic acid. Zones 2—6 were further resolved by paper electrophoresis and paper chromatography into a total of seventeen components. Zones 7 and 8 were homogeneous, but not zone 1, which contained 45% of the pyrimidine residues (attempts to fractionate it have so far been unsuccessful). After prolonged dialysis of the total hydrolysate of the dithioacetal against distilled water, only zone 1 remained in the dialysis bag.

The structures of the components whose identity appears certain are shown in Table 3. The homogeneity of most of the components with respect to their composition is supported by the analytical results and the fact that they could not be further resolved by chromatography or electrophoresis. Components 2B and 2C may have a different sequence from that indicated or be mixtures of substances of the same composition but different sequence. The terminal phosphate has been assigned to the 2-deoxy-*aldehydo*-D-ribose di(carboxymethyl) dithioacetal residue in view of the proposed fission mechanism and results of experiments on the action of snake venom diesterase on certain of the components (see following paper). It was unlikely that any of the components contained a 2-deoxy-*aldehydo*-D-ribose di(carboxymethyl) dithioacetal residue flanked on both sides by pyrimidine nucleotide residues since rehydrolysis of the components of each chromatographic zone did not alter their R_F values. The possibility that there occurred pyrimidine oligonucleotides carrying two terminal 2-deoxy-*aldehydo*-D-ribose di(carboxymethyl) dithioacetal residues was excluded, in the case of the identified components, by the analytical results, but such structures may be included in the unidentified components. The structures assigned to the components are supported by phosphorus, sulphur, and pyrimidine analyses (Table 3). The identities of zones 7 and 8 are also supported by their electrophoretic behaviour and of components 6A, 6B, and 6C by electrophoresis and enzymic degradation. Hydrolysis of component 2B with an acidic ion-exchange resin followed by treatment with phosphomonoesterase gave dithymidine phosphate, thus showing the presence, in this component, of an oligonucleotide with two adjacent thymine nucleotides. The structure of component 3B was doubtful since it was sulphur-free, but from the pyrimidine composition it was concluded that it contained at least three pyrimidine residues. Its structure as a trinucleoside diphosphate has been tentatively proposed in view of its position on the chromatograms, its behaviour on dialysis, and the fission mechanism which would be expected to give rise to sulphur-free fragments. Zones 4 and 5 were heterogeneous, containing at least 2 and 3 pyrimidine-containing fragments respectively, none of which have been identified. Zone 5 reacted with sprays specific for α-glycols, and it contained only 0·46 atom of phosphorus per atom of sulphur. Zone 4 contained more phosphorus than would be expected from its low pyrimidine content, and zone 6 contained sulphur which was not found in components 6A, 6B, and 6C. Thus the products from the degradation of the regions in the dithioacetal (I) which were free from pyrimidines, were probably in zones 4, 5, and 6. The analytical results suggested that zone 1 was mainly trinucleotides. Its non-dialysability against water indicated, but did not prove, the absence of material of lower molecular weight.

The nucleosides and the dinucleoside phosphates in the hydrolysate could have arisen from fission at both (A) and (B) in the dithioacetal (I), or by non-specific hydrolysis during the treatment of the deoxyribonucleic acid with mercaptoacetic acid. Since zone 1 must have consisted mainly of sulphur-containing fragments it appeared that these predominated over the sulphur-free fragments in the total hydrolysate, so that, in most cases, fission did not occur at both (A) and (B).

The main disadvantage of this method of degradation was the fission which accompanied the treatment with mercaptoacetic acid. This could have been specific hydrolysis similar to that occurring with alkali, but it was probably a non-specific hydrolysis of the main phosphodiester linkages. If this type of degradation could have been avoided, larger fragments might have been formed on alkaline hydrolysis. The results indicate, however, that in intact calf-thymus deoxyribonucleic acid there occur regions in which at least three pyrimidines are linked together. There was no indication of any simple uniform arrangement such as alternate purines and pyrimidines.

Experimental

Degradation of Calf-thymus aldehydo*Apurinic Acid Di(carboxymethyl) Dithioacetal with Dilute Alkali.*—The dithioacetal (I) (40 mg.) was dissolved in sodium hydroxide solution (pH 12·4; 30 ml.), aliquot parts were taken for the estimation of phosphorus, and three further samples (9 ml.) each dialysed against consecutive volumes (130 ml.) of sodium hydroxide (pH 12·4) at 37° for a total of 3 days. The dialysis was then similarly continued against M-sodium chloride at 16° for 3 days and finally against 2M-sodium chloride at 16° for 3 days. Calf-thymus deoxyribonucleic acid was treated in the same way. The phosphorus contents of the dialysates and that of the material remaining in the dialysis bags were determined. The results are recorded in Table 1.

Table 1. *Distribution of phosphorus in the alkaline hydrolysates of calf-thymus* aldehydo-*apurinic acid di(carboxymethyl) dithioacetal* (I) *and of calf-thymus deoxyribonucleic acid.*

	Total phosphorus (%) in				
	sodium hydroxide dialysates			M- and 2M-NaCl	Residue in
	1st	2nd	3rd	dialysates	dialysis bag
Dithioacetal (I)	29	14	2·5	48	4
Deoxyribonucleic acid	1·5	0·1	0·0	0·0	97

Fractionation of the Alkaline Hydrolysate of the Dithioacetal (I).—An aqueous solution of the dithioacetal (I) was adjusted to pH 12·4 with N-sodium hydroxide, kept at 37° for 26 hr., neutralised with dilute acetic acid, and freeze-dried. An aqueous solution of the product was used in fractionations.

Paper electrophoresis at pH 3·4, 4·9, 6·5, and 10·0 did not give adequate fractionation. Several solvent systems were investigated for paper chromatography. Development of the chromatograms with propan-2-ol (70), water (30), and ammonia (*d* 0·88; 6 parts) for 2 days on Whatman No. 3 paper gave five zones (detected by ultraviolet photography). When development was for only 16 hr., three additional zones were found, while zones 1—5 were only partly resolved. The R_F values of zones 6—8 were measured, and the R_C value of each zone calculated (R_C = distance moved by component/distance moved by cytidylic acid). These values are given in Table 2. Each zone, when chromatographed again in the same solvent gave a spot with a similar R_C value to the above. Eluates of the zones were rehydrolysed at pH 12·3 at 37° for 2 days. No changes in the R_C values were observed. Attempts were made to locate the phosphates of 2-deoxy-*aldehydo*-D-ribose di(carboxymethyl) dithioacetal, which would be expected from the alkaline hydrolysis of the dithioacetal (I). These would not be detectable by the ultraviolet photographic technique, so the following sprays were used. (1) A Dische spray, similar to that used by Buchanan,[3] detected all the eight zones revealed by photography, but no additional components. (2) After being sprayed with ammoniacal silver nitrate (2%), the chromatograms were heated at 105° for 5—10 min. Two brown spots developed; one just above zone 5 (due to sodium acetate), and one which coincided with zone 6. (3) The sugar phosphate spray of Hanes and Isherwood [4] detected zones 1—6, but not zones 7 and 8. No additional zones were detected. (4) Buchanan's spray for 1 : 2-glycols [5] gave a faint spot which coincided with zone 5. (5) A 0·3% solution of lead tetra-acetate in benzene gave a spot which coincided with zone 5.

Fractionation by Dialysis.—After the alkaline hydrolysate of the dithioacetal had been dialysed against running tap-water for 3 days and then repeated changes of distilled water for 2 days, only zone 1 remained in the dialysis bag. This material contained 4·2 atoms of phosphorus per 2 atoms of sulphur. When zone 1 prepared by paper chromatography was dialysed against distilled water, some of the material diffused slowly through the dialysis bag.

Determination of the Phosphorus : Sulphur Ratios of the Chromatographic Zones.—When zone eluates were analysed for sulphur,[6] very high control values (50% of the sulphur content of the eluates) were obtained. As these were due to the presence of sulphate in the paper which could not be completely removed by washing before chromatography, methods for removing

[3] Buchanan, *Nature*, 1951, **168**, 1091.
[4] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[5] Buchanan, Dekker, and Long, *J.*, 1950, 3162.
[6] Jones and Letham, *Analyst*, 1956, **81**, 958.

the interfering sulphate were developed. The first consisted of precipitating the sulphate with 4-amino-4′-chlorodiphenyl [7] and determining the sulphur : phosphorus ratio of the supernatant liquids. This method could be applied to all zones, but there was a slight precipitation of zone 1 with the 4-amino-4′-chlorodiphenyl. The second method, which was applicable to zones 1—3, was to precipitate the components with silver, leaving the sulphate in solution. No decomposition of the components occurred during this process.

The various zones were separated on Whatman No. 3 paper which had been previously washed with water, eluted with water, freeze-dried, and then analysed for phosphorus and sulphur by the above methods. The results are recorded in Table 2.

Determination of the Pyrimidine Composition of the Chromatographic Zones.—Pyrimidine analyses of the chromatographic zones and of various other fractions described later, were carried out as follows : the material was eluted from the paper with distilled water, the eluate freeze-dried, and the residue hydrolysed with " AnalaR " formic acid at 175° for 1 hr. The pyrimidines were then separated on Whatman No. 1 paper with propan-2-ol–hydrochloric acid,[8] eluted with 0·1N-hydrochloric acid, and estimated spectrophotometrically.

TABLE 2. *Properties of the chromatographic zones.*

Zone	1	2	3	4	5	6	7	8
R_C	0·04	0·15	0·35	0·59	1·15	1·9	2·8	3·1
R_F	—	—	—	—	—	0·42	0·61	0·69
Atoms of phosphorus per 2 atoms of sulphur (*a*) *	3·94	3·10	1·90	1·43	0·46	3·2	S and P absent	
(*b*) *	3·74	2·94	1·96	—	—	—	—	—
5-Methylcytosine (10^{-2} moles) per mole of cytosine	4·8	5·2	7·9	4·0	3·6	4·5	5·4	—

(*a*, *b*) Control values reduced by (*a*) precipitation with 4-amino-4′-chlorodiphenyl, and (*b*) precipitation of components with silver.

TABLE 3. *Structures of the components in the alkaline hydrolysate of calf-thymus* aldehydo-*apurinic acid di(carboxymethyl) dithioacetal.*

Component (zone)	% of total pyrimidines	Atoms of P per 2 atoms of S	Mols. of pyrimidines per atom of P: Cytosine	Thymine	Proposed structure *
8	3·0	P and S absent	Absent	Present	T
7	2·0	P and S absent	Present	Absent	C
6A	4·5	S absent	0·95	1·04	T–P–C
6B	2·0	,,	0·00	1·94	T–P–T
6C	1·5	,,	1·96	0·00	C–P–C
5	5·0	0·46	—	—	Heterogeneous
4	2·0	1·43	—	—	,,
3A	9·0	1·93	0·00	0·52	T–P–S–P
3B	2·0	S absent	Thymine/cytosine = 1·94		T–P–T–P–C (?)
3C	3·0	2·08	0·47	0·00	C–P–S–P
2A	5·0	2·90	0·00	0·69	T–P–T–P–S–P
2B	3·0	3·88	0·27	0·51	T–P–T–P–C–P–S–P
2C	10	3·09	0·32	0·33	T–P–C–P–S–P
2D	1·5	2·10	0·48	0·00	C–P–S–P
2E	<1	—	Contains both bases		—
2F	<1	S absent	Only cytosine present		—
1	45	3·94; 3·74	—	—	Heterogeneous
1 †	—	4·20	Thymine/cytosine = 1·48		,,

* T = Thymidine residue. C = Deoxycytidine residue. S = 2-Deoxy-*aldehydo*-D-ribose di-(carboxymethyl) dithioacetal residue. P = Phosphate residue. † Prepared by dialysis.

Zones 1—6 contained both thymine and cytosine, zone 7 contained only cytosine, and zone 8 only thymine. Since zones 1—6 were heterogeneous, quantitative analyses for cytosine and thymine were not carried out, but the distribution of 5-methylcytosine in the various zones was investigated. The results are in Table 2.

Further Resolution of the Chromatographic Zones.—Samples of the material from the various

[7] Belcher, Nutten, and Stephen, *J.*, 1953, 1334.
[8] Wyatt, *Biochem. J.*, 1951, **48**, 584.

zones were prepared by elution from a large number of sheets of Whatman No. 3 paper, and the eluates concentrated and freeze-dried. The zones were then resolved as follows :

Chromatography. When developed with *iso*butyric acid–ammonium *iso*butyrate (Magasanik *et al.*[9]), zone 1 streaked badly without showing resolution, zones 2, 3, 6, 7, and 8 were not resolved, zone 5 was separated into three components, and zone 4 into two components (all the papers examined by ultraviolet photography).

Electrophoresis. The zones were subjected to electrophoresis on Whatman No. 3 paper at 900 v for 3 hr. (apparatus : see Foster [10]) at pH 3·3; zone 2 separated into six components, and zones 3 and 6 each gave three. Zone 1 was not further fractionated under these conditions but gave a long streak. Zones 7 and 8 were not further resolved at pH 3·3, 4·8, or 9·0.

In order to determine the proportion of the total pyrimidine residues present in each component, the chromatograms and electrophoretograms were cut transversely into 0·5 cm. strips, and the components were eluted with phosphate buffer of pH 6·4. The optical densities of the eluates were read at 276 mμ (the wavelength at which the molecular extinctions of thymidylic acid and deoxycytidylic acid coincide), appropriate controls being used to allow for the presence of optically absorbing material in the paper. From these results the values given in Table 3 were calculated.

Identification of the Components.—Zones 7 and 8. These were not resolved by any method tried and were free from sulphur and phosphorus. Zone 7 contained cytosine but not thymine, and zone 8 contained thymine but not cytosine. They gave positive reactions with the cysteine–sulphuric acid reagent,[3] showing the presence of a deoxypentose moiety. Zones 7 and 8 were identical chromatographically and electrophoretically with authentic deoxycytidine and thymidine respectively.

Zone 6. Although zone 6 contained sulphur, components 6A, B, and C, isolated by electrophoresis, did not. These components were not attacked by prostate phosphomonoesterase under conditions which rapidly dephosphorylated nucleotides. Therefore, they did not have a terminal phosphate group. These and the analytical results (Table 3) showed that 6A was thymidine–deoxycytidine phosphate, 6B was dithymidine phosphate, and 6C was di(deoxycytidine) phosphate. These conclusions were supported by the electrophoretic behaviour of the components. At pH 3·3, component 6B moved to the anode more slowly than thymidylic acid; 6A had about the same mobility as deoxycytidylic acid; and 6C moved to the cathode, but more slowly than deoxycytidine.

Zones 2 and 3. After the components had been subjected to paper electrophoresis a second time in order to free them from small amounts of the adjacent components, they were analysed for phosphorus, sulphur, and pyrimidines. From the results (Table 3) it was possible to assign the structures suggested in Table 3. The agreement between the experimental and theoretical values was sufficient to show that the components were homogeneous with respect to pyrimidine, phosphorus, and sulphur content. Components 3A, 3C, 2A, and 2D were considered to be as indicated, but components 2B and 2C could have had other sequences. The presence of the sequence, thymidine–phosphate–thymidine in component 2B was demonstrated as follows : Component 2B was dissolved in water, Zeocarb 225 (H form; ½ vol.) added, and the suspension heated on a boiling-water bath for 3 hr. The resin was filtered off and the solution treated with phosphomonoesterase at pH 5·2 and 37°. Separation of the product by paper chromatography showed the presence of dithymidine phosphate and thymidine.

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[9] Magasanik, Vischer, Doniger, Elson, and Chargaff, *J. Biol. Chem.*, 1950, **186**, 37.
[10] Foster, *Chem. and Ind.*, 1952, 1050.