505. *The Nucleotide Sequence in Deoxypentosenucleic Acids. Part I. The Action of Mercaptoacetic Acid on Calf-thymus Deoxyribonucleic Acid.*

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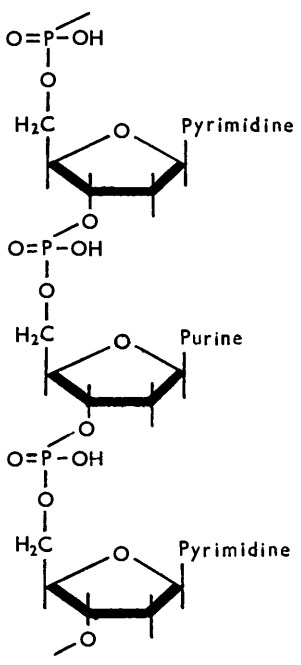
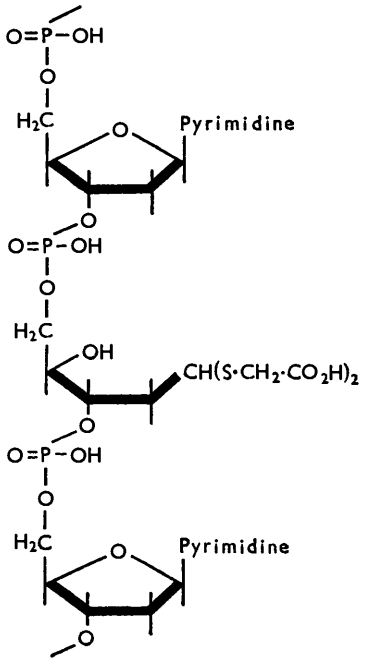
By treatment of calf-thymus deoxyribonucleic acid with mercaptoacetic acid, the purines were removed and replaced by carboxymethylthio-groups. The reaction did not go to completion unless anhydrous zinc chloride and sodium sulphate were also present (at 37°). Since only 6·2% of the phosphorus of the deoxyribonucleic acid was rendered dialysable, the product was suitable for studies of its degradation by alkali and hence for determination of the sequence of the pyrimidine nucleotides.

The enzymic degradation of deoxyribonucleic acid has been extensively studied.[1,2,3] Deductions from these results concerning the nucleotide sequence in the deoxyribonucleic acid have, however, not been very fruitful, owing to the obscure nature of the enzymic specificity and to the possibility of exchange reactions, similar to those occurring during action of ribonuclease.[4] In the present investigations, therefore, a chemical method for the specific degradation of deoxyribonucleic acids has been developed. Since deoxy-



(I)



(II)

pentosenucleic acids, in the form usually isolated from cells, are mixtures of different polynucleotides,[5,6] it is not possible to deduce a detailed nucleotide sequence from degradative studies. However, useful information of a more general character, *e.g.*, whether purines and pyrimidines are found in any given arrangement with respect to each other, can

[1] Smith and Markham, *Nature*, 1952, **170**, 120; *Biochim. Biophys. Acta*, 1952, **8**, 350.
[2] Zamenhof, Chargaff, and Brawerman, *J. Biol. Chem.*, 1950, **187**, 1.
[3] Overend and Webb, *J.*, 1950, 2746.
[4] Heppel and Whitfield, *Biochem. J.*, 1955, **60**, 1; Heppel, Whitfield, and Markham, *ibid.*, p. 8.
[5] Chargaff, Crampton, and Lipshitz, *Nature*, 1953, **172**, 289.
[6] Brown and Watson, *Nature*, 1953, **172**, 339.

be obtained. From preliminary experiments [7] it was deduced that in calf-thymus deoxyribonucleic acid there occur regions in which at least three pyrimidine nucleotides are adjacent.

Since removal of the purines from deoxyribonucleic acid with dilute acid liberates 2-deoxy-D-ribose residues which are partly in the *aldehydo*-form,[8] simultaneous treatment with a thiol [9] should give a dithioacetal (II; where the thiol is mercaptoacetic acid, $HS{\cdot}CH_2{\cdot}CO_2H$). In this product a hydroxyl group would be adjacent to the 3′- and 5′-phosphate residues, hence cyclic phosphate formation of the type known to render pentose-

FIG. 1.

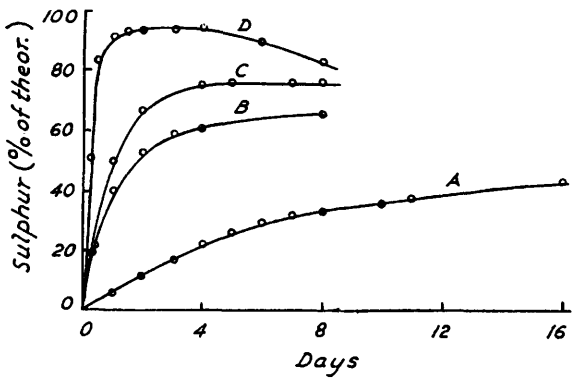


FIG. 2.

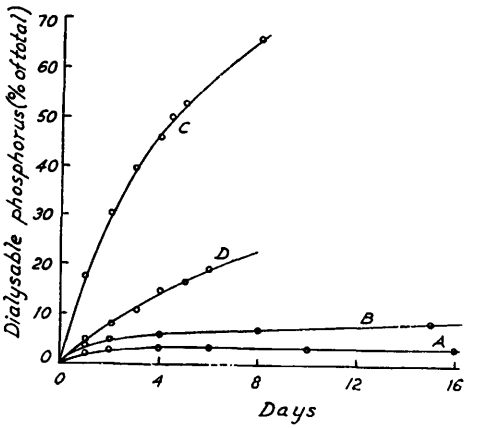
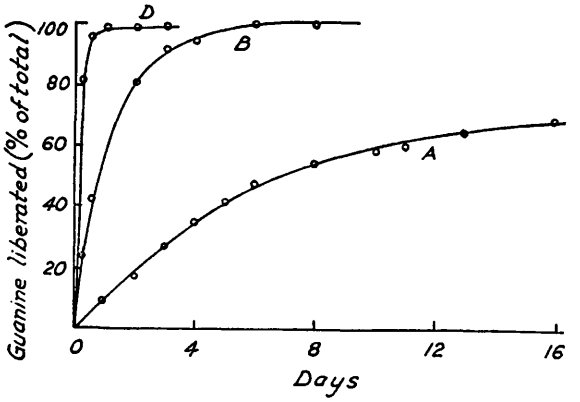


FIG. 3.



FIGS. 1—3. *The reaction of mercaptoacetic acid with calf-thymus deoxyribonucleic acid, A, at 16°; B, at 36°; C, at 62°; D, in the presence of zinc chloride and sodium sulphate at 37°.*

nucleic acids labile to alkali [10] would be possible. On alkaline hydrolysis of the dithioacetal, fission would be expected to occur at the internucleotide linkages, which in the intact deoxypentosenucleic acid were to purine nucleotides, but not at those between pyrimidine nucleotides. There would thus arise pyrimidine oligonucleotides, the separation and identification of which would give information about the sequence of the pyrimidine nucleotides in the deoxypentosenucleic acid. The sequence of the purine nucleotides might then be inferred, by assuming that in intact deoxypentosenucleic acid adenine is linked by hydrogen bonds to thymine, and guanine to cytosine.[11]

Mercaptoacetic acid ($HS{\cdot}CH_2{\cdot}CO_2H$) was selected for this work because it readily

[7] Jones and Letham, *Biochim. Biophys. Acta*, 1954, **14**, 438.
[8] Chong-Fu Li, Overend, and Stacey, *Nature*, 1949, **163**, 538.
[9] Kent, *Nature*, 1950, **166**, 442.
[10] Brown and Todd, *J.*, 1952, 52.
[11] Watson and Crick, *Nature*, 1953, **171**, 737.

dissolved deoxyribonucleic acids and was very reactive towards aldehydes, producing dithioacetals without the addition of a catalyst.[12] It would be expected, therefore, that mercaptoacetic acid would produce a dithioacetal from deoxyribonucleic acid with little degradation of the main phosphodiester " backbone." Mercaptoacetic acid, alone, was however not suitable: it did not react completely with commercial calf-thymus deoxyribonucleic acid at 16°, 36°, or 62° (Figs. 1—3), although at 36° there was complete liberation of guanine. At 62° large amounts of dialysable phosphorus were produced and the sulphur content of the product was only slightly higher than that from the reaction at 36°. No advantage was obtained by removing the purines from deoxyribonucleic acid with dilute acid,[13] and treating the resulting apurinic acid with mercaptoacetic acid.

When the apurinic acid was treated with mercaptoacetic acid in the presence of fused zinc chloride and anhydrous sodium sulphate at 37°, the sulphur content of the product was very near to the theoretical value. When applied to the reaction with calf-thymus deoxyribonucleic acid, these catalysts accelerated both the rate of purine release and the rate of incorporation of sulphur, the former being complete well before the latter. Products with sulphur contents close to the theoretical were obtained and only 6·2% of the total phosphorus was rendered dialysable. Over 90% of this dialysable phosphorus was

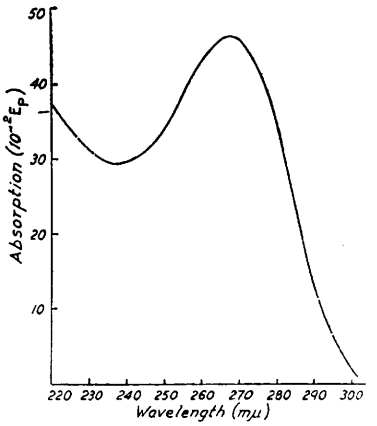


FIG. 4. *Ultraviolet absorption (extinction per g.-atom of phosphorus, E_P) of calf-thymus* aldehydo-*apurinic acid di(carboxymethyl) dithioacetal.*

" organic phosphorus." It appeared that free thymine and cytosine were not formed in appreciable quantities but small amounts of thymine and cytosine were isolated from the formic acid hydrolysate of the dialysable material.

While this investigation was in progress, the production of a diethyl dithioacetal from herring-roe deoxyribonucleic acid, by use of ethanethiol and hydrochloric acid, was reported by Lucy and Kent.[14] Comparative experiments with this reagent were carried out on calf-thymus nucleic acid. Treatment with ethanethiol and 10N-hydrochloric acid at 0° gave an insoluble gum into which sulphur was incorporated. When the sulphur content was 60% of the theoretical for complete incorporation, 18% of the total phosphorus had already been rendered dialysable. This reagent, therefore, caused greater degradation of the main phosphodiester linkages than did mercaptoacetic acid in the presence of the catalysts. In a recent paper [15] Kent, Lucy, and Ward have described their method in greater detail: they report only 10% of a non-dialysable dithioacetal. Only limited information about nucleotide sequence could be expected from the examination of such a small fraction of the intact deoxyribonucleic acid.

The product of the reaction of mercaptoacetic acid, zinc chloride, and sodium sulphate with calf-thymus deoxyribonucleic acid (sample 2) was essentially free from purines and contained the theoretical amount of sulphur. The product obtained from sample 1 (which

[12] Bongartz, *Ber.*, 1888, **21**, 478.
[13] Tamm, Hodes, and Chargaff, *J. Biol. Chem.*, 1952, **195**, 49.
[14] Lucy and Kent, *Research*, 1953, **6**, 49s.
[15] Kent, Lucy, and Ward, *Biochem. J.*, 1955, **61**, 529.

was less pure than sample 2) contained 95% of the theoretical amount of sulphur and gave a faint colour with Schiff's reagent indicating the presence of about 4% of the free aldehyde groups found in apurinic acid. The absorption spectrum of the dithioacetal (II) (Fig. 4) was similar to that of apurinic acid.[13] These properties showed that the reaction in the presence of the catalysts proceeded in the manner indicated. It has been suggested that mercaptoacetic acid may react with deoxyribonucleic acid in other ways,[15] but this is excluded because there were 4% of free aldehyde groups in the product in which dithioacetal formation was only 95% complete (as shown by sulphur analysis), which shows that there was little tendency to formation of a monothioacetal. Therefore, since the sulphur contents of the products were close to the theoretical for complete replacement of the purines it may be concluded that there was little reaction of mercaptoacetic acid with other groups in the molecule. In the absence of the catalysts, it appeared that either some aldehyde groups were less reactive than others, or thioglycosides were formed from some 2-deoxy-D-ribose residues. The production of dialysable phosphorus during the reaction indicated that some fission of the main internucleotide linkages had occurred. However, since 90% of the material was non-dialysable, it was considered to be suitable for the proposed structural studies which are reported in the following paper.

Experimental

Nitrogen was determined by Ma and Zuazaga's method [16] and phosphorus by that of Jones, Lee, and Peacocke.[17] Sulphur was measured by a micro-adaptation of the method of Belcher *et al.*[18] The compound, containing about 0·35 mg. of sulphur, was heated at 280—290° for 3 hr. in a sealed tube with sodium chloride (6 mg.) and concentrated nitric acid (0·3 ml.). The nitric acid was removed in a current of warm air, and the residue dissolved in water. Phosphate was precipitated as ammonium magnesium phosphate, which was used for the determination of phosphorus. Sulphate was precipitated from the acidified supernatant liquid as 4-amino-4'-chlorodiphenyl sulphate, which was centrifuged with a trace of cetrimide (Cetavlon) to effect a clean separation, and titrated with dilute aqueous sodium hydroxide. The method gave results within 1% of the theoretical with benzaldehyde di(carboxymethyl) dithioacetal, D-glucose diethyl dithioacetal, and D-glucose diethyl dithioacetal in the presence of deoxyribonucleic acid.

Deoxyribonucleic Acids.—Preliminary experiments were carried out on a sample of commercial calf-thymus deoxyribonucleic acid which had been freed from protein by Sevag's method.[19] It contained 1·00 mole of adenine and 0·80 mole of guanine per 4 atoms of phosphorus (Found: N, 14·6; P, 9·0%). Some cytosine had been deaminated to uracil but no pentose was present. The deoxyribonucleic acids used for the final experiments were isolated from calf thymus by Mirsky and Pollister's method [20] but with deproteinisation by detergent.[21, 22] They contained no detectable sulphur. The compositions are tabulated. The base composition was determined in triplicate by the method of Laland *et al.*[23] The nucleic acids were dried at 110° at 20 mm. for 2 hr. before treatment with mercaptoacetic acid.

Composition of calf-thymus deoxyribonucleic acid.

			Moles of bases per 4 atoms of phosphorus:			
Sample	N (%)	P (%)	adenine	guanine	cytosine *	thymine
1	13·8	8·14	1·05	0·83	0·83	1·06
2	15·0	8·96	1·09	0·85	0·88	1·11

* Includes 5-methylcytosine.

Reaction of Calf-thymus Deoxyribonucleic Acid with Mercaptoacetic Acid.—Three solutions of commercial calf-thymus deoxyribonucleic acid (0·6 g) in mercaptoacetic acid (98%; 10 ml.) were kept at 16°, 36°, and 62° severally. Aliquot parts (0·5 ml.) were withdrawn at intervals

[16] Ma and Zuazaga, *Ind. Eng. Chem. Anal.*, 1942, **14**, 280.
[17] Jones, Lee, and Peacocke, *J.*, 1951, 623.
[18] Belcher, Nutten, and Stephen, *J.*, 1953, 1334.
[19] Sevag, *Biochem. Z.*, 1934, **273**, 419.
[20] Mirsky and Pollister, *Proc. Nat. Acad. Sci., U.S.A.*, 1942, **28**, 344.
[21] Marko and Butler, *J. Biol. Chem.*, 1951, **190**, 165.
[22] Jones and Marsh, *Biochim. Biophys. Acta*, 1954, **14**, 559.
[23] Laland, Overend, and Webb, *J.*, 1952, 3224.

from each solution and diluted with ether (5 ml.), and the resulting precipitates centrifuged, washed with ether, dried, and dissolved in distilled water (2—3 ml.). The solutions were transferred quantitatively to washed dialysis bags and dialysed for 118 hr. at 0° against two changes of distilled water (200 ml.). The solutions in the dialysis bags were freeze-dried and the products analysed for sulphur. Samples of the dialysable material from each precipitate were adjusted to pH 1 with hydrochloric acid, and the concentrations of adenine and guanine determined by measurement of the optical extinctions at 249 and 262·5 mμ. A slight correction to the values was made to allow for the absorption of small amounts of mercaptoacetic acid (estimated from the sulphur contents). From these results, the rates of liberation of guanine from the deoxyribonucleic acid were calculated, it having been previously shown that guanine dissolved in mercaptoacetic acid was almost completely precipitated (98%) by 10 vols. of ether. The precipitation of adenine, however, was incomplete and variable so that the liberation of adenine could not be calculated. As a check, the adenine and guanine contents of some of the non-dialysable fractions were determined. They corresponded with the dialysable guanine values and showed that the rates of liberation of guanine and adenine were about equal. Samples of the dialysate were also taken for the determination of dialysable phosphorus. The guanine liberated during the reaction at 62° was not measured owing to the production of large quantities of dialysable phosphorus and hence pyrimidines. The results are recorded in Figs. 1—3.

Reaction of Calf-thymus Apurinic Acid with Mercaptoacetic Acid.—(a) *Wtihout catalysts.* Apurinic acid (80 mg., prepared according to Tamm *et al.*[13]) was treated with mercaptoacetic acid at 16°. The sulphur content of the product, and the dialysable phosphorus, were measured at intervals as described above. The results were :

Time (days)	2	6	12
Sulphur (% of theoretical)	61·5	61·7	61·2
Dialysable phosphorus (% of total P)	4·2	7·1	9·9

(b) *In the presence of zinc chloride and sodium sulphate.* Apurinic acid (60 mg.) was dissolved in mercaptoacetic acid (4 ml.), freshly fused zinc chloride (0·4 g.) and anhydrous sodium sulphate (0·3 g.) (cf. Hauptmann [24]) were added, and the suspension was shaken at 16° for 10 days. The product was precipitated with ether and suspended in acetate buffer (μ 0·2; pH 6·0; 10 ml.), and the suspension dialysed (18 hr.) against distilled water. Glycine (1 g.) was added to the suspension in the dialysis bag, and the resulting clear solution dialysed (18 hr.) against 1% glycine solution, followed by running tap-water (18 hr.) and finally distilled water. The product obtained after freeze-drying of the solution contained 84·7% of the theoretical sulphur content. The reaction was repeated at 37° for two days, yielding a product containing 98% of the theoretical sulphur content.

Reaction of Calf-thymus Deoxyribonucleic Acid with Mercaptoacetic Acid in the Presence of Catalysts.—Calf-thymus deoxyribonucleic acid (sample 1; 141 mg.) was dissolved in mercaptoacetic acid (5 ml.) by shaking at room temperature. Fused zinc chloride (0·5 g.) and anhydrous sodium sulphate (0·3 g.) were added (the zinc chloride precipitated some of the deoxyribonucleic acid), and the suspension was shaken at 37°. Aliquot parts (1 ml.) were removed at intervals and the products precipitated by the addition of ether (10 ml.). The precipitates were washed with ether and suspended in acetate buffer pH 6, to which glycine (0·2 g.) was added, and the suspensions were dialysed against changes (200 ml.) of 0·1% glycine solution. Glycine (to 10%) was added to the solutions in the dialysis bags, the solutions were centrifuged to remove small quantities of guanine, the dialysis was continued against tap-water and then distilled water, and the material freeze-dried. The sulphur content of the product, the dialysable phosphorus, and the guanine were estimated as before. To the last there were added the guanine which remained insoluble in the dialysis bags. The results are shown in Figs. 1—3. The dialysable " inorganic phosphorus " was :

Time (days)	1	2	3	4	6
Inorganic phosphorus (% of total dialysable P)	4·3	6·0	7·1	6·9	5·7

The product obtained when the reaction had proceeded for 36 hr. was isolated. It contained 3·59 atoms of sulphur per 4 atoms of phosphorus and had the following composition (moles of base per 4 atoms of phosphorus) : adenine, 0·01; guanine 0·00; thymine 1·17; cytosine 0·81. The ultraviolet absorption spectrum at pH 7·2 is shown in Fig. 4 (E_P at 267 mμ = 4650). The

[24] Hauptmann, *J. Amer. Chem. Soc.*, 1947, **69**, 562.

ultraviolet photographs of the chromatograms developed with the butanol solvent of Laland *et al.*[23] showed a component travelling slightly faster than, but not completely separated from, thymine. This material was a derivative of mercaptoacetic acid. To correct for the interference due to the presence of this substance, a blank containing mercaptoacetic acid equivalent to that present in the nucleic acid derivative was hydrolysed with formic acid and chromatographed. To detect free aldehyde groups in the product, the supersensitive Schiff's reagent of Tobie [25] was used. This indicated that about 4% of the reducing groups were free (calf-thymus apurinic acid being used as a standard).

Calf-thymus aldehydo*Apurinic Acid Di(carboxymethyl) Dithioacetal.*—Calf-thymus deoxyribonucleic acid (sample 2; 1 g.) was dissolved in mercaptoacetic acid (45 ml.), fused zinc chloride (5·5 g.) and anhydrous sodium sulphate (3·5 g.) were added, and the mixture was shaken at 37° for 34 hr. Ether (450 ml.) was then added, the precipitate centrifuged, washed with ether, dried, and shaken with acetate buffer (μ 0·1; pH 6·0; 100 ml.) containing glycine (5 g.). The suspension was dialysed against changes of 0·5% glycine solution at 4° and centrifuged. The undissolved fraction was shaken with 5% glycine solution (100 ml. amounts) until only guanine remained. The combined solutions were dialysed against running tap-water, then against distilled water, and freeze-dried. The product (0·90 g.) contained 3·96 atoms of sulphur (102% of theoretical), 0·01 mol. of adenine, no guanine, 1·19 mols. of thymine, 0·84 mol. of cytosine, and 0·04 mol. of 5-methylcytosine per 4 atoms of phosphorus.

Examination of the Dialysable Material.—Calf-thymus deoxyribonucleic acid (sample 2; 0·5 g.) was treated with mercaptoacetic acid, zinc chloride, and sodium sulphate as above and dialysed against several changes of distilled water (without glycine). The solutions containing the material which had diffused through the dialysis bags were combined, concentrated by evaporation under reduced pressure, adjusted to pH 6, and freeze-dried. Direct hydrolysis of the product with formic acid, followed by paper chromatography, caused bad streaking owing to the large amount of salt (zinc chloride and sodium sulphate) present. This was overcome as follows: The material was packed as a column in a glass tube, and formic acid percolated through it. The first 0·6 ml. of this solution, which contained almost all the ultraviolet-absorbing material and much less salt, was hydrolysed with formic acid and the products were separated by paper chromatography. Thymine and cytosine were present in the molar ratio of 1·20 : 1 (cf. the ratio of 1·32 : 1 in the intact nucleic acid).

Examination of the Material not Precipitable by Ether.—The ether was removed by evaporation from the supernatant liquid obtained after ether-precipitation of the dithioacetal, and the solution examined by paper chromatography in the usual way. Two ultraviolet-absorbing components were detected; a large spot having R_F 0·88 (butanol solvent of Laland *et al.*[23]) was due to mercaptoacetic acid or a reaction product of mercaptoacetic acid and a spot which contained only adenine. Cytosine, thymine, and pyrimidine nucleotides were absent. Since thymine was not precipitated by ether under the conditions used above and cytosine only incompletely precipitated, it appeared that free cytosine and thymine were not produced by the reaction of mercaptoacetic acid with deoxyribonucleic acid.

Reaction of Calf-thymus Deoxyribonucleic Acid with Ethanethiol and Hydrochloric Acid.—Calf-thymus deoxyribonucleic acid (sample 2; 60 mg.) was shaken at 0° for 7 days with ethanethiol (10 ml.) containing hydrochloric acid (*d* 1·16; 0·12 ml.). The deoxyribonucleic acid was thus gradually transformed into a gum which did not dissolve. Ether (several volumes) was added, then removed by decantation, and the residual gum was washed several times with ethanol, dissolved in acetate buffer (pH 6), and dialysed against changes of distilled water. The dialysable phosphorus was found to be 92% of the total. The non-dialysable product obtained on freeze-drying contained sulphur but there was not sufficient material for a complete analysis. The above reaction was then repeated but for only 16 hr. The non-dialysable material contained 4·2% of sulphur (2·38 atoms of sulphur per 4 atoms of phosphorus; 61% of theoretical) and 18% of the total phosphorus had become dialysable during the reaction.

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[25] Tobie, *Ind. Eng. Chem. Anal.*, 1942, **14**, 405.