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Sequences of Consecutive Cytosine Deoxynucleotides in Deoxyribonucleic Acid

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In previous work, up to seven consecutive thymine deoxynucleotides have been found in calf thymus DNA (Burton & Petersen, 1960; Petersen, 1963*a*), but sequences of more than four consecutive cytosine deoxynucleotides have not been detected (Petersen, 1963*a*; Hall & Sinsheimer, 1963; Spencer & Chargaff, 1963). It was important to investigate the occurrence of pentadeoxycytidylate sequences because their absence would have strongly suggested the absence of CCC (and GGG) coding triplets (see Burton, Lunt, Petersen & Siebke, 1963). By using large samples of DNA we have been able to isolate and identify pentadeoxycytidylate sequences from the three deoxyribonucleic acids we have examined. Some of these results have already been briefly reported (Petersen, 1963*b*; Burton *et al.* 1963).

In the present paper we also include some observations on the degradation of DNA by aqueous formic acid at 30° and also by 0.1 M-sulphuric acid at 100°. The latter experiments were undertaken because tetradeoxycytidylate sequences had not been found by this method (Spencer & Chargaff, 1963).

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MATERIALS AND METHODS

Deoxyribonucleic acid. DNA was prepared from calf thymus by the method of Kay, Simmons & Dounce (1952), and from herring testis by the method of Emanuel & Chaikoff (1953). *Micrococcus lysodeikticus* DNA was prepared essentially by the method of Marmur (1961). The bacteria were grown at 30–35° with aeration in a medium containing 1% of tryptone, 0.5% of yeast extract, 0.5% of NaCl and 0.2% of glucose. Antifoam A (Hopkin and Williams Ltd.) was added as required. The cells (approx. 280 g. wet wt.) from 30 l. batches were suspended in 0.15 M-NaCl, pH 8 (1.5 ml./g. wet wt.). Lysozyme (0.7 mg./l.) was added and the cells were lysed at 37° with gentle stirring. To obtain satisfactory yields of DNA we found it necessary to add EDTA after lysis, 0.5 vol. of 0.3 M-EDTA, pH 8.0, containing NaCl (0.15 M) being used. The procedure of Marmur (1961) was then followed. The product contained much RNA, even after the ribonuclease treatment. Most of the RNA was removed by adsorption on to charcoal (Zamenhof & Chargaff, 1951). The final DNA was contaminated with non-nucleotide material, probably polysaccharide, and still contained 3.3% of the total phosphate as RNA P, as determined by the method of Webb (1956).

Ion-exchange resins. Dowex 1 (X8) and Dowex 50 (both 200–400 mesh) were washed as described by Burton & Petersen (1960). DEAE-Sephadex A-25, medium grade

(Pharmacia Ltd., Uppsala, Sweden), was freed of fines by decantation and washed as recommended by the manufacturers.

Urea. Reagent-grade urea (British Drug Houses Ltd., Poole, Dorset) was purified by passing an 8M solution several times through a mixture (1:5) of decolorizing charcoal (Harrington Brothers Ltd., London) and Celite 545 on a Buchner funnel, until the extinction at 260 μ m was below 0.05. This stock solution was diluted to sp.gr. 1.113 (7M) as required.

Prostatic phosphomonoesterase. This was prepared as described by Burton & Petersen (1960) and was from the batch used by Petersen (1963a).

Bacterial alkaline phosphomonoesterase. Chromatographically purified enzyme suspended in ammonium sulphate solution (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was mixed with 2 vol. of 0.01M-MgCl₂, dialysed against 0.01M-tris-HCl buffer, pH 8.0, containing MgCl₂ (0.01M) and heated at 85–90° for 10 min.

Lysozyme. This was from British Drug Houses Ltd.

Apurinic acid. Herring DNA was incubated with aq. 66% (v/v) formic acid at 30° for 17 hr. The solution was then diluted with 0.5 vol. of water and shaken four times with 2.5 vol. of ether. The aqueous solution was then passed at 0° through a column of Dowex 50 (H⁺ form) to remove free purines and the column was washed with water until the extinction at 260 μ m was negligible. This treatment with Dowex 50 was completed in 2 hr. The combined eluate and washing were freeze-dried to give solid apurinic acid in the acidic form. Part of this was used for one ultracentrifuge run (Fig. 4) and for base analysis. The remainder was dissolved in water, neutralized with NaOH and freeze-dried. The resulting powder contained 14% of moisture (determined by drying for 3.5 hr. *in vacuo* at 65° over P₂O₅). On a dry-weight basis it contained 10.5% of P. Base analysis (for details see Burton, 1960) indicated 29.6 moles of thymine, 22.5 moles of cytosine, 0.97 mole of 5-methylcytosine and 1.5 moles of guanine/100 g.atoms of P. Adenine was not detected and at least part of the guanine was free, as indicated by paper chromatography in aq. 70% (v/v) propan-2-ol. The colorimetric diphenylamine reaction (Burton, 1956) indicated the presence of 0.485 mole of reactable deoxyribose/g.atom of P.

Ultracentrifuge measurements. The apurinic acid or its sodium salt was dissolved in 0.2M-acetic acid-sodium acetate buffer, pH 5.2, and examined in the Spinco model E analytical ultracentrifuge at 20.0 $\pm$ 0.1° by using schlieren optics. Molecular weights were evaluated by the integration method of Engelberg (1963). The partial specific volume was found to be 0.53 by measuring the density of a 5% solution in the acetate buffer, a 250 μ l. pipette being used as a pycnometer.

Phosphorus estimations. Inorganic P and total P were determined as described by Burton & Petersen (1960).

Paper electrophoresis. This was carried out in formate buffer, pH 2.7, as described by Petersen (1961, 1963a).

EXPERIMENTAL AND RESULTS

Isolation of cytosine oligonucleotides from deoxyribonucleic acid

Samples of DNA (0.5–1.5 g.) were degraded to release the pyrimidine nucleotides as products of

general formula $\text{Py}_n\text{P}_{(n+1)}^*$ by means of 66% (v/v) formic acid containing 2% (w/v) of diphenylamine (Burton & Petersen, 1960). The reaction mixtures were then mixed with 1 vol. of water and extracted twice with 2 vol. of ether to remove the bulk of the diphenylamine. Remaining formic acid was removed by repeated cycles of evaporation to a syrup in a rotary-film evaporator (bath temp. 35°) and addition of water. The solution was then made alkaline (approx. pH 9) with ammonia and adsorbed on to a column (27 cm. $\times$ 2.5 cm. diam.) of Dowex 1 (formate form; X 8). Free purines were washed off by 5 mM-formic acid and the cytosine oligonucleotides were then eluted as a single peak with 0.9M-formic acid (Burton *et al.* 1963). Inorganic orthophosphate was eluted just before the cytosine-containing peak, but no thymine-containing nucleotides were eluted. The combined peak was freed of formic acid as above, and chromatographed on DEAE-Sephadex (HCO₃⁻ form) with a linear gradient of ammonium bicarbonate, pH 8.6 (Fig. 1a).

Five peaks were clearly separated, representing runs of up to five deoxycytidylate units. The fractions from each peak were pooled and desalted on the rotary-film evaporator, water being added and dried down repeatedly until all traces of ammonium bicarbonate were removed. On paper electrophoresis, each peak showed only one component which was identified from its ultraviolet-absorption spectrum, phosphorus content and mobility on paper electrophoresis at pH 2.7 both before and after terminal dephosphorylation (Table 1). The amount of each component was determined from the ultraviolet absorption at 267 μ m of the pooled fractions for the appropriate peak.

Chromatography in 7M-urea. Tests with the purified C₄P₅ and C₆P₆ fractions obtained above suggested that the recovery of the oligonucleotides from Dowex 1 might not be quantitative. This was also found when DEAE-Sephadex (formate form) was used instead of Dowex 1 (formate form). We therefore repeated the separation of the calf-thymus DNA nucleotides with 7M-urea added to all the solutions and using DEAE-Sephadex for both column separations. Urea is known (Tomlinson & Tener, 1963) to improve the resolution and recovery of certain oligonucleotides by decreasing secondary binding effects.

A digest of calf-thymus DNA (0.6 g. of DNA) containing urea (7M) was adsorbed at pH 5–6 on to

* Abbreviations. Compositions of pyrimidine deoxynucleotide compounds are indicated by the following symbols: C, deoxycytidine; Py, any pyrimidine deoxynucleoside; Pu, any purine deoxynucleoside; p, orthophosphate residue esterified with nucleosides. The prefix 'd' is omitted since all nucleosides are of the deoxyribose series.

Fig. 1. Separation of cytosine deoxynucleotides obtained from calf-thymus DNA. (a) Separation of the products obtained from 3 g. of DNA on a column (31 cm. $\times$ 0.9 cm. diam.) of DEAE-Sephadex A-25, medium grade (HCO_3^- form). The gradient of ammonium bicarbonate, pH 8.6, is indicated by the broken line. (b) Separation of the products obtained from 1.2 g. of DNA by elution with ammonium bicarbonate containing urea (7M). The mixture of cytosine deoxynucleotides was adsorbed on to a column (8 cm. $\times$ 2 cm. diam.) of DEAE-Sephadex A-25, coarse grade (HCO_3^- form). This column was then equilibrated with 7M-urea, pH 8.6, and attached to a column (45 cm. $\times$ 1 cm. diam.) of DEAE-Sephadex A-25, medium grade (HCO_3^- form). The nucleotides were eluted with ammonium bicarbonate, pH 8.6, the gradient being shown by the broken line; 7M-urea was present throughout.

a column (38 cm. $\times$ 2 cm. diam.) of DEAE-Sephadex (formate form) previously equilibrated with 7M-urea. The purines were washed from the column with 0.02M-formic acid containing urea (7M), and cytosine oligonucleotides were eluted with formic acid in a linear gradient from 0 to 1M in 2.1 l., containing urea (7M) throughout. Inorganic phosphate was eluted in the range 0.15–0.4M-formic acid and the cytosine oligonucleotides appeared as a single peak at 0.45–0.9M-formic acid. The fractions containing cytosine were pooled, freed of formic acid and taken up in water so that the urea concentration was approx. 2M. This solution was made alkaline (pH 9) with ammonia and chromatographed on DEAE-Sephadex (HCO_3^- form), sequences from Cp_2 to C_5P_6 being clearly resolved (Fig. 1b).

In this experiment, the amounts of material in the various sequences were determined by measuring the areas under the peaks of the elution pattern.

Table 2 gives the relative amounts of the various deoxycytidine compounds obtained from calf-thymus, herring-testis and *M. lysodeikticus* DNA by the different isolation procedures. Chromatography in the presence of urea appears to give higher yields of C_4P_5 and C_5P_6 . We have chosen to express the amounts relative to the amount of C_2P_3 since in our experience the frequencies of the dicytidylate sequences are more accurately known from our previous data (Burton, 1960; Petersen, 1963a) than are any of the other frequencies.

Ultraviolet-absorption spectra. The analytical data in Table 1 indicate that the cytosine oligodeoxynucleotides show no appreciable hypochromicity in acid solution. More spectral data are given in Table 3, and they indicate that at pH 7 the larger oligonucleotides show a slight but detectable hypochromicity which is more pronounced at 280 $m\mu$ than at 260 $m\mu$.

Table 1. Analytical data for cytosine deoxynucleotides

The fractions used were obtained from (a) calf-thymus DNA and from (b) *M. lysodeikticus* DNA. Cytosine was determined from the u.v. absorption of the fractions, ignoring hypochromicity and taking a molar extinction coefficient for deoxycytidylic acid of 9900 at 267 $m\mu$ and pH 1–2. The electrophoretic mobilities (all determined on fractions from *M. lysodeikticus* DNA) are corrected for endosmotic flow, determined from the movement of deoxycytidylic acid, which is assumed to have zero net charge.

Peak (cf. Fig. 1a)	Organic P:cytosine ratio			Relative electrophoretic mobility at pH 2.7			Identity
	Found		Calc.	Towards anode	Towards cathode after monoesterase treatment		
	(a)	(b)					
1	1.86	1.96	2.0	(1.0)	(1.0)		Cp ₂
2	1.54	1.47	1.50	0.83	0.50		C ₂ P ₃
3	1.26	1.30	1.33	0.76	0.42		C ₃ P ₄
4	1.30*	1.23	1.25	0.70	0.27		C ₄ P ₅
5	1.22*	1.12	1.20	0.67	0.27		C ₅ P ₆

* After purification by paper electrophoresis.

Action of phosphomonoesterase. Samples of the purified cytosine oligonucleotides (containing 1–2 μ moles of cytosine) in 0.3 ml. of water, adjusted to pH 5.6, were incubated at 37° with 0.05 ml. (26 units) of prostatic phosphomonoesterase. Toluene was added to prevent bacterial contamination. After 24 hr. another 0.05 ml. of enzyme was added and incubation continued for a further 12 hr. C_5P_6 was similarly incubated with 0.05 ml. of the bacterial alkaline phosphatase in the presence of 0.07 M-triethylamine bicarbonate, pH 7.6. The enzyme digests were deproteinized with chloroform, concentrated and examined by paper electrophoresis at pH 2.7. Each of the five oligonucleotides was completely dephosphorylated by these treatments to give a single ultraviolet-absorbing product. In no instance was there any evidence of degradation to smaller nucleotides.

A sample of prostatic phosphatase prepared by a different method (Ostrowski & Tsugita, 1961) gave satisfactory dephosphorylation of C_4P_5 to C_4P_3 , but the slower action on C_5P_6 was accompanied by extensive decomposition. Several ultraviolet-absorbing products were formed, the major one having the mobility of C_3P_2 . This type of

prostatic phosphatase preparation has not been used for any of the experiments that have been described in our previous papers.

Stability of the tetracytidylate compound to the acid conditions of degradation procedures. Samples of C_4P_5 were treated either with 66 % (v/v) formic acid for 22 hr. at 30° or with 0.1 M-sulphuric acid for 30 min. at 100° and then examined by paper electrophoresis. In both instances only one component was detected and this had the same mobility as the original C_4P_5 . With the amounts of material used, a small amount of decomposition would not have been detected. These tests indicate that the previous difficulties encountered in detecting the tetra- and penta-deoxycytidylate sequences (see the introduction section) are not due to extensive decomposition by the acid conditions used in the degradation of the DNA.

Degradation of deoxyribonucleic acid by sulphuric acid

Shapiro & Chargaff (e.g. 1957, 1963) have studied pyrimidine sequences in DNA by degrading the nucleic acid in 0.1 M-sulphuric acid at 100°. To investigate this degradation, we have measured the

Table 2. *Amounts of cytosine deoxynucleotides obtained from various deoxyribonucleic acids by the methods described in the text*

The separation methods used were: A, elution from Dowex 1 (formate form) with 0.9 M-formic acid, followed by elution from DEAE-Sephadex with ammonium bicarbonate; B, elution from DEAE-Sephadex with 0.1 M-formic acid in 7 M-urea, followed by elution from DEAE-Sephadex with ammonium bicarbonate in 7 M-urea; C, paper electrophoresis and chromatography on paper (Petersen, 1963a); D, paper chromatography (Burton, 1960). Amounts are expressed as moles of cytosine relative to C_2P_3 . Values obtained in previous studies are given for comparison.

Source of DNA	Amount taken (g.)	Separation method	Sequence				
			Cp_2	C_2P_3	C_3P_4	C_4P_5	C_5P_6
			Amount of cytosine deoxynucleotide				
Calf thymus	1.5	A	—	(1.0)	0.36	0.13	0.010*
	3	A	2.27	(1.0)	0.45	0.11	0.016*
	1.2	B	1.77	(1.0)	0.37	0.16	0.035
	1.1	B	—	(1.0)	0.43	0.19	0.053
	—	C	1.97	(1.0)	0.38	0.16	<0.015
Herring testis	1.8	A	2.90	(1.0)	0.32	0.04	~0.01*
	2.5	C	2.91	(1.0)	0.32	0.05	<0.015
<i>M. lysodeikticus</i>	0.6	A	1.30	(1.0)	0.38	0.09	0.006
	—	D	1.41	(1.0)	0.35	—	—

* After further purification by paper electrophoresis.

Table 3. *Ultraviolet-absorption spectra of cytosine deoxynucleotides from calf-thymus deoxyribonucleic acid*

The values are extinctions relative to $E_{260\text{ m}\mu}$ (pH 1–2) 1.0 before and after further purification by paper electrophoresis.

Sequence	Before electrophoresis				After electrophoresis			
	<i>E</i> (pH 1–2)		<i>E</i> (pH 7)		<i>E</i> (pH 1–2)		<i>E</i> (pH 7)	
	260 m μ	280 m μ	260 m μ	280 m μ	260 m μ	280 m μ	260 m μ	280 m μ
Cp_2	(1.0)	2.13	1.18	1.25	(1.0)	2.09	1.11	1.26
C_2P_3	(1.0)	2.17	1.20	1.25	—	—	—	—
C_3P_4	(1.0)	2.19	1.18	1.19	(1.0)	2.26	1.20	1.23
C_4P_5	(1.0)	2.06	1.15	1.15	—	—	—	—
C_5P_6	—	—	—	—	(1.0)	2.20	1.08	0.99

Fig. 2. Release of inorganic phosphate from DNA by sulphuric acid and the effect of subsequent treatment with diphenylamine in aq. formic acid. Herring-testis DNA in 0.100M-H₂SO₄ (20 mg. of DNA/ml.) was heated for 2 hr. at 100° with frequent shaking until dissolved (3 min.), samples being removed into 3 vol. of water. P_i and total P were measured on these solutions and, also, 1.0 ml. portions were mixed with 0.1 ml. of 2N-NH₃ and 2.2 ml. of 3% (w/v) diphenylamine in 98% (v/v) formic acid. After incubation at 30° for 2 or 17 hr., 4 ml. of water was added and the mixture extracted three times with 20 ml. of ether. The P_i and total P were measured on the aqueous phase. The points show % P_i on sulphuric acid treatment alone (○), or after subsequent diphenylamine treatment for 2 hr. (▲) or for 17 hr. (●).

release of P_i during this acid treatment and on subsequent treatment with diphenylamine in aqueous formic acid at 30° (Fig. 2). At all stages diphenylamine can release considerably more P_i and, after both treatments, the amount of P_i is greater than that released by the diphenylamine-formic acid mixture without the prior sulphuric acid treatment. The significance of this experiment is discussed below.

Action of aqueous 66% (v/v) formic acid on deoxyribonucleic acid

Herring-testis DNA was incubated with aqueous formic acid under the conditions of the diphenylamine degradation except that no diphenylamine was present. The resulting apurinic acid was isolated and characterized as described in the Materials and Methods section. On sedimentation in the ultracentrifuge at 59780 rev./min. it gave a single boundary, but the material was clearly polydisperse because the sedimentation coefficient at the boundary fell with time (Fig. 3) and the molecular weights determined at lower speeds by the method of Archibald (1947) changed with the time of centrifuging. Values obtained at the meniscus fell with time (Fig. 4) and those at the cell bottom increased (cf. Smith, Wood & Charlwood, 1956). On extrapolation to zero time, the data indicate a mean molecular weight of about 57000

Fig. 3. Sedimentation velocity of apurinic acid obtained by treating DNA with aqueous formic acid. The ordinate scale is log x , where x is the distance (cm.) of the maximum gradient of refractive index from the centre of the rotor. The abscissa scale is time at top speed (59780 rev./min.). The sodium salt (0.2%) in 0.2M-acetate buffer, pH 5.2, was used. The slope of the broken line corresponds to a $S_{20,w}$ value of 1.8S.

Fig. 4. Molecular weight of apurinic acid obtained by treating DNA with aqueous formic acid. The molecular weight was calculated from conditions at the meniscus. ○, Solution (0.8%) of the free acid in 0.2M-acetate buffer, pH 5.2; 11573 rev./min. ●, Solution (0.2%) of the sodium salt in same buffer; 7447 rev./min. Data for the cell bottom gave higher values for the molecular weight, extrapolating to 50000–60000 at zero time, but these values have not been plotted because they are less reliable.

(Fig. 4). Molecular weights of 10000–27000 have previously been reported for apurinic acid prepared by other methods (Tamm & Chargaff, 1953; Smith *et al.* 1956; Wood & Smith, 1956).

DISCUSSION

The occurrence of sequences of five consecutive cytosine nucleotides is of particular interest in connexion with the triplet code because, as dis-

cussed by Burton *et al.* (1963), five consecutive deoxycytidylate residues constitute the shortest sequence that unequivocally contains a CCC triplet. We have found sequences of up to five consecutive deoxycytidylate residues in both calf-thymus and *M. lysodeikticus* DNA. Less of the tetracytidylate sequence was found in herring-testis DNA and the pentacytidylate one was only just detectable. Some of the elution patterns obtained from calf-thymus DNA also suggested the presence of C_6P_7 (Fig. 1b). Our previous failure to detect pentadeoxycytidylate sequences (Petersen, 1963a) is probably due to the low content of this sequence in the deoxyribonucleic acids studied and to the smaller amount of material used in the paper-chromatographic separations than in the present experiments. We cannot satisfactorily explain why Spencer & Chargaff (1963) failed to find the tetracytidylate sequence in calf-thymus DNA. From our tests it is not extensively destroyed by the acid conditions of the degradation. Shapiro & Chargaff (1963) have cited unpublished experiments in which C_4P_5 was detected.

It was surprising to find so little C_5P_6 in the cytosine-rich DNA from *M. lysodeikticus*. However, chromatographic procedure A (Table 2) was used for this sample of DNA, and the experiments with calf-thymus DNA suggest that this procedure gives low yields of C_4P_5 and C_5P_6 . It is also conceivable that any losses were relatively larger with the *M. lysodeikticus* DNA because less of this DNA was used than of the calf-thymus DNA.

Compared with calf-thymus DNA, herring-testis DNA gives many more single cytidine residues but fewer tetracytidylate sequences. Burton *et al.* (1963) have discussed these differences in terms of a model based on the triplet code. The differences would be explained if calf-thymus DNA had more Pu-C-C and C-C-Pu triplets but fewer (Pu or Py)-Pu-C or C-Pu-(Pu or Py) triplets.

Comparison of diphenylamine-formic acid degradation and hot-sulphuric acid degradation. Pyrimidine nucleotides of the general formula $Py_nP_{(n+1)}$ may be obtained from DNA by treatment with either hot dilute mineral acids (Levene & Jacobs, 1912; Dekker, Michelson & Todd, 1953; Cohn & Volkin, 1957; Shapiro & Chargaff, 1957, 1963; Spencer & Chargaff, 1963) or aqueous formic acid and diphenylamine at 30° (Burton & Petersen, 1957, 1960). Both degradations probably occur by β -elimination reactions (Brown, 1963), but the diphenylamine reaction has the advantage that the aromatic amine allows the degradation to proceed to completion under conditions that do not cause appreciable breakdown of the $Py_nP_{(n+1)}$ products. When DNA is treated with 66 % (w/v) formic acid for 17 hr. at 30° in the absence of an aromatic amine no P_1 is released (Burton & Petersen, 1960), and

only about 0.4 % of the phosphodiester links are broken because the resulting apurinic acid has a weight-average molecular weight of about 57 000 (Fig. 4). Even these few breaks probably occur at the sugar residues with a free aldehydic group at C-1 and not at the phosphodiester links joining two pyrimidine deoxynucleotides.

We have previously given extensive evidence for the specificity of the diphenylamine-formic acid degradation and its freedom from interfering side reactions (Burton & Petersen, 1960). To summarize: (1) the initial release of P_1 (presumed to be interpurine P) reaches a definite constant value; (2) once this value has been reached, no more phosphodiester linkages are broken; (3) the amount of initial P_1 (interpurine P) is equal to the amount of residual diester P (interpyrimidine P), as is to be expected from the equimolar amounts of purines and pyrimidines; (4) exchange reactions have not been detected. For separating the products we have relied mainly on paper chromatography and paper electrophoresis after treatment with phosphomonoesterase. Spencer & Chargaff (1963) and Michelson (1963) have pointed out that any phosphodiesterase in the monoesterase preparation would be a source of error in this procedure. However, Burton & Petersen (1960) had already eliminated this possibility as a significant source of error by isolating some of the mono- and dipyrimidine compounds by anion-exchange chromatography without the phosphatase treatment and showing that the amounts agreed closely with those of the corresponding dephosphorylated compounds obtained by paper chromatography. Moreover, the time-course of the action of the phosphomonoesterase gave no indication of any contaminating diesterase or deoxyribonuclease activity and, in the present paper, we give additional evidence that the type of phosphomonoesterase preparation is satisfactory. For several types of DNA (Burton, 1960) our values for the amounts of interpurine P agree closely with the frequencies of purine-purine nearest neighbours as determined by the DNA polymerase (Josse, Kaiser & Kornberg, 1961; Swartz, Trautner & Kornberg, 1962).

There are, however, some appreciable differences between our results and those obtained in studies of the partial hydrolysis of DNA by 0.1 M-sulphuric acid at 100°. Later results obtained by this method (Rudner, Shapiro & Chargaff, 1962; Spencer & Chargaff, 1963) agree better with ours than do the earlier results of Shapiro & Chargaff (1957, 1960b) for the same deoxyribonucleic acids. Even so, there are still some large differences, most noticeably among the amounts of the monopyrimidine fragments. For example, Spencer & Chargaff (1963) report that the thymine:cytosine ratio in this fraction from calf-thymus DNA is 2.4; our results

consistently give 1.6–1.7 (Burton & Petersen, 1960; Burton, 1960; Petersen, 1963a), and Hall & Sinsheimer (1963), who also used the diphenylamine degradation, found 1.44. The amounts of monopyrimidine fragments from *Escherichia coli* DNA as reported by Rudner *et al.* (1962) are only about 72 % of those reported by Burton (1960).

These differences might be at least partly due to incomplete release or secondary destruction of pyrimidine sequences by the sulphuric acid hydrolysis. In recent papers (Spencer & Chargaff, 1963; Shapiro & Chargaff, 1963) most importance is given to the amounts of products formed after hydrolysis for 35 min., even though only about half of the P_i is released from the interpurine positions by this time (Fig. 2; see also Tsumita & Chargaff, 1958; Shapiro & Chargaff, 1960a). Unfortunately, direct information is not available to indicate if the pyrimidine sequences are completely released from the deoxyribose residues that were originally constituents of the purine deoxyribonucleotides. It is known that the amounts of several products change markedly on prolonging the time of hydrolysis (Shapiro & Chargaff, 1957, 1960a, 1963), but with the available data it is not possible to decide what contributions are made by the various conceivable processes.

A measure of the extent of secondary destruction is given by the fact that diphenylamine in aqueous formic acid can release more of the interpurine P from sulphuric acid-treated DNA, the final P_i being greater than that released by the diphenylamine reagent alone (Fig. 2). The extra P_i increases with the time of treatment with sulphuric acid but is hardly affected by prolonging the diphenylamine treatment from 2 to 17 hr. Hence it must arise by breakdown of the $Py_nP_{(n+1)}$ compounds during the sulphuric acid treatment. Most probably, this P_i comes from terminal phosphomonomer positions, and the observed amount at 35 min. could then arise by 9 % of the $Py_nP_{(n+1)}$ compounds losing one terminal phosphate group. The full effects of all modes of secondary destruction might be larger because of hydrolysis at non-terminal linkages.

SUMMARY

1. Sequences of four and five consecutive cytosine deoxynucleotides have been detected in DNA from calf thymus, herring testis and *Micrococcus lysodeikticus*.

2. Some observations are reported on the degradation of DNA by 0.1 M-sulphuric acid at 100°.

3. Treatment with aq. 66 % (v/v) formic acid at 30° for 17 hr. converted DNA into an apurinic acid with a weight-average molecular weight of 57 000.

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