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The Frequencies of Certain Sequences of Nucleotides in Deoxyribonucleic Acid

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The degradation of deoxyribonucleic acid by 66% (v/v) formic acid containing 2% of diphenylamine, at room temperature (Burton, 1956; Burton & Petersen, 1957), appears to be a valuable reaction for the study of certain sequences in the poly-deoxyribonucleotide chains. In this paper we describe the properties of the reaction and a simple chromatographic method by which the frequencies of a considerable number of pyrimidine sequences in deoxyribonucleic acid can be measured on, if necessary, as little as 10–20 mg. of the nucleic acid.

MATERIALS AND METHODS

Deoxyribonucleic acid. This was prepared from calf thymus by extracting the nucleoprotein with 10% (w/v) NaCl and subsequent treatment with sodium dodecyl sulphate (Chargaff, 1955). A sample was freeze-dried to constant weight and found to contain 6·3% of P. Base analysis by hydrolysis in 7·5N-perchloric acid and paper chromatography in propan-2-ol–conc. HCl–water (65:20:10, by vol.; Wyatt, 1955) gave the following molar proportions (mean ± S.E.M.); adenine 28 ± 0·3, guanine 20·9 ± 0·4, cytosine 21·4 ± 0·4, thymine 28·3 ± 0·6, 5-methylcytosine approx. 1. Between 90 and 95 moles of bases were recovered/100 g.atoms of P. These values were obtained from a total of 10 analyses on two hydrolysates.

Apurinic acid. This was prepared from deoxyribonucleic acid (DNA) according to Tamm, Hodes & Chargaff (1952).

Diphenylamine. This was either analytical-reagent grade or a less-pure grade that was recrystallized several times from aqueous ethanol.

Formic acid. Analytical-reagent grade (98–100%) was used.

Calcium bis(p-nitrophenyl)phosphate. This was prepared according to Privat de Garilhe & Laskowski (1955).

Ion-exchange resins. Dowex-1 was nominally 8 or 10% cross-linked and 200–400 mesh. The finer particles were removed by decantation after suspension in water, the resin was packed into a column and washed with 2N-NaOH, water and 3N-HCl until the extinction coefficient of the eluate was less than 0·01 at 260 mμ. Formate and acetate forms were prepared from the chloride form as described by Sinsheimer (1954). Dowex-50 (200–400 mesh) was cleaned according to Moore & Stein (1951). The Dowex-1 was kept clean until required by slowly percolating a solution of the corresponding ammonium salt (0·01M) through the resin.

Paper for two-dimensional chromatography. Sheets of Whatman no. 1 paper for chromatography (47 cm. × 53 cm.) were serrated along the short edge and washed for about 15 hr. with N-HCl by allowing the acid to run along the paper and off the serrated edge in a tank for descending chromatography. They were then similarly washed with water for about 30 hr. and dried in air at room temperature.

Snake-venom phosphodiesterase. This was prepared from *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A.) by the method of Cohn, Volkin & Khym (1957), including a second acetone fractionation. An increase of 0·13 in the extinction coefficient at 440 mμ was given by 0·1 ml. of the preparation under the conditions of Cohn *et al.* (1957).

Spleen phosphodiesterase. This was prepared according to Heppel & Hilmoe (1955) and dialysed against 0·1M-NaCl and then against water to remove pyrophosphate. The final preparation contained 30 units of activity/ml. (units as defined by Heppel & Hilmoe).

Prostatic phosphomonoesterase. Hypertrophic human prostate glands were dissected free of connective tissue and

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fat and the enzyme was then extracted and purified by salt fractionation. For one preparation purified by the method of Markham (1955), the ratio of monoesterase to diesterase activity was 35. Preparations by two other methods (Kerr & Chernigoy, 1957; Davidson & Fishman, 1959) gave a ratio of 1700. Each of these preparations was further purified by chromatography on 2% cross-linked Dowex-50, adsorption of the enzyme at pH 5·2 or 5·5 and elution with buffer at pH 5·7 (Boman, 1955; Privat de Garilhe, Cunningham, Laurila & Laskowski, 1957). The active fractions were dialysed against water at 4° until free of phosphate and concentrated by freeze-drying to give solutions with about 700 units of monoesterase/ml. Before dialysis, sufficient stabilizing protein (egg albumin) was added to give a final concentration of 0·5%. The ratio of monoesterase to diesterase activity was then $4–7 \times 10^4$.

Monoesterase activity was measured by incubating a sample of enzyme at 37° in a total volume of 3·2 ml. of solution containing 1·6 mM-disodium *p*-nitrophenylphosphate and buffered at pH 5·6 with 6·2 mM-acetic acid–56 mM-sodium acetate. After a suitable time the reaction was stopped and the colour of the free *p*-nitrophenol was developed by addition of 0·2 ml. of aq. 2N-NH_3 soln. One unit of activity is defined as the amount of enzyme causing an increase of unity in the extinction coefficient at 400 mμ in 1 min., compared with a control sample containing no enzyme. Diesterase activity was measured similarly but with the calcium bis-(*p*-nitrophenyl)phosphate as substrate and stopping the reaction with aq. 2N-NH_3 soln. containing 0·1M-tetrasodium ethylenediaminetetra-acetate (EDTA). The EDTA was included to prevent the precipitation of calcium phosphate.

Inorganic phosphate. The procedure of Berenblum & Chain (1938) as modified by Bartley (1953) has been used for the reaction mixtures containing amine and formic acid. For samples that had been degraded and extracted with ether, the more convenient method of Chen, Toribara & Warner (1956) was used with a total volume of 4 ml. Both methods gave the same values on these mixtures. For the eluate from paper chromatograms, the method of Chen *et al.* was scaled to a total volume of 0·4 ml.

Total phosphorus. Samples containing about 1 μmole of phosphate were measured into a 150 mm. × 14 mm. Pyrex test tube and a small alundum chip was added. The tubes were supported vertically in a tin-plated can and dried by an infrared lamp. The ashing fluid was conc. H_2SO_4–60% perchloric acid (3:2, v/v) saturated with ammonium molybdate as a catalyst; 0·5 ml. of this reagent was added to each tube and the contents were ashed by gentle boiling, which was continued for 3–5 min. after the mixture became clear. When cool, the contents of the tubes were diluted to 25 ml. and phosphate was measured on 2 ml. by the method of Chen *et al.* (1956) with a total volume of 4 ml.

To measure the P content of spots on paper chromatograms a suitable portion of the eluate containing 1–15 μm-moles of phosphate was measured into a 90 mm. × 7 mm. hard-glass test tube and dried under an infrared lamp. A mixture of 3 vol. of conc. H_2SO_4 and 2 vol. of 60% perchloric acid was saturated with ammonium molybdate and 12·5 μl. was measured into each tube and allowed to drain to the bottom. The tubes were heated in an oil bath at about 180° for 4 min. and cooled. The colorimetric reagent was prepared just before use by mixing 15 vol. of 17 mM-EDTA with 2 vol. of 10% ascorbic acid and 2 vol. of 5% ammonium molybdate. Of this, 0·38 ml. was added to each tube and immediately mixed with its contents. The samples were incubated at 37° for 2–3 hr. and the extinction was measured at 820 mμ against that of a reagent blank in cells of 0·4 ml. capacity and 1 cm. light path. The reading was proportional to the phosphate content, 10 μm-moles giving a reading of about 0·77. The EDTA was included because it prevents the development of a spurious blue colour.

Monoesterified phosphate. The solution containing about 0·4 mM-phosphate was made 0·01–0·02M with respect to EDTA, adjusted to pH 5·6 and incubated at 37° with 0·05–0·1 vol. of phosphomonoesterase until dephosphorylation was complete (usually overnight). Protein was precipitated by 0·5M-perchloric acid and inorganic phosphate was estimated on the solution according to Chen *et al.* (1956).

Spectrophotometric measurements. These were made with a Beckman model DU quartz spectrophotometer in cells of 1 cm. light path and with correction for cell blanks. The readings obtained with this instrument were identical with those obtained on two similar ones.

Desalting. Several methods were used for removing salt from the fractions obtained after ion-exchange chromatography. Formic acid and ammonium formate were removed by sublimation on a freeze-drying apparatus at about 40°. Hydrochloric acid was removed from most of the fractions by adsorbing the nucleotides onto Nuchar charcoal (West Virginia Pulp and Paper Co., New York 17, N.Y., U.S.A.). The charcoal was then washed with water and the nucleotides were eluted with 50% ethanol containing 2% (v/v) of conc. aq. NH_3 soln. Ammonia and ethanol were then removed in a rotary evaporator. The trinucleotide products were freed of HCl by dialysing against distilled water for about 2 hr. in dialysis tubing that had been purified by heating in several changes of water (Hughes & Klotz, 1956). The nucleotides themselves diffused only very slowly.

Nomenclature of pyrimidine nucleotides

The system of abbreviations described by Privat de Garilhe & Laskowski (1956) has been used with the slight modification that, since all the products described here are deoxyribose derivatives, the symbol (*d*) is omitted. C, T and M represent deoxycytidine, thymidine and 5-methyldeoxycytidine respectively. p represents orthophosphate esterified with these nucleosides. If p appears on the left of the nucleoside symbol it represents a 5′-phosphate, if on the right a 3′-phosphate. When p appears between two capital letters it represents the 3′:5′-phosphodiester bridge linking the two nucleosides.

EXPERIMENTAL AND RESULTS

Effects of amines on the liberation of inorganic phosphate from deoxyribonucleic acid and apurinic acid

Table 1 shows the effect of a number of amines on the liberation of inorganic phosphate from DNA in 66% (v/v) formic acid. No aliphatic amine caused any detectable release of phosphate, but all the primary and secondary aromatic amines tested

were effective except for 2:4-dinitroaniline and 4-nitrodiphenylamine, which were very poorly soluble in the reaction mixture. Only very small amounts of phosphate (0·3 and 1·4 % respectively) were released by the slightly soluble 2-nitro and 2:4-dinitro derivatives of diphenylamine. Triphenylamine was not effective. Of the amines tested, diphenylamine was the most effective and *p*-nitroaniline was almost as good, followed by *p*-aminoaniline and 1-naphthylamine.

Table 1. *Inorganic phosphate released from calf-thymus deoxyribonucleic acid incubated with various amines in acid solution*

Two volumes of a 3 % solution of the amine were added to 1 vol. of aq. DNA (0·6 mg./ml.). Inorganic phosphate was measured after 6·5 hr. at 30°. No phosphate was released with formic acid alone or with benzylamine, dibenzylamine, ethanolamine or triethylamine.

Amine	Inorganic P (% of DNA P)
Aniline	10·5
o-Nitroaniline	7·1
m-Nitroaniline	13
p-Nitroaniline	23
o-Aminoaniline	3·4
m-Aminoaniline	0·5
p-Aminoaniline	18·4
o-Hydroxyaniline	8·5
m-Hydroxyaniline	4·3
p-Chloroaniline	8
p-Methoxyaniline	6·4
Diphenylamine	25
1-Naphthylamine	16·7
2-Naphthylamine	11·7
Triphenylamine	0

The release of phosphate from apurinic acid is more rapid than it is from DNA, but again diphenylamine was the most effective of the amines that were tested. Table 2 shows the action on apurinic acid of diphenylamine and of some other amines.

The reaction is complicated by a side reaction in which the amines are converted into inactive derivatives by the formic acid. This was not particularly serious with diphenylamine because a solution of 3 % of diphenylamine in formic acid retained about one-third of its original activity when it was stored at room temperature for 4 days. Except in this one experiment, the amines were dissolved in the formic acid shortly before use.

Apart from aqueous acetic acid, no satisfactory alternative solvent has yet been found. As shown in Table 2, the degradation is much slower if half of the formic acid is replaced by *N*-dimethylformamide. Small amounts of phosphate were liberated in various mixtures of dilute HCl and dimethylformamide.

Fig. 1 shows the appearance of inorganic phosphate from calf-thymus DNA. The reaction is complete after about 16 hr. and the amount of inorganic phosphate then remains constant at 27·0–28·5 % of the total phosphate. The lability of the linkages between deoxyribose and the purines and the relative stability of those involving the pyrimidines suggest that the inorganic phosphate arises from phosphodiester groups joining two purine nucleosides (Burton, 1956). The pyrimidine nucleosides are expected to remain as nucleotides of

Table 2. *Release of inorganic orthophosphate from apurinic acid*

To an aqueous solution of apurinic acid (8·7 m-moles of P/l.) was added 2 vol. of a 3 % solution of the amine in either (A) formic acid or (B) a mixture of equal volumes of formic acid and *N*-dimethylformamide. *N*-Phenyl-2-naphthylamine was not completely dissolved in mixture (A). Samples containing *p*-aminodiphenylamine had to be extracted with ether before estimation of the inorganic phosphate. No detectable phosphate was liberated when no amine was added.

(A) Formic acid

Time (hr.)	Inorganic P (% of total)			
	Diphenylamine	*p*-Nitroaniline	*N*-Phenyl-2-naphthylamine	*p*-Aminodiphenylamine
0·25	25	14	—	—
0·33	25·5	—	9·3	18
0·50	25·5	20·5	—	—
1	27	—	21	23
5	27	25	26	22
24	27·5	29	—	—

(B) Formic acid and *N*-dimethylformamide

Time (hr.)	Inorganic P (% of total)		
	Diphenylamine	*N*-Phenyl-1-naphthylamine	*N*-Phenyl-2-naphthylamine
0·5	10·5	5	—
1·0	16·2	8·5	—
5	26·5	23	17
24	27·7	26	27

the general structure Py_np_{n+1} (where Py is a pyrimidine nucleoside and p is a mono- or di-esterified phosphate group). Since the molar amounts of purine and pyrimidine bases in DNA are equal, the amount of diesterified phosphate between adjacent purine nucleosides must equal the amount between the pyrimidine nucleosides. Thus the amount of inorganic phosphate that is released in the degradation by diphenylamine should equal the amount of organic phosphate that remains after the exhaustive treatment of the pyrimidine nucleotide products with a specific phosphomonoesterase. As shown in Table 3, this expectation was fulfilled.

In another experiment, samples were taken after degradation with diphenylamine for 16, 24 and 40 hr. Each sample was then diluted with an equal volume of water and extracted with ether to remove the diphenylamine and most of the formic acid. They were then treated with phosphomonoesterase and the final amount of inorganic phosphate was found to be 72·8, 73·8 and 72·2 % of the total phosphate in the respective samples. Prolonged incubation with the diphenylamine and formic acid does not therefore cause any further increase in the amount of terminal-phosphate groups and so it does not lead to non-specific splitting of interpyrimidine linkages. Further evidence for this was obtained by incubating DNA in 66 % (v/v) formic acid for 24 hr. at 30° and then adding solid diphenylamine and incubating for a further 24 hr. The inorganic phosphate values before and after phosphomonoesterase were 25 % and 72·8 % respectively.

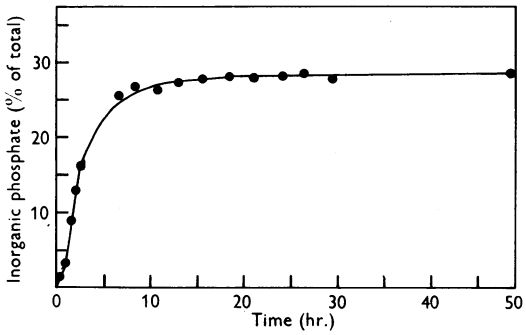

Fig. 1. Release of inorganic phosphate from DNA. Aqueous DNA (0·6 mg./ml.) was incubated at 30° with 2 vol. of a 3% solution of diphenylamine in formic acid.

Table 3. *Action of prostatic phosphomonoesterase on the products obtained by degrading deoxyribonucleic acid with diphenylamine*

DNA was degraded with diphenylamine in formic acid and extracted with ether. An amount containing 5·6 μ-moles of total P was incubated with 370 units of phosphomonoesterase in 15 ml. of 0·15 M-acetate buffer plus 0·07 M-EDTA, pH 5·6, at 37°. Inorganic phosphate was measured on 1 ml. samples after deproteinizing with perchloric acid.

Time (hr.)	Inorganic phosphate (% of total P)
0	28
2	65·7
3	69
4	72
6	71·2
10	72

Separation of the pyrimidine nucleotide products

Burton & Petersen (1957) described a preliminary separation of the products by chromatography on Dowex-1 chloride, eluting them with HCl. This led to the separation of thymidine and deoxycytidine 3′:5′-diphosphate (pCp and pTp) and the dinucleotide containing cytidine, thymidine and three phosphate groups (CTp_3). Data on these fractions are included in Table 5. Improved separations were, however, obtained by eluting the compounds from Dowex-1 formate by mixtures of ammonium formate and formic acid and such an experiment is here described in detail.

A solution of 1 g. of calf-thymus DNA in 150 ml. of water was incubated for 17 hr. at 30° with 300 ml. of a 3 % (w/v) solution of diphenylamine in formic acid. To remove formic acid and diphenylamine, the mixture was poured into an equal volume of water, extracted five times with about 5 vol. of ether and the aqueous layer was then extracted with ether in a continuous liquid–liquid extraction apparatus for about 4 hr. Solid NaOH was added to the ether in the extraction flask to absorb formic acid. The aqueous phase was made alkaline with ammonia and passed through a column of Dowex-1 formate, 17 cm. × 2·3 cm. diam., which adsorbed the purines and the phosphorus-containing compounds. The free purines were washed from the resin with 10·8 l. of 5 mM-formic acid, and the pyrimidine nucleotides were then eluted with the ammonium formate–formic acid mixtures shown in Fig. 2. Fractions (10 ml.) of the eluate were collected and the extinction coefficients of the fractions were measured at 267 mμ against a blank solution that contained the same concentrations of ammonium formate and formic acid. The linear gradients were obtained as described by Parr (1954). The overall recovery of ultraviolet absorption was 90 %, representing about equal recovery of the purines and pyrimidines of the original digest.

The fractions were pooled into the eight groups shown in Table 4. Most of these were purified by rechromatography on Dowex-1, with either the

chloride form and elution with HCl or the formate form and elution with ammonium formate and 0·05 M-formic acid. The re-adsorption of large volumes for rechromatography was conveniently accomplished by adsorbing the alkaline material on to a short wide column (approx. 4 cm. × 2·5 cm. diam.) and attaching this to the top of a longer column (16–24 cm. × 1 cm. diam.) before beginning the elution. Each purified component was concentrated and freed of salt before further characterization.

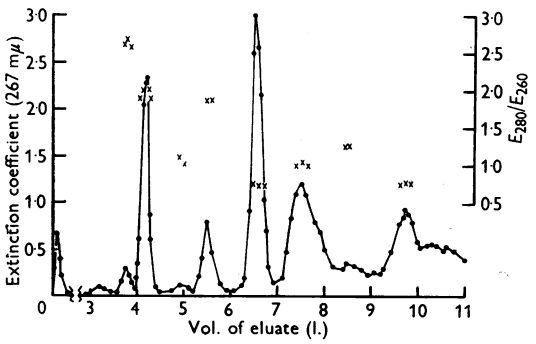

Fig. 2. Separation of pyrimidine nucleotides on Dowex-1 formate. The free purines had previously been washed from the resin with 10·8 l. of 5 mM-formic acid. The pyrimidine nucleotides were eluted with mixtures of formic acid and ammonium formate: 0–0·7 l., 0·01 M-formic acid–0·05 M-ammonium formate; 0·7–2·78 l., 0·01 M-formic acid containing a linear gradient in ammonium formate concentration from 0·06 to 0·25 M; 2·78–2·92 l., 0·01 M-formic acid–0·05 M-ammonium formate; 2·92–12·07 l., 0·1 M-formic acid containing a linear gradient in ammonium formate concentration from 0·06 to 0·56 M in the first 5 l. and from 0·56 to 2·0 M in the remaining 4·15 l. ●, Extinction coefficient at 267 mμ; ×, ratio of extinction at 280 mμ to that at 260 mμ (E_{280}/E_{260}) after adjusting to pH 1·0–2·0. See Tables 4 and 5 for the identities of the components.

Tables 4 and 5 summarize most of the pertinent details of these products and of their rechromatography. Further details are as follows.

Group 1. Adenine and guanine were detected after paper chromatography in propan-2-ol–HCl (Wyatt, 1955). The fraction contained only 0·008 % of the total P applied to the column.

Group 2. Ammonium formate was removed from a portion of this peak by sublimation *in vacuo* and the residue was hydrolysed by heating with 0·1 ml. of 7·5 N-perchloric acid for 1 hr. at 100°. Paper chromatography in propan-2-ol–HCl (Wyatt, 1955) showed the presence of two spots, identified as 5-methylcytosine and cytosine respectively on the basis of their chromatographic position and ultraviolet absorption at 280 and 260 mμ. The ratio of 5-methylcytosine to cytosine was 4·7 to 1.

Group 3. This fraction was eluted as a sharp peak with a constant ratio of E_{280} to E_{260} and in a similar position to that found by Shapiro & Chargaff (1957*b*) for deoxycytidine 3′:5′-diphosphate. The material was accidentally lost, but its identity is confirmed by the fact that the amount obtained from the DNA was closely similar to that which was previously obtained by separation on Dowex-1 chloride (see Table 5).

Table 4. *Details of the rechromatography of pyrimidine nucleotides*

Group	Position in Fig. 2 (l.)	E_{280}/E_{260} (pH 1·0–2·0)	Percentage of u.v. absorption at 267 mμ	Rechromatography: Eluent	Rechromatography: Gradient	Recovery (%)	Principal constituents
1	0–3·4	—	0·78	—	—	—	Adenine guanine
2	3·4–3·95	2·64	0·52	—	—	—	Mp_2
3	3·95–4·7	1·96	3·0	—	—	—	Cp_2
4	5·1–6·0	1·6	1·7	Formic acid (0·05 M)–ammonium formate (gradient)	0·2–0·4 M in 1 l.	—	C_2p_3
5	6·0–6·95	0·8	2·9	Formic acid (0·05 M)–ammonium formate (gradient)	0·3–0·55 M in 1·5 l.	95	Tp_2
6	6·95–8·4	1·26	5·0	HCl	0·01–0·04 M in 3 l.	*c.* 100	CTp_3 C_2Tp_4
7	8·4–9·2	1·38	2·2	HCl	0·01–0·04 M in 4 l.	*c.* 100	C_2Tp_4
8	9·2–11·1	0·99	7·9	HCl	0·07–0·12 M in 3·5 l.	*c.* 100	$C_2T_2p_5$ T_2p_3

Table 5. *Pyrimidine nucleotide phosphates separated and identified by anion-exchange chromatography*

For evaluation of the amounts, see text. The values for the molar-extinction coefficient at 280 mμ (ϵ_{280}) were determined from the u.v. absorption and the phosphorus content.

Product	Pyrimidine (moles/ 100 g.atoms of P)	E_{280}/E_{260} (pH 1·0–2·0)	Percentage of total P as end group: Found	Percentage of total P as end group: Expected	$10^{-3}\,\epsilon_{280}$/mole
		Separation on Dowex-1 chloride			
pMp	0·3	—	—	—	—
pCp	3·7	2·0	—	—	14·0
pTp	5·5	0·69	—	—	6·4
CTp_3	5·2	1·25	—	—	—
		Separation on Dowex formate			
pMp	0·5	—	95	100	—
pCp	3·7	1·86	—	—	—
pTp	6·1	0·69	100	100	6·8
pCpCp	1·8	1·9	86	67	27·3
pTpTp	—	0·73	62	67	—
pCpTp, pTpCp	3·2, 2·2	1·23	67	67	19·5
C_2Tp_4	1·23 (group 6)	1·46	60	50	—
	2·03 (group 7)	1·46 (pCpTpCp)	52	50	—
		1·46 (pTpCpCp)	45	50	—
		1·46 (pCpCpTp)	60	50	—

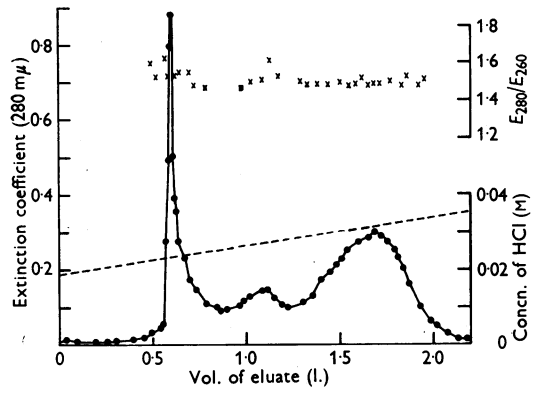

Fig. 3. Separation of C_2Tp_4 isomers on Dowex-2 chloride by gradient elution with HCl. Rechromatography of group 7. ●, Extinction coefficient at 280 mμ; ×, E_{280}/E_{260}; - - - -, concentration of HCl. The three isomers are eluted in the order pCpTpCp, pTpCpCp and pCpCpTp.

Group 4. On rechromatography it gave one sharp peak with a small earlier peak which was assumed to be deoxycytidylic acid or pCp arising by spontaneous decomposition of the main material.

Group 5. On rechromatography it gave one sharp peak with a small earlier peak assumed to be thymidylic acid that had been formed by decomposition of pTp.

Group 6. This gave three peaks on rechromatography. The first (at 0·5–0·8 l.) was a mixture that was not resolved on further chromatography on Dowex-1 formate. The second peak, at 1·4–2·0 l., was identified as C_2Tp_4 and the third peak, at 2·1–2·7 l., was identified as CTp_3.

Although Cohn & Volkin (1957) have separated the two isomers of CTp_3 by chromatography on Dowex-1 chloride, we were not able to obtain a separation under similar conditions. The proportions of the two isomers were determined by the method of Privat de Garilhe *et al.* (1957), in which the material is dephosphorylated by phosphomonoesterase and the remaining CpT and TpC compounds are separated by paper chromatography.

Group 7. The principal component of this group (C_2Tp_4) became partially separated into its three isomers when chromatographed on Dowex-1 chloride (Fig. 3).

The identity of each isomer was established by degrading the dephosphorylated material with either spleen or snake-venom diesterases. These two enzymes catalyse the hydrolysis of the 5′- and 3′-phosphoester bonds respectively. The three C_2Tp_2 isomers obtained after phosphomonoesterase treatment would therefore yield the following products when incubated with these enzymes:

	snake venom		spleen diesterase	
T + pC + pC	←	TpCpC	→	Tp + Cp + C
C + pT + pC	←	CpTpC	→	Cp + Tp + C
C + pC + pT	←	CpCpT	→	Cp + Cp + T

Material from each peak was treated with phosphomonesterase and heated at 100° for 15 min. to inactivate the enzyme. The solutions were centrifuged and concentrated by a current of air at 37°. A portion was adjusted to pH 8–9 by adding aq. N-NH_3 soln. and incubated at 37° for 6 hr. with 0·1 ml. of the snake-venom enzyme in 1·5–2·0 ml. of solution containing 0·01 M-magnesium acetate. Another portion was adjusted to pH 6·7 and

similarly incubated with 0·1 ml. of the spleen diesterase in the presence of 0·01 M-sodium tartrate, which was added to suppress the monoesterase activity. The reaction mixtures were deproteinized by shaking with $CHCl_3$, concentrated and chromatographed on Whatman no. 1 filter paper with an ascending solvent mixture of 2 N-formic acid–6 M-NH_4Cl–propan-2-ol (15:15:70, by vol.). Since the preparations of diesterases contained some monoesterase activity, both nucleosides were produced in each incubation mixture. However, a spot with the absorption spectrum and phosphorus content of thymidylic acid was found only in the hydrolysates of the first and third peaks by the snake enzyme and of the first and second peaks by the spleen enzyme. The isomers had therefore been eluted from the column in the order pCpTpCp, pTpCpCp and pCpCpTp.

The solvent used for paper chromatography is suitable for use with mixtures containing appreciable amounts of salt. The solvent runs in two phases. The faster one is free of chloride and contains the thymidine spot. Deoxycytidine is concentrated at the interphase. Thymidylic acid and deoxycytidylic acid move as separate spots in the slower phase, with the thymidylic acid in front.

Group 8. This appeared to be a complex mixture and only one peak, T_2p_3 (2·5–3·5 l.), had a constant ratio between the absorptions at 280 and 260 mμ. The T_2p_3 obtained (1·6 moles/100 g.atoms of P) was probably only a fraction of the T_2p_3 in the original mixture.

Two-dimensional chromatography on paper

To separate the complex mixture of products we have investigated less laborious alternatives to column chromatography and have been able to obtain good separations by two-dimensional paper chromatography after removing the terminal-phosphate groups (cf. Smith & Markham, 1952). By these methods it is possible to study the frequencies of a variety of sequences with only about 20 mg. of DNA. The degradation was performed with 88 mg. of DNA, but the same number of chromatograms could have been run with less than 30 mg. DNA was dissolved in water (4 mg./ml.), degraded with diphenylamine and extracted with ether as described above. Most of the purines were removed by passing the solution through a 4 cm. × 1 cm. diam. column of Dowex-50 (H^+ form). The column was washed with 100–200 ml. of water until the extinction coefficient of the washings was less than 0·05. The combined eluate and washings were adjusted to pH 5·6 by adding N-NaOH, concentrated in a rotary evaporator (bath temp. 40°) and transferred to a suitable tube in a final volume of 6 ml. Then, 0·15 ml. of 0·2 M-EDTA, adjusted to pH 5·6 with NaOH, was added to act as a buffer and also as an activator of the phosphatase. The solution was incubated at 37° with about 200 units of prostatic phosphomonoesterase until it was certain that the release of inorganic phosphate had stopped (60 hr.). The final amount of inorganic phosphate was 73 % of the total P. The solution was shaken vigorously with a little $CHCl_3$ and the denatured protein was centrifuged and washed with 1–2 ml. of water. The washings were added to the supernatant and the solution was concentrated in a current of air at 37° to 1·5 ml.

An amount containing about 7 μmoles of total phosphate was applied to each sheet of acid-washed paper and chromatographed first with propan-2-ol–water (70:30, v/v), which was allowed to drip off the serrated edge for about 3 hr. The papers were dried at room temperature and again developed with the same solvent in the same direction until the solvent just reached the serrated edge. The papers were dried and 2-methylpropan-2-ol–conc. HCl–water (11:1:3, by vol.) was run at right angles until the solvent front almost reached the edge of the paper. The chromatogram was then dried by standing for at least 18 hr. at room temperature. Contact prints were made on Ilford 50M document paper by the light of a Chromatalamp (Hanovia Ltd., Slough, Bucks.), with the shortest exposure needed to obtain satisfactory prints. A tracing of such a print is shown in Fig. 4.

The absorbing areas of the chromatograms were cut out and weighed to compare their areas. They were eluted by soaking in 4 ml. of 0·03 M-HCl for 30 min. The extinction coefficients were then measured at 260, 267 and 280 mμ. Three or four

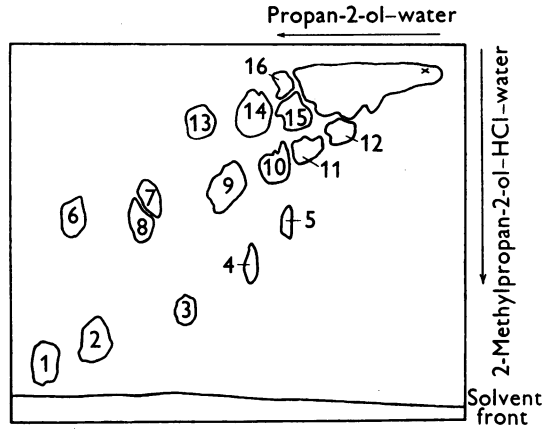

Fig. 4. Two-dimensional paper chromatogram of the products after degradation with diphenylamine and treatment wtih phosphomonoesterase. Solvents: propan-2-ol–water (70/30, v/v) and 2-methylpropan-2-ol–conc. HCl–water (11:1:3, by vol.). Tracing of photograph made in urltaviolet light. See Table 6 for the identities of the components.

blank areas of each chromatogram were similarly eluted as paper blanks. Except for the areas very close to the solvent front in the second solvent, the absorptions of these blanks were very similar per unit area of paper and so satisfactory blank corrections could be applied to the nucleotide eluates. The eluates from one of the chromatograms were used for determining total and inorganic phosphate. Most of the paper blank was due to inorganic phosphate. The results are summarized in Table 6.

The identities of the thymidine and deoxycytidine spots (numbers 1 and 6 in Fig. 4) were confirmed by paper-chromatographic comparisons and by their ultraviolet-absorption spectra at pH 1·5, 7·0 and 13·0. Thymidine and its nucleotides have a value of about 0·7 for the ratio of extinction at 280 mμ to that at 260 mμ at pH 1·5 whereas deoxycytidine and its nucleotides have values of about 2. The proportions of thymine to cytosine in the various components were obtained by comparing their ratio of extinctions at 280 and 260 mμ with the values calculated for various mixtures of integral molar proportions of thymidine and deoxycytidine. The total pyrimidine content of each component was obtained from the extinction at 267 mμ as described below. The ratio of phosphorus to pyrimidine served to decide whether a component was a di- or tri-nucleotide or a higher oligonucleotide. Chain lengths were assigned to the higher oligonucleotides on the basis of their chromatographic positions. Thus the row of spots 1–5 appears to be the homologous series of thymidine and the thymidine nucleotides of chain lengths 2–5. Spots 6, 13 and 16 are the homologous series of cytidine and the di- and tri-cytidine nucleotides. Spots 7 and 8 are the two isomers CpT and TpC, and the row of spots 7 plus 8 and 9–12 appear to be the homologous series of nucleotides containing only one cytidine and increasing amounts of thymidine. Similarly, the row from 13 to 15 appears to be the series with two cytidine residues and 0, 1 or 2 thymidine residues.

Spots 7 and 8 were clearly separated and, on hydrolysis with spleen diesterase and paper chromatography as described above, spot 7 gave a material with the ultraviolet-absorption and phosphate content of cytidylic acid (Cp). No thymidylic acid (Tp) was detected. Spot 8 gave thymidylic acid but no cytidylic acid. The two materials were therefore the two isomers CpT and TpC respectively.

The total amount of material applied to the chromatograms was obtained either by measurement of the volume of solution first applied or, more conveniently, by eluting the inorganic phosphate and determining it colorimetrically. The inorganic phosphate ran in the neighbourhood of spots 2 and 3 in the first solvent and in front of them in the second solvent. The two methods agreed very closely. For three chromatograms, the amounts of organic P for the identified spots were calculated from the ultraviolet absorption as described below. The organic P in the unidentified ultraviolet-absorbing areas was determined directly. The respective total recoveries were 74, 83 and 90 % of the organic P applied to the paper.

For comparison, Table 6 includes the amounts of the various nucleotides that are expected if the nucleotides are randomly arranged in the DNA. These random values were calculated according to Jones, Stacey & Watson (1957), taking 21·4 moles

Table 6. *Dephosphorylated pyrimidine nucleotide products separated by two-dimensional paper chromatography*

Spot no.	Identity	E_{280}/E_{260} Found	E_{280}/E_{260} Theory for mixture of T and C	Organic P/pyrimidine Found	Organic P/pyrimidine Theory	Amount (moles of pyrimidine/100 g.atoms of DNA P)	Mean	Theory for random
1	T	0·70	0·70	0	0	6·17, 6·3, 6·15, 6·2	6·2	7·07
2	T_2p	0·68	0·70	0·52	0·5	2·6, 2·65, 2·6, 2·6	2·6	4·00
3	T_3p_2	0·67	0·70	0·65	0·67	1·34, 1·48, 1·2, 1·21	1·3	1·70
4	T_4p_3	0·70	0·70	0·81	0·75	0·6, 0·57	0·6	0·64
5	T_5p_4	0·70	0·70	—	0·8	0·27	(0·3)	0·23
6	C	2·2	2·0	0	0	3·82, 3·84, 3·82, 3·75	3·8	5·35
7	CpT	1·25	1·24	0·54	0·5	5·03, 5·2, 5·01 {3·06	3·1	3·02
8	TpC	1·25	1·24	0·51	0·5	5·03, 5·2, 5·01 {1·95	2·0	3·02
9	CT_2p_2	1·03	1·04	0·67	0·67	2·7	2·7	3·85
10	CT_3p_3	0·95	0·95	0·77	0·75	1·6	1·6	1·94
11	CT_4p_4	0·85	0·89	0·7	0·8	0·75	0·7	0·86
12	CT_5p_5	0·83	0·86	0·88	0·83	0·49	0·5	0·35
13	C_2p	2·06	2·0	0·52	0·5	1·78, 1·80, 1·75	1·8	2·29
14	C_2Tp_2	1·5	1·46	0·65	0·67	2·6	2·6	2·92
15	$C_2T_2p_3$	1·25	1·24	0·78	0·75	1·9	1·9	2·20
16	C_3p_2	1·89	2·0	0·67	0·67	0·7	0·7	0·76

of cytosine and 28·3 moles of thymidine per 100 g.-atoms of DNAP. Differences of less than 0·3-mole/100 g.atoms of P are not regarded as being significant.

Evaluation of the amounts of nucleotides

Because the measurement of ultraviolet absorption is more reproducible and easier than is the determination of total phosphorus, the amounts of the various nucleotides in Tables 5 and 6 have been determined from their ultraviolet absorption. There are, however, some conflicting values for the molar-extinction coefficients. We have measured the millimolar-extinction coefficients of thymidine 5′-phosphate and 3′:5′-diphosphate at pH 1·0–2·0, obtaining their concentrations from the phosphorus content. For the materials supplied by the California Corporation for Biochemical Research (Los Angeles 63, Calif., U.S.A.) our values are 9·3 and 9·0 respectively at 260 mμ and 10·2 and 9·8 at 267 mμ. For the samples of thymidine diphosphate isolated in this work (Table 4) we obtained values of 9·3 and 9·9 at 260 mμ. The values for thymidylic acid are at least 10% higher than those of Volkin & Cohn (1954) but they agree closely with those of Sinsheimer (1954) and of Shapiro & Chargaff (1957*a*). The values for thymidine 3′:5′-diphosphate at 260 mμ are appreciably higher than those of 8·64 and 8·2 previously reported by Shapiro & Chargaff (1957*a*) and by Cohn & Volkin (1957) respectively.

We have also examined the effect of enzymic dephosphorylation on the ultraviolet absorption of these two nucleotides. At 267 mμ, any change was less than 1% at either pH 1·5 or 5·6 and so their extinction coefficients are, in fact, virtually the same and also very close to that of thymidine itself. Likewise, when deoxycytidine 5′-phosphate was dephosphorylated the change was less than 1% at pH 1·5.

The amounts of the nucleotides of thymine or cytosine have accordingly been evaluated by using a value of 9·9 for the extinction coefficient per m-mole of total pyrimidine/l. at 267 mμ and pH 1·0–2·0. This value is reasonably consistent with the above-mentioned values for the two thymidine compounds and also with data for thymidine, cytidine and deoxycytidine (Fox & Shugar, 1952), for deoxycytidine 5′-phosphate and for its 3′:5′-diphosphate (Shapiro & Chargaff, 1957*a*) as well as for the 2′- and 3′-phosphates of cytidine (Loring, Bortner, Levy & Hammell, 1952). The same extinction coefficient has been used for the various oligonucleotides of cytosine and thymine. Although the absorption by oligonucleotides is usually less than that by a mixture of the component mononucleotides, it appears that this hypochromic effect is only about 3% for nucleotides containing only thymine or cytosine or both (Privat de Garilhe & Laskowski, 1956; Gilham & Khorana, 1958). The values in Tables 5 and 6 support the view that this hypochromic effect can be neglected for the present purposes. For 5-methylcytidine 3′:5′-diphosphate the coefficient of 3·4 at 260 mμ and pH 1–2 has been used. This was taken to be the same as that for the 5′-phosphate and was obtained from the data of Sinsheimer (1954) and of Volkin & Cohn (1954). The material isolated had a value of 2·64 instead of 3·15 for the ratio of extinction at 280 mμ to that at 260 mμ. As mentioned above, base analyses indicated that it was mixed with about 18% of the analogous cytosine compound.

Possibility of exchange reactions

Although it seems unlikely that the proportions of the different products can be appreciably affected by any exchange reactions occurring during the degradation or the enzymic dephosphorylation, such a possibility cannot be discounted without direct evidence. The following experiments with ^{32}P-labelled nucleotides indicate that the products do not undergo any appreciable degree of exchange by reactions that involve their phosphodiester groups.

Dr L. I. Fessler kindly gave us the insoluble fractions from *Escherichia coli* that had been grown on 1·25 l. of a synthetic growth medium containing $Na_2H^{32}PO_4$ (1 mc = 0·1 m-mole/l.) as the sole source of P and crushed in a bacterial press (Hughes, 1951). These fractions were suspended in water, shaken vigorously with octanol and $CHCl_3$ and centrifuged. The supernatant was poured into ethanol, the fibrous precipitate was washed with 80% ethanol, suspended in water and the insoluble material was removed by centrifuging. Colorimetric estimation (Burton, 1956) indicated the presence of 5·8 μmoles of DNA P in the solution. It was treated with 0·4 N-NaOH for 6 hr. at 38° to degrade the ribonucleic acid. Calf-thymus DNA (10 mg.) was added as carrier and the DNA was precipitated by neutralization with HCl, addition of perchloric acid to a final concentration of 0·1 N and the addition of 0·3 vol. of ethanol. This ^{32}P-labelled DNA was washed with a mixture of 20 ml. of ethanol and 4 ml. of 2·5 N-perchloric acid at 0°. It was then degraded with diphenylamine and formic acid, extracted with ether and chromatographed on Dowex-1 essentially as for Fig. 2 but with a smaller column and smaller volumes. The four principal groups of nucleotides were: 1, pCp; 2, mainly C_2p_3; 3, mainly Tp_2; 4, a complex mixture similar to group 6 of Fig. 2 and Table 4. The fractions were concentrated and freed of salt by adsorption on charcoal as described in the Materials and Methods section.

A portion of each ^{32}P-labelled fraction was added to about 4 mg. of DNA and the mixture was treated with diphenylamine and dephosphorylated with phosphatase essentially as described above, but omitting to remove the purines by Dowex-50. Two-dimensional chromatograms were run and the radioactive areas detected by X-ray film. These patterns were compared with those on chromatograms obtained by first dephosphorylating the ^{32}P-labelled fraction and then adding to it some DNA that had already been treated with diphenylamine and phosphatase. Each fraction gave a similar pattern of radioactivity after both treatments.

The clearest results were given by the Cp_2 fraction, because this did not contain any other component. It was completely dephosphorylated by the enzyme and virtually all the radioactivity was found in the inorganic phosphate area even when the Cp_2 had been mixed with the DNA for treatment with diphenylamine and then the phosphatase. Thus during these reactions none of its phosphorus had been converted into phosphodiester groups and so neither of the following two reactions had taken place:

$$\text{-Py-p} + {}^{32}\text{p-C-}{}^{32}\text{p} \rightarrow \text{-Py-}{}^{32}\text{p-C-}{}^{32}\text{p} + \text{p}$$

$$^{32}\text{p-C-}{}^{32}\text{p} + \text{p-Py-} \rightarrow {}^{32}\text{p-C-}{}^{32}\text{p-Py-} + \text{p}$$

Although the following reaction is not directly excluded,

$$\text{-Py-p} + {}^{32}\text{p-C-}{}^{32}\text{p} \rightarrow \text{-Py-p-C-}{}^{32}\text{p} + {}^{32}\text{p}$$

its occurrence is unlikely since it should have been accompanied by the second of the two excluded reactions. The absence of radioactivity in the diester groups is thus an indication that none of these exchange reactions has occurred. This conclusion is, of course, supported by the apparent similarities between the two radioautographs obtained with each of the other three fractions.

DISCUSSION

As would be expected, the first stage in the degradation of DNA is the removal of the purines. This is shown by the fact that the release of phosphate from apurinic acid occurs much sooner than from intact DNA. The mechanism of the subsequent release of phosphate remains obscure. Acid conditions are necessary. The reactions presumably involve the condensation of the amine with the aldehyde groups of the apurinic acid and it is possible to visualize a series of electron shifts along the deoxyribose chain resulting in the elimination of the phosphoester groups at first from the 3 position and then from the 5 position. The immediate product does not, however, seem to be stable and it undergoes further reactions. Under the conditions of the colorimetric reaction (Burton, 1956) it gives an intense blue colour but this does not happen in the formic acid mixture, where the colour is much fainter and is not intensified when the products are transferred to the conditions of the colorimetric reaction.

The evidence presented in this paper shows that the degradation of DNA by diphenylamine effectively results in the quantitative removal of the purine nucleoside components yielding inorganic orthophosphate, pyrimidine nucleoside 3′:5′-diphosphates and pyrimidine di- and oligonucleotide fragments with phosphomonoester groups at each end (Py_np_{n+1}). Although other reactions such as acid hydrolysis (Cohn & Volkin, 1957; Shapiro & Chargaff, 1957*a*, *b*) or mercaptalation and alkaline hydrolysis (Kent, Lucy & Ward, 1955; Jones & Letham, 1956; Jones, Letham & Stacey, 1956*a*, *b*) also give similar or analogous pyrimidine mono-, di- and oligo-nucleotide fragments, the diphenylamine reaction appears to go to completion without the occurrence of either appreciable side reactions or exchange processes. The diphenylamine reaction is therefore of much more value and it is very suitable for studying the patterns of nucleotide sequences in DNA.

The amounts of inorganic phosphate released from calf-thymus DNA, both with and without subsequent treatment with phosphatase, indicate a slight bias in favour of interpurine (-Pu-p-Pu-) and interpyrimidine (-Py-p-Py-) sequences, for they occur to the extent of 27–28 % of the DNA phosphorus instead of the 25 % expected on a random arrangement. By difference, the -Py-p-Pu- or -Pu-p-Py- sequences therefore represent 22–23 % of the total DNA phosphorus.

The amounts of the nucleotides obtained by ion-exchange chromatography (Table 5) agree closely with those obtained by the paper-chromatographic method (Table 6). The results also demonstrate a bias in favour of polypyrimidine sequences because the amount of pyrimidines in the mono- and di-nucleotide fragment is 39 % of the total, leaving 61 % to be accounted for as trinucleotides and higher oligonucleotides. On a random arrangement, 50 % would occur in each group. Perhaps the clearest evidence that the arrangement is not random is the considerable difference between the amounts of the two sequential isomers, pCpTp and pTpCp.

The amounts of the mono- and di-pyrimidine fragments recovered in this work are much higher than those obtained by Shapiro & Chargaff (1957*b*) after hydrolysing DNA in 0·1 M-sulphuric acid at 100° for 30 min.

The runs of four and five thymine nucleotides with a purine nucleotide at each end are found in amounts which are somewhat similar to those which would occur in a randomly arranged poly-

nucleotide. These findings have a bearing on the feasibility of certain codes which have been suggested as conceivable relations between the sequences of the nucleotides in DNA and those of the amino acids in proteins (Crick, Griffith & Orgel, 1957; Golomb, Welch & Delbrück, 1958).

SUMMARY

1. A number of aromatic amines have been found to induce the release of inorganic orthophosphate from deoxyribonucleic acid in aqueous 66% (v/v) formic acid at 30°. The reaction with diphenylamine, the most effective of the amines tested, has been studied in detail.

2. The purines are first removed and then both phosphoester bonds attached to the exposed deoxyribose residues are broken. Phosphodiester groups joining two purine nucleoside residues are converted into inorganic phosphate. Sequences of pyrimidine nucleotides appear quantitatively as nucleoside 3′:5′-diphosphates and as di- and oligonucleotides with terminal phosphate groups at both ends.

3. After enzymic removal of the terminal phosphate groups, many of the pyrimidine oligonucleotides can be separated by two-dimensional chromatography on paper, thus providing a simple method for the study of the frequencies of certain sequences of nucleotides.

4. In calf-thymus deoxyribonucleic acid there is a slight bias in favour of three or more consecutive pyrimidine nucleotides. These amount to about 61% of the total pyrimidines instead of the 50% for a random arrangement.

5. Evidence is given that runs of four and five thymine nucleotides with a purine nucleotide at each end occur in amounts which are somewhat similar to those of a random arrangement.

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