66. *The Nucleotide Sequence in Deoxyribonucleic Acids. Part VI.* The Preparation and Reactions of Permanganate-oxidised Deoxyribonucleic Acid.*

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Treatment of deoxyribonucleic acids with potassium permanganate at pH 9 at 37° for 19 hours oxidised the cytosine, thymine, and guanine groups to ureido-residues. The oxidised deoxyribonucleic acid was slowly hydrolysed by N-potassium hydroxide at 37° to urea and oligonucleotides which contained both adenine and ureido-groups, but urea was not completely liberated at this temperature or at 60°. Similar treatment at 100° for 1 hour, however, liberated all the urea and gave in addition, adenine, deoxyadenosine, 5′-(deoxyaden-3′-yl) deoxyadenosine, deoxyadenosine-3′,5′-diphosphate, and adenine-containing oligonucleotides.

It has been reported that treatment of deoxyribonucleic acid (I) with potassium permanganate at pH 9 at 37° oxidised all the bases except adenine, and that the ease of oxidation is in the order cytosine > thymine > guanine ≫ adenine.[1] From experiments with model compounds [2] it was expected that the thymine and guanine groups would give ureido-groups and that the cytosine groups would give both ureido and biuret groups. It was further expected, in view of the known alkali-lability of glycosylureas,[3] that the oxidised deoxyribonucleic acid (II) would break down under alkaline conditions to urea and a product (III) which would be of similar structure to apurinic acid. The product (III) would then break down further to give adenine-containing oligonucleotides.[4] The present paper gives details of the determination of the structure of the permanganate-oxidised deoxyribonucleic acid and of its reaction with alkali.

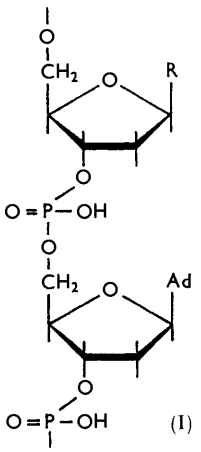
Oxidation of calf-thymus deoxyribonucleic acid with potassium permanganate at 37° for 19 hours gave compound (II) in 90% yield. This did not diffuse through a dialysis membrane and contained about 95% of the adenine groups of the original deoxyribonucleic acid and no detectable guanine, cytosine, or thymine groups.

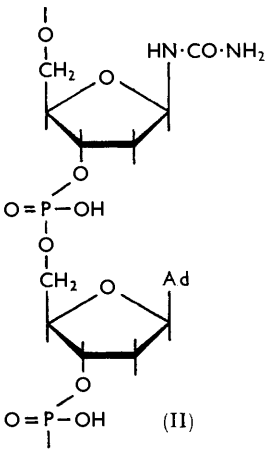
The material which diffused through the dialysis membrane during the isolation of compound (II) contained guanidine which had been produced from the guanine residues. The nitrogen content of compound (II) indicated that it contained only adenine and

* Part V, *J.*, 1961, 1903.

[1] Bayley and Jones, *Trans. Faraday Soc.*, 1959, **55**, 492.
[2] Benn, Chatmara, and Jones, *J.*, 1960, 1014; Chatamara and Jones, *J.*, 1963, 811; Jones and Walker, *J.*, 1963, 3554.
[3] Baron and Brown, *J.*, 1955, 2855; Benn and Jones, *J.*, 1960, 3837.
[4] Bayley, Brammer, and Jones, *J.*, 1961, 1903.

ureido-groups and no biuret. This was confirmed by the facts that upon acid hydrolysis essentially all the non-adenine nitrogen was converted into urea, and no biuret was formed. Biuret was not hydrolysed to urea under the conditions used. Compound (II) reacted with the Dische diphenylamine reagent; 88% of total 2-deoxyribose residues were


(I)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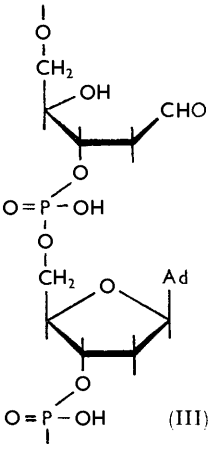
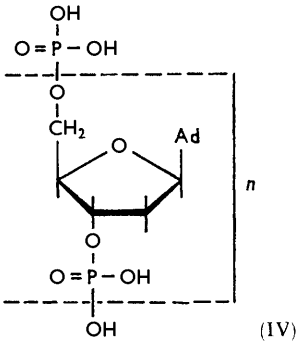
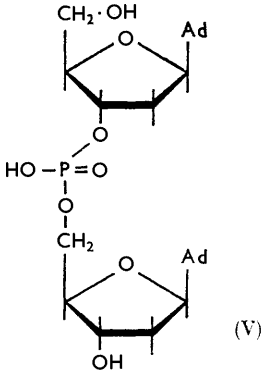

(III)

R = Guanine, cytosine, or thymine residue.
Ad = Adenine residue.

detected. This showed that the ureido-groups were liberated by the acid of the Dische reagent and that most of the 2-deoxyribose residues had not been affected by the permanganate.

The ureido-groups in compound (II) were only slowly hydrolysed by N-alkali, at 37°, 56% being liberated after about 10 days. A previous report,[1] that oligonucleotides containing adenine but no ureido-groups were formed under these conditions, was due to unsuspected ultraviolet-absorbing impurities in the adenine fraction, and must be withdrawn. After treatment with N-alkali at 37° for 7 days the non-degraded material was isolated. This was qualitatively similar to the starting material (II), but the ratio adenine : urea was greater. This fraction, upon treatment again with alkali, was slowly hydrolysed with liberation of urea.


(IV)


(V)

Treatment of compound (II) with N-alkali at 60° for several days also did not completely liberate the urea and, when deoxyribonucleic acid was similarly treated, considerable degradation of the bases occurred. The conditions used by Habermann [5] for the alkaline

[5] Habermann, *Biochim. Biophys. Acta*, 1962, **55**, 999; *Coll. Czec. Chem. Comm.*, 1963, **28**, 510.

degradation of apyrimidinic acid, namely, 0·3N-alkali at 100° for 1 hr., also did not completely degrade compound (II) to adenine-containing oligonucleotides and urea. However, when compound (II) was treated with N-alkali at 100° for 1 hr. complete liberation of urea was effected. There were produced also adenine-containing products and ultraviolet-absorbing compounds presumably derived from the 2-deoxyribose residues. These were separated on a column of diethylaminoethylcellulose, and the adenine-containing components were identified by their adenine and phosphorus contents, their behaviour on paper chromatograms, the nature of the products formed on treatment with phosphomonoesterase, and their relative position of elution from the diethylaminoethylcellulose column. The following were present: adenine, deoxyadenosine, deoxyadenosine-3′,5′ diphosphate (IV; $n = 1$), 5′-(deoxyaden-3′-yl)deoxyadenosine (V), and oligonucleotides of the general formula $(\text{deoxyadenosine})_n$ $(\text{phosphate})_{n+1}$ (IV). Studies on the mechanism of this degradation and its use to determine the distribution of adenine residues in deoxyribonucleic acids will be described later.

Experimental

Analytical Methods.—Nitrogen was determined either by a micro-Kjeldahl procedure [6] or by a colorimetric method based on the production of Indophenol Blue by the action of sodium hypochlorite on phenol in the presence of ammonia.[7] Phosphorus was determined by Jones, Lee, and Peacocke's method.[8] The base contents of the deoxyribonucleic acids and their oxidised products were determined by hydrolysis with formic acid and chromatographic separation of the bases and their estimation spectrophotometrically as described by Wyatt.[9] The 5-methylcytosine contents were not measured. Urea was determined by separating it from other constituents by paper chromatography in butan-1-ol–ethanol–water (4 : 1 : 5), elution with water, and determination of the nitrogen content. Appropriate corrections were applied for partial recovery from the paper and for the nitrogenous impurities in paper. 2-Deoxyribose was determined by the Dische diphenylamine reaction.[10]

Deoxyribonucleic Acids.—These were isolated as their sodium salts from calf thymus and from herring sperm by Marko and Butler's method.[11] They contained less than 0·6% of protein and no detectable ribonucleic acid. Their composition was as follows:

Deoxyribonucleic acid	N (%) *	P (%) *	Moles of base/4 g.-atoms of P			
			Adenine	Thymine	Guanine	Cytosine
Calf thymus	—	9·12	1·10	1·01	0·83	0·82
Herring sperm	16·2	9·18	1·11	1·07	0·91	0·80

* Material dried at 110° *in vacuo* for 3 hr.

Oxidation of Deoxyribonucleic Acids with Potassium Permanganate.—Sodium deoxyribonucleate (3 g.) and sodium hydrogen carbonate (28 g.) were dissolved in water (620 ml.), heated to 37°, added to potassium permanganate (3 g.) in water (620 ml.) also heated to 37°, and the mixture was kept at 37° for exactly 19 hr. Solid sodium metabisulphite was then added until the solution became colourless. The mixture was filtered and dialysed against distilled water at 0° for 5 days and the non-diffusable fraction passed down a column (100 × 3 cm.) of Zeokarb 225 (Na^+), which was then washed with water until no more ultraviolet-absorbing compounds were eluted. The eluate was dialysed against distilled water for 5 days and then freeze-dried to give " oxidised deoxyribonucleic acid " (II). The yield was 81% based on the phosphorus content. This was improved to 93% in later experiments. The products had the following composition:

Oxidised deoxyribonucleic acid	N/P (w/w)	Moles of adenine/4 g.-atoms of P
Calf thymus (av. of 3 samples)	1·29 ± 0·04	1·01 ± 0·04
Herring sperm (av. of 3 samples)	1·35 ± 0·02	1·06 ± 0·04

[6] Ma and Zuazaga, *Ind. Eng. Chem., Analyt. Edn.*, 1942, **14**, 280.
[7] Holbrook and Jones, unpublished results.
[8] Jones, Lee, and Peacocke, *J.*, 1951, 623.
[9] Wyatt, *Biochem. J.*, 1951, **48**, 584.
[10] Dische, *Mikrochemie*, 1930, **8**, 4.
[11] Marko and Butler, *J. Biol. Chem.*, 1951, **190**, 165.

No guanine, cytosine, or thymine was detected. The 2-deoxyribose content was determined by the use of Dische's diphenylamine reagent but with 20 minutes' heating instead of the 10 min. recommended. The calf-thymus oxidised deoxyribonucleic acid gave a value of 0·88 mole of 2-deoxyribose per g.-atom of P.

The diffusable material obtained during the first dialysis, in the experiments with herring-sperm deoxyribonucleic acid, was examined by paper chromatography in butan-1-ol–acetic acid–water (4 : 1 : 5) and butan-1-ol–ethanol–water (4 : 1 : 5). By the use of the ninhydrin–sodium hydroxide spray,[12] considerable amounts of guanidine were detected and a trace of urea was detected with the fructose–hydrochloric acid spray.[2] Protamine and arginine were oxidised by permanganate and several guanidine derivatives were detected in the products, in addition to guanidine itself. These were not present in the oxidation products of the deoxyribonucleic acids, so it was concluded that the guanidine did not arise from protein impurities.

Hydrolysis of Oxidised Deoxyribonucleic Acid.—(*A*) *Acidic.* Oxidised herring-sperm deoxyribonucleic acid [N, 12·2; P, 9·0% (dry wt.), H_2O, 18%; 1·11 moles adenine/4 g.-atoms P] (3·2 mg.) in N-hydrochloric acid (6 ml.) was kept at 37° for 24 hr. A sample (0·3 ml.) was neutralised with dilute aqueous sodium hydroxide and applied to washed Whatman No. 1 chromatography paper. The chromatogram was developed with butan-1-ol–ethanol–water (4 : 1 : 5); the position of adenine was detected by ultraviolet photography and the positions of urea and biuret by the use of markers which were detected with the appropriate sprays. Adenine and biuret ran together in this solvent. The urea spot and that corresponding to adenine and biuret were eluted with 0·1N-hydrochloric acid (20 ml.) for 24 hr. at 37°. In the adenine–biuret spot there were 84 μg. of adenine-nitrogen (calc. from the adenine content determined from the optical extinction at 262·5 mμ) and 84·1 μg. of total nitrogen. Biuret was therefore absent. In the urea spot there were 100·3 μg. of urea-nitrogen. This was 87% of the non-adenine-nitrogen of the oxidised deoxyribonucleic acid. Since the recovery of urea from paper chromatograms was 80—90% this corresponded to essentially complete recovery of the non-adenine nitrogen.

(*B*) *Alkaline.* (*a*) *N*-Alkali at 37°. A solution of oxidised herring-sperm deoxyribonucleic acid (40 mg.) in N-potassium hydroxide (2 ml.) was kept at 37°. Samples were taken at intervals, neutralised with analytical-grade Amberlite IR-120 (H^+), and analysed for urea. Appropriate corrections were made for the partial hydrolysis of urea by alkali. The results were:

Time (hr.)	23	63	119	230
Ureido-residues (%) converted into urea	30·8	33·6	47·7	56·5

Oxidised calf-thymus deoxyribonucleic acid (400 mg.) was similarly hydrolysed for 7 days. The solution was neutralised with perchloric acid, the potassium perchlorate centrifuged off, and the solution dialysed until no more ultraviolet-absorbing material diffused. The non-diffusable material was freeze-dried to give a brown solid (98 mg.) [N, 8·3; P, 7·7% (moist material); 1·50 moles adenine/4 g.-atoms P; adenine-nitrogen/total nitrogen, 0·74]. Acid hydrolysis of this product gave adenine and urea. The latter accounted for all of the non-adenine-nitrogen of the material. This non-diffusable fraction was slowly hydrolysed by N-alkali at 37° to give urea (47% after 7 days).

(*b*) *N*-Alkali at 60°. Oxidised herring-sperm deoxyribonucleic acid (50 mg.) in N-potassium hydroxide (10 ml.) was kept at 60°. After 1 and 7 days, respectively, samples were withdrawn and neutralised with perchloric acid. The potassium perchlorate was centrifuged off and the supernatant liquids were adjusted to pH 4 with acetic acid, and a solution of lanthanum acetate (also at pH 4) was added dropwise until no further precipitation occurred. The precipitates were dissolved in sodium hydrogen carbonate solution, and the resulting precipitates of lanthanum carbonate centrifuged off. The adenine nitrogen : total nitrogen ratios of the solutions were 0·63 and 0·47 after hydrolysis for 1 and 7 days, respectively.

When deoxyribonucleic acid was treated with alkali in a similar manner, there was, after 7 days, considerable degradation of the bases. The base composition of the product was (moles of base/4 g.-atoms P): guanine, 0·79; adenine, 0·53; cytosine, 0·04; uracil, 0·50; thymine, 1·13.

(*C*) N-*Alkali at* 100°. Oxidised calf-thymus deoxyribonucleic acid (80 mg.) in N-potassium hydroxide (12 ml.) was heated at 100° in the absence of oxygen for 1 hr., then adjusted to pH

[12] Jones and Thompson, *J. Chromatography*, 1963, **10**, 248.

8·6 with perchloric acid, and potassium perchlorate was centrifuged off. The inorganic phosphorus content of the solution [13] was 30% of the total phosphorus.

Half of the solution was adjusted to pH 4 and lanthanum acetate added to precipitate the oligonucleotide fraction which was washed and redissolved as described above. This fraction was hydrolysed with N-hydrochloric acid at 100° for 1 hr. When the hydrolysate was subjected to paper chromatography in propan-2-ol–ammonia (*d* 0·88)–water (70 : 6 : 30) and examined for the presence of urea,[14] none could be detected. The method was capable of detecting $<0{\cdot}5\%$ of the total urea which could be liberated from the oxidised deoxyribonucleic acid.

The other half of the solution was diluted to 10 ml. with 0·01M-ammonium hydrogen carbonate (pH 8·6) and fractionated on a column of diethylaminocellulose (22 × 1 cm.) by the use of a linear gradient (0·01M ⟶ 0·5M, in 1 l.) of ammonium hydrogen carbonate (flow rate, 30 ml./hr.; 5-ml. fractions collected). Nine ultraviolet-absorbing fractions (A—I, Figure) were obtained. The adenine in each fraction was determined by hydrolysis with N-hydrochloric acid at 100°, followed by chromatographic separation from impurities and spectrophotometric estimation as is usual for a base analysis of a nucleic acid.[9] The fractions were identified as follows:

(*A*) Most of the ultraviolet absorption of this fraction was due to a phosphorus-containing compound that did not contain adenine. By paper chromatography and spectrophotometry, free adenine and deoxyadenosine were shown to be present also.

(*B*) This fraction was eluted from the column at the position expected for a deoxyadenosine monophosphate, but it contained a negligible amount of adenine. B had $\lambda_{max.}$ 250 mμ in water and was presumably a degradation product of the 2-deoxyribose residues.

(*C*) This contained both adenine and phosphorus. Its behaviour on paper chromatography and the position of elution from the column were identical with those of 5′-(deoxyaden-3′-yl)-deoxyadenosine (ApA).[15]

(*D*) Paper chromatography in propan-2-ol–ammonia (*d* 0·88)–water (70 : 6 : 30) showed the presence of a major adenine and phosphorus-containing component (R_F 0·11) and a minor phosphorus-containing component. The former contained 1·99 g.-atoms of phosphorus per mole of adenine and liberated all its phosphorus as inorganic phosphate on treatment with prostatic phosphomonoesterase.[16] It was therefore deoxyadenosine-3′,5′ diphosphate.

(*E*) Upon paper chromatography in the propan-2-ol–ammonia solvent, this gave only one component (R_F 0·085). It contained 1·94 moles of adenine per 3 g.-atoms of phosphorus. 66% of the phosphorus was liberated as inorganic phosphate on treatment with phosphomonoesterase. The compound was therefore bisdeoxyadenosine triphosphate (pApAp).

(*F*) Chromatography in the propan-2-ol–ammonia solvent gave three phosphorus-containing components, only one of which (R_F 0·035) contained adenine. This contained 2·94 moles of adenine per 4 g.-atoms of phosphorus, and 50% of the phosphorus was liberated as inorganic phosphate on treatment with phosphomonoesterase. It was therefore trisdeoxyadenosine tetraphosphate (pApApAp). Its behaviour on paper chromatography and its position of elution from the column agreed with this structure.

(*G* and *H*) From their positions of elution from the column these were probably tetrakisdeoxyadenosine pentaphosphate$_5$ and pentakisdeoxyadenosine hexaphosphate, respectively (see below). They were dialysed against distilled water for 2 days (recovery 66 and 76%, respectively) and analysed for adenine and phosphorus fraction. (*G*) contained 3·8 moles of adenine per 5 g.-atoms of phosphorus and (*H*) contained 4·8 moles of adenine per 6 g.-atoms of phosphorus.

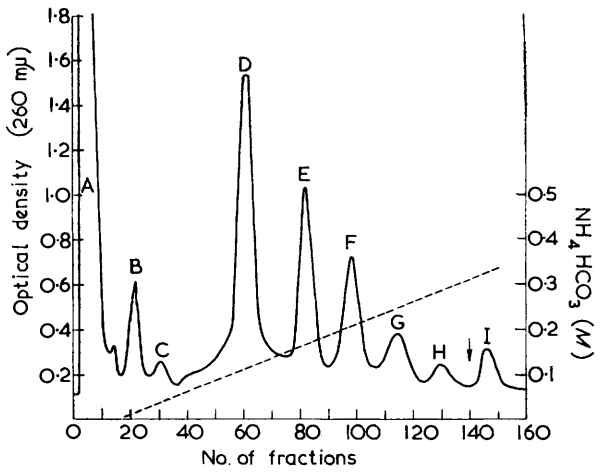
(*I*) This was a mixture which was eluted from the column with M-ammonium hydrogen carbonate as a dark brown solution.

When the number of phosphate groups per molecule in a particular fraction was plotted against the number of moles of ammonium hydrogen carbonate passed down the column to the point of maximum elution of the fraction, a straight line was obtained. This was the case with the alkaline hydrolysate of the oxidised deoxyribonucleic acid and with this hydrolysate after treatment with phosphomonoesterase. After treatment with this enzyme the fraction ApA increased by an amount which corresponded to the content of pApAp in the original

[13] Fiske and Subbarow, *J. Biol. Chem.*, 1925, **66**, 375.
[14] Hubener, Bode, Mollat, and Wehner, *Z. physiol. Chem.*, 1952, **290**, 136.
[15] Heppel, Whitfield, and Markham, *Biochem. J.*, 1955, **60**, 8.
[16] Burton and Petersen, *Biochem. J.*, 1960, **75**, 17.

hydrolysate. Similarly the fraction ApApA was produced in an amount which corresponded with fraction F (pApApAp).

(*D*) 0·3N-*Alkali at* 100°. The procedure described above was repeated but with 0·3N-alkali. Fractionation on the diethylaminoethylcellulose column gave results qualitatively



Fractionation of the products of the alkaline degradation of permanganate-oxidised deoxyribonucleic acid (40 mg.) on a column (22 × 1 cm.) of diethylaminoethylcellulose. 5-ml. fractions were collected. The arrow shows the commencement of elution with M-ammonium hydrogen carbonate.

similar to those shown in the Figure. However, the amount of material in fraction I was very much larger and this indicated that degradation was incomplete.

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