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Studies on the Nucleotide Arrangement in Deoxyribonucleic Acids. XI. Selective Removal of Cytosine as a Tool for the Study of the Nucleotide Arrangement in Deoxyribonucleic Acid*

Herman S. Shapiro and Erwin Chargaff

ABSTRACT: When calf thymus deoxyribonucleic acid (DNA) is treated with nitrous acid at pH 3.35, complete deamination takes place. The resulting product is not dialysable and retains about 96% of the original DNA phosphorus. It contains the initial proportions, characteristic of the starting DNA, of thymine and uracil (from cytosine), and about 80% of the original adenine in form of hypoxanthine, whereas most of the xanthine (from guanine) is found as the free purine. Apurinic acid and oligonucleotides derived from DNA can be treated similarly. From such preparations of deamino-DNA or of deaminated apurinic acid, uracil can be eliminated selectively by treatment with hydroxylamine at pH 10 as shown by the analysis of several products. The application of these procedures to the study of the arrangement of thymidylic acid and of its oligonucleotides in DNA is illustrated by a few examples, namely, the determination of the proportion of position isomers in a dinucleotide consisting of thymidylic and deoxycytidylic acids and the liberation of the thymidylic acid runs of lengths 1–5 occurring in calf thymus DNA.

An unambiguous solution of the problem of the nucleotide sequence of a cellular deoxyribonucleic acid (DNA) is not likely to occur in the near future. The very great length of the chains composed of a very small number of different building blocks, the absence of enzymes of a narrow specificity, the lack of generally applicable methods for the determination of end groups, and the existence of many lacunas in our understanding of even the primary structure of these high polymers all combine to render practical only the most general statistical interpretation of the nucleotide arrangement. This has been discussed before (Chargaff, 1963; Shapiro *et al.*, 1965).

This communication attempts to remove one of the difficulties encountered in the study of the pyrimidine nucleotide runs in DNA. A naturally occurring polynucleotide chain is composed of alternating runs of purine and pyrimidine nucleotides of different length and composition. Differential degradative procedures permit the separate isolation of either the purine or the pyrimidine sequences of a DNA. Thus, the acid degradation of DNA or the alkaline degradation of apurinic acid afford a mixture of pyrimidine isostichs (Shapiro and Chargaff, 1964) of the general structure[1] Py_np_{n+1}, whereas by the alkaline degradation of apyrimidinic acid the corresponding purine isostichs, Pu_np_{n+1}, become available (Chargaff *et al.*, 1963, 1965). In all instances, either the purines flanking the pyrimidine runs or the pyrimidines flanking the purine runs must be removed before the intact sequences are liberated by a series of β-elimination reactions (Shapiro and Chargaff, 1957).

The oligonucleotides, which result from the chromatographic separation of the hydrolysates, will in many cases consist of mixtures of position isomers. For instance, the fraction CT_2p_4 can be prepared (Spencer and Chargaff, 1963; Shapiro and Chargaff, 1963); but in most cases it will presumably comprise the three

* From the Cell Chemistry Laboratory, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York. *Received May 31, 1966.* This work was supported by a research grant from the National Institutes of Health, U. S. Public Health Service. Paper X of this series is in press (Rudner *et al.*, 1966).

[1] The following abbreviations are used: Pu, purine deoxyribonucleoside; Py, pyrimidine deoxyribonucleoside; A, deoxyriboadenosine; I, deoxyriboinosine; G, deoxyriboguanosine; C, deoxyribocytidine; U, deoxyribouridine; T, thymidine; O, deoxyribose. Esterified phosphate is indicated as p, placed on the right of the symbol when linked to the 3′-hydroxyl and on the left when linked to the 5′-hydroxyl. When no specified sequence of an oligonucleotide fraction is implied, the appropriate symbols are followed by the number of phosphate groups in the particular fraction. Thus, CTp_3 can indicate a mixture of the position isomers pCpTp and pTpCp.

TABLE I: Composition of Calf Thymus DNA and of Degradation Products under Study.[a]

No.	Prepn	Moles/100 g-atoms of DNA Phosphorus							Act. Recov (%)	Molar Ratio of Thymine: Cytosine or Thymine: Uracil
		Adenine	Hypoxanthine	Guanine	Xanthine	Cytosine	Uracil	Thymine		
1	DNA	27.9	—	21.7	—	22.1	—	28.4	98.3	1.29
2	Apurinic acid	—	—	—	—	21.2	—	28.8	49.6	1.36
3	Deamino-DNA, prepn 1	—	22.7	—		—	20.5	29.5	47.6	1.44
4	Deamino-DNA, prepn 2	—	23.7	—		—	20.2	29.8	49.5	1.48
5	Deamino-DNA, prepn 3	—	23.4	—		—	20.0	30.0	47.5	1.50
6	Deamino-DNA, prepn 3	—	21.5	—	1.26					

[a] The analytical methods used are described in the Experimental Section. The hypoxanthine component listed in expt 3–5 had the spectral characteristics of this purine, but may have included a small amount of xanthine. The figures on actual recovery refer to all bases in 1 and to the pyrimidines only in 2–5. The molar proportions were corrected for a 100% recovery in 1 and for a 50% recovery in 2–5.

isomers pCpTpTp, pTpCpTp, and pTpTpCp. In longer oligonucleotide runs the number of position isomers will, of course, increase enormously. Only the shortest mixed oligonucleotide CTp_3 appears to be amenable to analysis by enzymic (Shapiro and Chargaff, 1957) or chromatographic (Burton and Petersen, 1960) procedures, permitting the determination of the frequency of pCpTp and pTpCp; and the comparison of the relative frequencies of pCpTp and pTpCp with those of pApGp and pGpAp has, in fact, provided a direct test of the polarity of the complementary chains of a DNA (Chargaff *et al.*, 1965).

It is quite clear that a method allowing the removal of one of the two base constituents of a mixed oligonucleotide fraction will, in many cases, facilitate the estimation of its isomeric components. In addition, it may be of interest for the consideration of the potential informational content of a DNA molecule, since it permits the estimation not only of those homologous pyrimidine oligonucleotide sequences that are interjacent between purines, but also of those flanked by another pyrimidine.

Hydroxylamine has proved a useful reagent in the study of the structure of ribonucleic acid (RNA) (Verwoerd *et al.*, 1963) by virtue of its ability to bring about, at pH 10, a specific degradation of the uracil component. The application of this reaction to DNA, following the deamination of the cytosine component of the latter to uracil, forms the subject of this communication.

The DNA preparations used were obtained from calf thymus. Treatment with nitrous acid at pH 3.3 led to products, here referred to as "deamino-DNA," in which the original thymine component was recovered in unchanged proportions, as was the uracil corresponding to the original cytosine complement, and in which most of the initial adenine was present as hypoxanthine (77–85% of initial adenine). On the other hand, the products retained only about 6% of the guanine component in forms of xanthine. The deamination was complete, as adenine, guanine, and cytosine were no longer demonstrable. The phosphorus recovery in terms of initial DNA phosphorus was about 96%. It is obvious that under the conditions of deamination observed here, namely, pH 3.3 at 37° for 72 hr, the glycosidic bond of xanthine was largely cleaved, whereas that of hypoxanthine mostly survived.[2] This is borne out by model experiments on the action of HNO_2 on deoxyadenylic and deoxyguanylic acids which will be found in the Experimental Section. Under our conditions, a DNA sequence of the type of -pApGpCpTp- would, hence, be converted to -pIpOpUpT-. Analytical data on the DNA and the products of its deamination are given in Table I.

The treatment of deamino-DNA with hydroxylamine yielded products in which the thymine component was retained in virtually unchanged concentration, whereas the uracil component had been eliminated; a conclusion based on both spectroscopic and analytical evidence (compare Figure 1). The same series of reactions,

[2] The effect of HNO_2 on DNA, as a function of pH and of other conditions, is quite complex and deserves a detailed study, especially in view of the mutagenic action of this reagent. The conditions for deamination chosen here are based on unpublished studies by Dr. P. Rüst in this laboratory.

TABLE II: Enzymic and Chemical Degradation of Dinucleotide Mixture pCpT and pTpC.

Expt No.	Agent[a]	Components Produced	Moles/100 Moles of Dinucleotide	Molar Proportions of pCpT:pTpC
1	Phosphatase	CpT + TpC		
	Phosphodiesterase	C	75.1	
		pT	73.9	2.9
		T	24.9	
		pC	26.1	
2	HNO_2	pUpT + pTpU		
	Phosphatase	UpT + TpU		
		U	73.7	
	Phosphodiesterase	pT	73.8	2.8
		T	26.3	
		pU	26.2	
3	HNO_2	pUpT + pTpU		
	NH_2OH, benzaldehyde	pOpT + pTpO		
	H_2SO_4	pT	76.6	3.3
		pTp	23.4	

[a] In each experimental step the products were isolated before being subjected to the next agent listed.

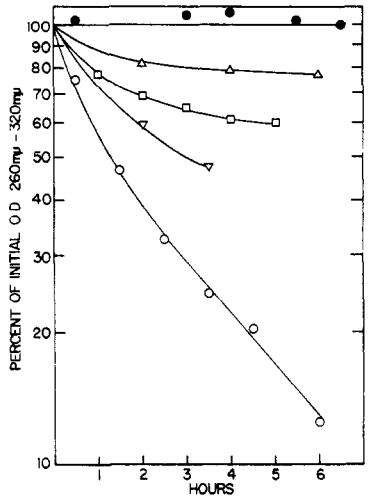

FIGURE 1: Rate of destruction of pyrimidine chromophores by hydroxylamine at pH 10 and 37°. The per cent decrease of optical density (difference between readings at 260 and 320 mμ) was determined in 20-μl portions, diluted with 4 ml of 0.1 N HCl, after heating at 100° for 10 min. ●, thymidine 5′-phosphate; ○, deoxyuridine 5′-phosphate, ▽, dinucleotide UTp_2; △, deamino-DNA, preparation 1; □, deaminated apurinic acid. See text for details.

deamination and degradation of uracil, was also carried out with apurinic acid (Tamm *et al.*, 1952). These reaction products could rightly be given the now discarded designation "thymic acid."

The potential usefulness of these procedures is illustrated here by two examples. The first has to do with the estimation of the proportions of sequence isomers. We limit ourselves here to the simplest case, a mixed dinucleotide, but hope to describe other applications later. The dinucleotide studied was a mixture of pCpT and pTpC isolated from DNA by the action of barley deoxyribonuclease (Brawerman and Chargaff, 1954). Three experiments performed with this mixture are shown in Table II. (a) The consecutive action of phosphomonoesterase and, after the removal of this enzyme, of phosphodiesterase yielded 5′-thymidylic acid and deoxycytidine in equal amounts indicative of the proportion of pCpT present in the initial mixture; the quantities of 5′-deoxycytidylic acid and thymidine similarly related to the proportion of pTpC. (b) The same series of reactions applied to the dinucleotide deaminated previously by means of HNO_2 yielded corresponding quantities of pT and U and of pU and T. (c) The same deaminated dinucleotide, *i.e.*, pUpT and pTpU, was treated with hydroxylamine and then hydrolyzed with sulfuric acid. Since the deaminated dinucleotide mixture consisted, after the destruction of uracil, of a mixture of pOpT and pTpO, the quantities of thymidine 5′-phosphate and thymidine 3′,5′-diphosphate, respectively, set free by β elimination, were indicative of the proportions of the two sequence isomers, pCpT and pTpC, present originally in the dinucleotide. The molar ratio of pCpT:pTpC was found as 2.9, 2.8, and 3.3 in the three experiments.

The estimation of the relative abundance of all thymidylic acid runs of lengths 1–5 present in a DNA

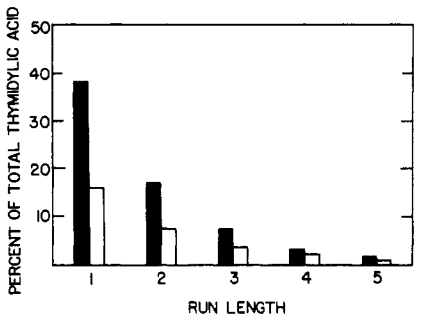

FIGURE 2: Liberation of thymidylic acid runs of calf thymus DNA (0.1 M H_2SO_4, 35 min, 100°). Black bars, hydrolysis of preparation Hx-3 (deamino-DNA preparation 3 treated with hydroxylamine and benzaldehyde). White bars, hydrolysis of calf thymus DNA (taken from Shapiro and Chargaff, 1964).

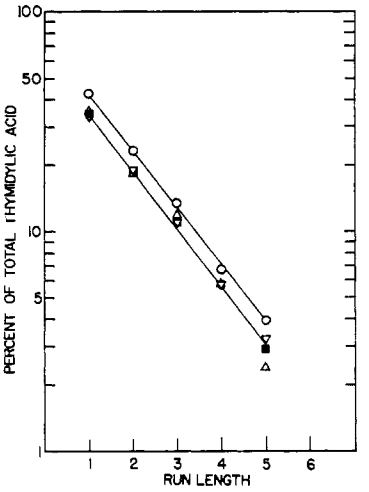

FIGURE 3: Liberation of thymidylic acid runs from preparation Hx-4 (deamino-DNA preparation 1 treated with hydroxylamine and benzaldehyde). Methods of degradation: (1) product (31.5 mg) heated in 2 ml of 0.1 M H_2SO_4 at 100° for (a) 35 min, Δ; (b) 60 min, ∇; (c) 120 min, ■. (2) Product (16 mg) heated in 60 ml of 66% HCOOH–2% diphenylamine at 35° for 18 hr, O.

provides another instance of the application of our procedure. This may prove a useful supplement to the studies of the distribution of pyrimidine isostichs in apurinic, and of purine isostichs in apyrimidinic, acids mentioned before. Let us, for example, consider the following arbitrary decanucleotide sequence of a DNA chain.

-pApTpGpTpCpTpTpTpCpG-
1 2 3 4 5 6 7 8 9 10

This sequence, or the corresponding apurinic acid segment, would during the usual acid degradation be cleaved at positions 1, 3, and 10, releasing 1 equiv of Tp_2 and 1 equiv of a hexanucleotide $T_4C_2p_7$. It would be extremely difficult to determine the presence of a pT triplet in this hexanucleotide, let alone the number of sequence isomers it may contain. On the other hand, the application of the degradative procedures discussed here leads to a product which on mild acid treatment will be also ruptured at positions 5 and 9 and will give rise to 2 equiv of Tp_2 (from positions 2 and 4) and to the triplet T_3p_4 (from positions 6–8).

It becomes thus possible to estimate the frequencies of the entire series of contiguous thymidylic acid stretches in a DNA regardless of the nature of the nucleotide units that flank them in the polymer chain. Figure 2 provides an example in the form of a histogram in which the abundance of all poly-pT units of lengths 1–5 in calf thymus DNA is compared with the proportions of the same units that occur interjacent between purine nucleotides. The differences between the corresponding bars indicate the poly-pT units that are flanked by pC in the DNA. In Figure 3 orienting experiments are shown in which the release of the thymine oligonucleotides from the same DNA is compared under different hydrolytic conditions, *viz.*, 0.1 M H_2SO_4 at 100° for 35, 60, and 120 min and formic acid–diphenylamine (Burton and Petersen, 1957). These data complete a previous comparative study of the purine-flanked pyrimidine isostichs (Shapiro and Chargaff, 1964). We hope to present, at a later occasion, a more comprehensive discussion of the all-thymine runs encountered in DNA preparations from different species.

Experimental Section

Materials. The DNA specimen employed as the starting material was prepared from calf thymus, with a few modifications (compare Chargaff, 1955), by the procedure of Kay *et al.* (1952), but with the substitution of 0.01 M Tris buffer (pH 7.5) for distilled water. The final preparation was stored in fibrous form under ethanol in the cold, after precipitation from a 1 M NaCl solution with 2 volumes of 95% ethanol, and samples were brought back into solution before use. The base composition of this DNA preparation and that of an apurinic acid specimen prepared from it (Tamm *et al.*, 1952) are listed in Table I.

The mixed dinucleotide preparation CTp_2 was isolated, as described previously (Shapiro and Chargaff, 1957), from a digest of calf thymus DNA with barley nuclease by chromatography on Dowex 1. The eluate was desalted by treatment with charcoal Darco G-60 and elution with ethanol–concentrated aqueous ammonia–water (25:1:24, v/v). The dinucleotide was precipitated from the concentrated solution with 95% ethanol. The mononucleotides used as test substances, deoxyribouridylic and thymidylic acids, were commercial preparations.

Hydroxylamine was prepared from the hydrochloride as described by Verwoerd *et al.* (1963). The concentration of the preparations recovered by distillation *in vacuo*, which was determined by titration with 1 N

TABLE III: Products of Deamination of Deoxyadenylic and Deoxyguanylic Acids.[a]

Nucleotide	Reaction Products	Mole % of Nucleotide after Reaction (hr) with Nitrous Acid 2	4.5	7.5	24	48	72
Deoxyadenylic acid	Deoxyinosinic acid				80	63	46
	Hypoxanthine				20	37	54
Deoxyguanylic acid	Deoxyxanthylic acid	44	14	7	0		
	Xanthine	56	86	93	100		

[a] See Experimental Section for conditions.

H_2SO_4, varied from 9 to 11.5 M. The hydroxylamine preparations were stable for at least 2 months when stored at −20°.

Analytical Procedures. For the determination of the base composition of intact DNA and of the partial degradation products the customary hydrolytic (formic acid), chromatographic, and spectrophotometric techniques were employed (Chargaff, 1955; Beaven *et al.*, 1955). The preparations listed in Table I as 1–5 and also the products of the treatment with hydroxylamine to be described below were analyzed essentially according to Chargaff *et al.* (1951). For the determination of hypoxanthine and xanthine in deamino-DNA preparation 3 (Table I, 6), 1 mg of the material was kept in 0.5 ml of dilute HCl of pH 1.6 for 24 hr at 37°. The neutralized solution was taken to dryness and 10-μl portions of the solution of the residue in 0.1 ml of H_2O were used for P analysis and paper chromatography in 1-butanol–diethylene glycol–water (4:1:1, v/v) in an NH_3 atmosphere (Vischer and Chargaff, 1948). The quantitive estimation was performed according to Kream and Chargaff (1952).

Phosphorus was determined by the method of King (1932). The liberation, chromatographic separation, and quantitative determination of the thymine oligonucleotides of lengths 1–5 have been described before (Shapiro and Chargaff, 1963).

Action of Hydroxylamine on Deoxyribouridylic and Thymidylic Acids. To 0.25-ml portions of 0.8% aqueous solutions of the nucleotides 0.5 ml of 11.5 M NH_2OH and 1 capillary drop of 2.4 N NaOH were added. The reaction mixtures which had a pH of 9.8 and were approximately 7.5 M with respect to NH_2OH, were kept at 37° with gentle rotation. At zero time and at subsequent intervals, 20-μl samples were withdrawn and heated, in small test tubes loosely closed with glass caps, with 4 ml of 0.1 N HCl for 10 min in a boiling water bath, after which the solutions were cooled and analyzed spectrophotometrically and by paper chromatography which bore out the destruction of pU and the survival of pT. The slight residual ultraviolet absorption of the reaction product of deoxyribouridylic acid after 4 hr was not that of the starting material, and the degradation was considered complete. The course of the reaction is shown in Figure 1. The degradation of uridylic acid and the stability of thymidylic acid have been noted before (Verwoerd *et al.*, 1963; Freese *et al.*, 1961).

Deamination of Deoxyadenylic and Deoxyguanylic Acids. The same conditions of deamination were employed as those chosen for the deamination of DNA (see next section). The concentration of deoxynucleotide used was 1 mg/ml of 0.65 M $NaNO_2$–HNO_2 of pH 3.35. Samples were incubated at 37° for various periods; when the solutions were taken to dryness and the residues were dissolved in ethanol–2 N ammonia (1:1, v/v) in the experiments with guanylic acid, in water in those with adenylic acid. Portions served for P analyses and for chromatographic separation in 1-butanol–diethylene glycol–water (4:1:1, v/v) in an NH_3 atmosphere. The decomposition of deoxyguanylic acid was very rapid, that of deoxyadenylic acid somewhat slower (see Table III). The rapid production of free xanthine is particularly noteworthy.

Deamination of DNA. The addition of 13.5 g of $NaNO_2$ to a solution of 500 mg of DNA in 250 ml of H_2O was followed by the adjustment of the solution to pH 3.35 by means of glacial acetic acid. The mixture, brought with water to a final volume of 300 ml, corresponding to an approximate molarity of 0.65 with respect to $NaNO_2$–HNO_2, was transferred to a flask, closed securely with a ground-glass stopper, and kept at 37° for 72 hr. It was then neutralized by dialysis in the cold against frequent changes of 0.5 M borate buffer of pH 7.3. After additional dialysis against running tap and distilled water, the deaminated product was recovered by the evaporation *in vacuo* of the frozen solution, accounting for approximately 96% of the initial DNA phosphorus. The composition of three preparations of the deamination product is listed as "deamino-DNA" in Table I.

Action of Hydroxylamine on Deaminated DNA. The removal of uracil from deamino-DNA (preparation 1) was followed spectroscopically, as described before in the case of the mononucleotides. Approximately 36 mg of the deaminated DNA (corresponding to 3.2

mg of organic P) was dissolved in 2 ml of 8 M NH_2OH and adjusted to pH 10 with 3 N KOH. The mixture was kept at 37° for 6 hr with rotation, and small samples were analyzed at intervals with the procedure noted before. The course of this reaction is also shown in Figure 1. A decrease of about 23% of the initial OD was recorded at the end of 6 hr, when the product was precipitated in the cold by the addition of 5 volumes of ethanol. The mixture was cooled to −20° and adjusted to a 1% concentration of trichloroacetic acid, and the precipitate was collected by centrifugation and washed with ethanol. It was dissolved in 0.5 ml of H_2O and, after extraction with ether, 0.5 volume of benzaldehyde was added to the solution which was kept at room temperature for 18 hr. After exhaustive extraction with ether the material was recovered by lyophilization. This preparation, designated Hx-1, was entirely free of uracil and contained 27.9 moles of thymine/100 g-atoms of DNA phosphorus.

In two other preparations, made by the same procedure and designated Hx-2 (from deamino-DNA preparation 1) and Hx-3 (from preparation 3) the corresponding thymine values were 25.5 and 24.4, respectively; they also were free of uracil. In both instances, the decrease in OD accompanying the removal of uracil amounted to 23%. Between 2.5 and 9% of the phosphorus present in the deamino-DNA serving as the starting material was not recovered in the product of the NH_2OH treatment insoluble in ethanol. In some preparations carried out on a larger scale (16 mg of organic P) the destruction of uracil was not complete: one specimen, made from deamino-DNA preparation 1, preparation Hx-4, contained, for instance, 29.2 mole % of thymine and 1.5 mole % of uracil.

Experiments were also conducted on the removal of uracil from deaminated apurinic acid. Deamino-DNA (preparation 2) was freed of purines by exposure to pH 1.4 at 37° for 48 hr (Tamm *et al.*, 1952) and the deaminated apurinic acid subjected to treatment with NH_2OH, a drop of 40% of the OD being recorded after 5 hr at 37° (Figure 1). The product isolated in the usual manner, analogous to the previously described oxime of apurinic acid (Tamm and Chargaff, 1953), was converted to the aldehydo form by treatment with benzaldehyde. It was recovered in a yield corresponding to 93% of the phosphorus of the starting material and was found to be entirely free of uracil and to contain 24.6 moles of thymine/100 g-atoms of organic P. It can, hence, be regarded as a degradation product of apurinic acid in which about 87% of the original thymidylic acid sequences of the DNA are preserved, alternating with the deoxyribophosphate runs that had been substituted with the adenine, guanine, and cytosine components.

Estimation of Sequence Isomers in Dinucleotide Mixture. A portion of the mixed dinucleotide preparation CTp_2 (50 OD_{260} units) was dissolved in 5 ml of 0.65 M $NaNO_2$–HNO_2 of pH 3.35 and the solution was kept at 37° for 72 hr. The mixture was concentrated to 1 ml and desalted by passage through a column (58 × 1 cm) of Biogel P-2 (supplied by Calbiochem, Los Angeles). Approximately 90% of the deaminated dinucleotide was eluted before saltbeg an to break through. The salt-free preparation of UTp_2 and the initial CTp_2 preparation were used in the subsequent experiments (compare Table II).

The content of sequence isomers in these preparations was first determined by enzymic analysis. The mixture of pCpTp and pTpCp was deprived of terminal phosphate groups by treatment with the alkaline phosphatase of *Escherichia coli* purified according to Fiers and Sinsheimer (1962) and the resulting mixture of CpT and TpC was subjected to the action of purified snake venom diesterase (Keller, 1964). The nucleosides, deoxycytidine and thymidine, and the nucleotides, thymidylic and deoxycytidylic acids, present in the digest were separated chromatographically with buffered isobutyrate (Magasanik *et al.*, 1950) and estimated spectrophotometrically.

The deaminated dinucleotide mixture, pUpT and pTpU, was analyzed similarly. Another portion (25 OD_{260} units) was treated with hydroxylamine (1 ml of 7.5 M NH_2OH, pH 10, 37°). After 3.5 hr the destruction of uracil that followed spectrophotometrically (compare Figure 1) was complete. The decrease in OD_{260} amounted to 53% of the initial value, in accordance with the known absorbances of pU and pT (Beaven *et al.*, 1955). The mixture was shaken at room temperature with a few drops of benzaldehyde for 18 hr and extracted with ether. The aqueous layer was adjusted to 0.1 M H_2SO_4 and heated at 100° for 35 min. The separation of thymidine 5′-phosphate and thymidine 3′,5′-diphosphate, which were produced by this series of reactions, was carried out on Whatman No. 1 filter paper in the arrangement described before (Shapiro and Chargaff, 1963), either two dimensionally with I. isopropyl alcohol–H_2O (7:3, v/v) in NH_3 atmosphere (Markham and Smith, 1952), II. isobutyric acid–0.5 N ammonia (5:3, v/v) at pH 3.7 (Magasanik *et al.*,

TABLE IV: R_F Values of Compounds Obtained during Degradation of Mixed Dinucleotide pTpC and pCpT.

Compound	Relative R_F Values[a]	
	Buffered Isobutyrate	Isopropyl Alcohol–NH_3
T	1.68	3.24
U	1.47	2.72
pT	1.00	1.00
pU	0.83	0.57
pTp	0.48	0.52
pUpT + pTpU	0.65	0.63
UpT + TpU	1.05	1.75
pOpT + pTpO	1.00	0.52
OpT + TpO	1.91	0.88

[a] The values are given relative to the mobility of thymidine 5′-phosphate taken as 1.00.

1950), or in the latter solvent alone. The relative mobilities of the various compounds are listed in Table IV. The recovery of dinucleotide-P in these experiments ranged from 70 to 80%.

Liberation of Thymidylic Acid Runs of DNA. These experiments were carried out with deamino-DNA preparations from which uracil had been eliminated by the action of hydroxylamine, as described before. For instance, 12.5 mg of preparation Hx-3 (thymine content, 24.4 mole %) was treated with 1.1 ml of 0.1 M H_2SO_4 for 35 min at 100°. The hydrolysate was neutralized and concentrated to a volume of 0.7 ml; 10-μl portions were used for phosphorus analysis and 60-μl portions for the separation of the thymidylic acid runs. Determinations were usually run in quadruplicate with the use of the two-dimensional arrangement described by Shapiro and Chargaff (1963). The hydrolysis with H_2SO_4 liberated 49.8% of the phosphorus as inorganic phosphate. The results of this experiment are shown in Figure 2.

Another preparation of the degradation product of DNA, preparation Hx-4 (29.2 mole % thymine and 1.5 mole % uracil), was used in comparative experiments in which the effect of heating at 100° for 35, 60, and 120 min in 0.1 M H_2SO_4 was compared with that of a mixture of 66% formic acid and 2% diphenylamine for 18 hr at 35° (Burton and Petersen, 1957). The experimental arrangement was described in a previous paper (Shapiro and Chargaff, 1964). The results of this experiment are represented graphically in Figure 3.

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