ACID DEGRADATION PRODUCTS OF DEOXYRIBONUCLEIC ACID*

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INTRODUCTION

The study of structural features of deoxyribonucleic acids (DNA) by isolation and characterization of the mono- and polynucleotide products of chemical attack has utilized, as an initial step, the lability of the purine-deoxyribose linkage to acid hydrolysis (VOLKIN *et al.*[1]; TAMM *et al.*[2]). In contrast to ribonucleic acids, in which essentially complete degradation to mononucleotides by mild alkali is almost a matter of definition, deoxyribonucleic acids yield readily to alkali—or to acid, for that matter—only after the purine bases are lost and the substances called apurinic acids (TAMM and associates[2,3]) are formed.

Apurinic acid, owing to the loss of the purine groups from the 1′ positions of deoxyribose residues, would be expected to have two labilities toward alkali or acid not possessed by the parent DNA molecule. Although DNA is denied the 2′-hydroxyl of ribonucleic acid (RNA) to which the alkali lability of that species is due (BROWN AND TODD[4,5,6]), the 4′-hydroxyl in each apurinated residue, formed upon opening of the furanose ring, is similarly available for cyclization. Consequently, alkali could produce a rupture of the polymer to leave the phosphates in question attached to expurine residues. This seems to be the sole mechanism of the alkali-catalyzed breakdown of mercaptoacetylated DNA (JONES AND LETHAM[7]; JONES *et al.*[8,9]) in which two carboxymethylthio groups form a thioacetal with the aldehyde liberated by the removal of each purine base. The mono-, di-, and trinucleotide products isolated (JONES *et al.*[8]) possess (1) one less phosphate than pyrimidine, with no sulfur, or (2) phosphate equal to pyrimidine, with two sulfur atoms per molecule (*i.e.*, one expurine residue is still attached). This is consistent with cyclization mechanisms involving the 4′-hydroxyl of the apurinic residues. From the standpoint of analysis however, the appearance of two products from each type of sequence in the original molecule is a complicating factor brought about by the two possibilities of cyclization, *i.e.*, between 3′ and 4′, or between 4′ and 5′ (JONES *et al.*[9]).

The other degradation mechanism for apurinic acids, which can occur in acid as well as in alkali, is that of β elimination (*i.e.*, cleavage at the bond two removed from the aldehyde group) (WHITFELD[10]; BROWN *et al.*[11]; BROWN AND TODD[6]). Such cleavage will leave on pyrimidine nucleosides those phosphates formerly located between pyrimidine and purine nucleosides and thus give rise to nucleotides containing one more phosphate than pyrimidine. The 3′, 5′-diphosphates of deoxycytidine

* Work performed under Contract No. W-7405-eng-26 for the Atomic Energy Commission, and presented at the Third International Congress of Biochemistry, Brussels, 1955 (*cf. Résumés des Communications*, p. 21).

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and of thymidine have been found in such digests (DEKKER *et al.*[12]). The elimination reaction may also take place in alkaline hydrolysis, as is shown by the alkaline degradation of ribose phosphates (KHYM *et al.*[13]), but the alkaline hydrolysis of mercaptoacetylated apurinic acid indicates a cyclization mechanism (JONES *et al.*[9]) as may be expected from acetals (BROWN AND TODD[5] [p. 327]). In acid, on the other hand, cyclization should be much less prominent than elimination (BROWN AND TODD[5,6]) and a single degradation product from each original sequence type could reasonably be anticipated. A simpler analytical problem might thus result than from alkaline hydrolysis, in which at least two products per sequence type were anticipated and found (JONES *et al.*[9]).

We have made a preliminary test of these arguments, by separating the acid degradation products of calf thymus DNA and identifying some of them.

EXPERIMENTAL

The calf thymus DNA was prepared by a method similar to that of MARKO AND BUTLER[14], except that the anionic detergent, aerosol OT*, was substituted for sodium dodecylsulfate. Hydrolysis was effected in boiling water in the presence of N HCl for varying lengths of time. The times of hydrolysis chosen for chromatographic analysis were 1 hour and 4 hours, corresponding to the plateaus in the formation of inorganic phosphate (see Fig. 1). Inorganic phosphate was determined by the method of GRISWOLD *et al.*[15].

The hydrolyzed material was diluted about 100-fold with water, made alkaline with ammonia, and absorbed on a Dowex-1 column (200–400 mesh, chloride form) (COHN[16]). Ammonium chloride (0.01 M) removed most of the adenine and guanine. Phosphate esters were then eluted by a series of gradient elutions, in which the concentration of HCl was increased linearly (not exponentially) in volumes of 2 liters each between the following limits: 0 N to 0.01 N; 0.01 N to 0.04 N; 0.04 N to 0.10 N; 0.10 N to 0.30 N. A linear increase in eluting concentration was achieved by allowing the solution at the higher concentration to flow into the solution at the lower concentration, with stirring, on its way to the column, with the volumes in the two containers remaining equal at all times (PARR[17]).

Peaks were isolated on the basis of recorded ultraviolet absorption characteristics (VOLKIN AND COHN[18]), neutralized or lyophilized, or both, further fractionated by chromatography (in HCl or in NH_4Cl) if necessary, and identified by (1) ion-exchange behavoir, (2) spectrophotometric behavior, (3) extinction coefficient based on phosphorus analysis and, (4) ratio of monoesterified phosphorus to total phosphorus (end-group analysis) as indicated by inorganic phosphate released by bone monoesterase (VOLKIN[19]).

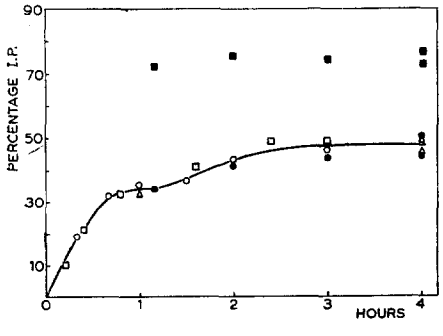

Fig. 1. Production of inorganic phosphate in the course of hydrolysis of DNA by N HCl at 100°, in percentage of total P present. Solid squares represent total inorganic phosphate after treatment of a digest (solid circles) with bone phosphatase.

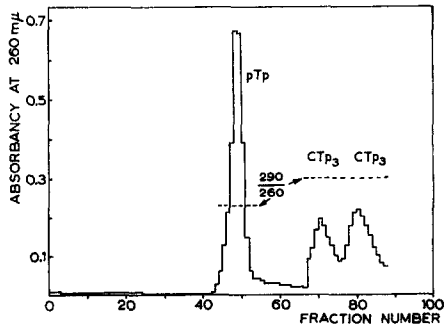

Fig. 2. Rechromatography of peak IVa (see Fig. 3) in NH_4Cl, increased linearly from 0.03 to 0.2 M (same column as in Fig. 3).

* Aerosol OT (dioctyl sodium sulfosuccinate) was obtained from Fischer Scientific Co.

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RESULTS

Although a large number of "peaks" were obtained in the first chromatographic analysis of the 1-hour and 4-hour hydrolyzates (see Fig. 3), few of these may be considered to be single substances. Hence the origins of the final products are designated groups I, II, III, IV, and V as indicated in Fig. 3. Most mixed peaks yielded well to rechromatography in HCl, but peak IV could be separated only at pH 5–6 (NH_4Cl). Rechromatography of peak IV is shown in Fig. 2.

The substances isolated and identified from the various peaks are listed in Table I, together with the criteria of identification. Also included are a few peaks for which only the ultraviolet absorbancy ratios are known.

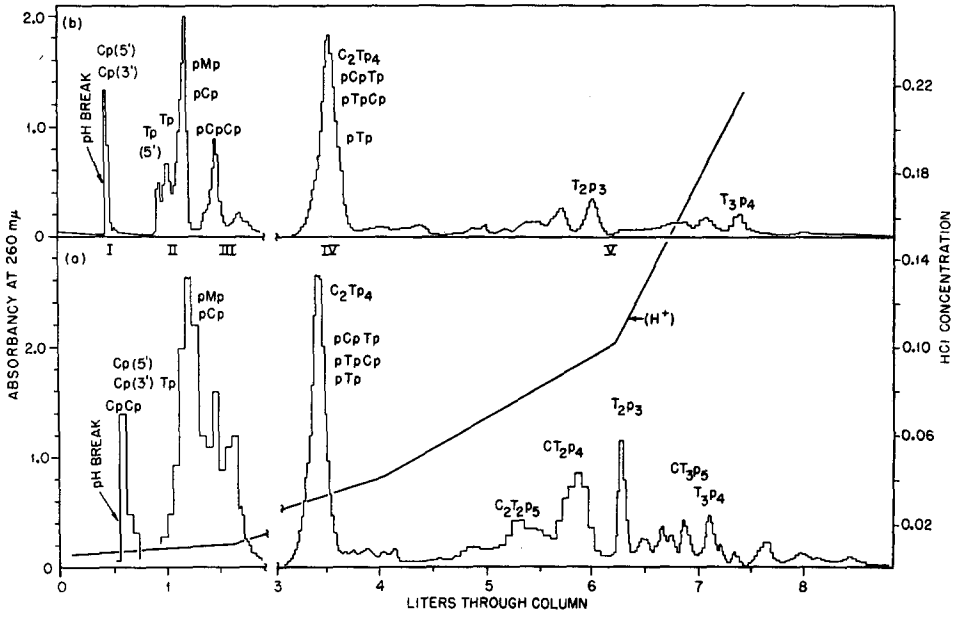

Fig. 3. Separation by linear gradient elution of the nucleotide products formed from 180 mg of calf thymus DNA by *N* HCl at 100° in 1 hour (a) and 4 hours (b). Column: Dowex-1, 200–400 mesh, *ca.* 1 cm^2 × 10 cm; HCl concentrations as shown. Identifications of substances made as described in text.

DISCUSSION

The appearance of mononucleotide diphosphates, dinucleotide triphosphates, and trinucleotide tetraphosphates, members of a Py_np_{n+1} series*, is in keeping with an elimination mechanism of hydrolysis as opposed to a cyclization mechanism, for the reasons set forth in the INTRODUCTION. The appearance of mononucleotide monophosphates (and there is suggestive evidence for higher analogs) may indicate a different mechanism, but it is possible that they arise from the slow hydrolysis of members of the Py_np_{n+1} series. Their origin cannot be defined on present evidence but it is apparent, from Fig. 1, that the sum of inorganic phosphate and monoesterified phosphate remains constant for 1 hour to 4 hours. In other words, monoester

* Abbreviations are given in the footnotes of Table I.

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phosphate disappears at the same rate that inorganic phosphate appears. This is consistent with the hypothesis that CpCp, for example, appears as a hydrolysis product of pCpCp rather than from, say, pTpCpCp, which would produce more

TABLE I

CHARACTERIZATION OF PRODUCTS OF ACID HYDROLYSIS OF DNA

Group[a]	Eluant[b]	Absorbancy ratios[c] (mμ) 280/260	290/260	End-p[d]	p[e]	Substance[f]
Ia, b	0.005 N HCl	2.12	1.53		7.2	Cp(5′)
	0.01 N Formic	2.00	1.30		7.5	Cp(3′)
Ia	0.002 N HCl / 0.1 N Formic	2.0	1.5	50–60	7.2	CpCp
IIa, b	0.005 N HCl	3.3	3.6		2	pMp
IIa, b	0.005 N HCl	2.0	1.5		3.15	pCp
IIa, b	0.005 N HCl	0.65	0.29	100	8.6	Tp
IIb	0.005 N HCl	0.74	0.28		8.5	Tp(5′)
IIa	0.005 N HCl	1.6	1.07			($C_3Tp_?$)
IIIb	0.01 N HCl	2.0	1.5	67	4.5	pCpCp
IIIa, b	0.01 N HCl	1.25	0.75			($CTp_?$)
IIIa	0.01 N HCl	1.10	0.66	40	10	
IVa, b	0.03 N HCl	1.50	1.0	50	6	C_2Tp_4
IVa, b	0.04 N HCl	1.25	0.75	67	7.5	pCpTp
IVa, b	0.04 N HCl	1.25	0.75	67	7.5	pTpCp
IVa, b	0.04 N HCl	0.70	0.25	100	4.1	pTp
IVa, b	0.03 N HCl	1.55	1.0			(C_2Tp_4)?
IVa	0.03 N HCl	1.8	1.25		7.5	
IVa, b	0.03 N HCl	1.38	0.90			
IVa, b	0.03 N HCl	1.17	0.73			
IVa, b	0.04 N HCl	0.75	0.32	67	8	
Va	0.08 N HCl	1.25	0.75	40	6.5	$C_2T_2p_5$[g]
Va, b	0.09 N HCl	1.05	0.57	50		CT_2p_4[h]
Va, b	0.10 N HCl	0.74	0.26	67	5.3	T_2p_3[i]
Va	0.16 N HCl	0.97	0.50	40		CT_3p_5
Va	0.18 N HCl	0.73	0.26	50		T_5p_4
Vb	0.09 N HCl	0.97	0.48			(CT_5)p?
Va	0.12 N HCl	1.25	0.75			(C_nT_n)p?
Vb	0.12 N HCl	0.90	0.43			—[j]
Va	0.15 N HCl	1.05	0.57	40(?)		(CT_2p_4)
Va	0.25 N HCl	0.80	0.31			

[a] a and b refer to 1- and 4-hour hydrolyzates, respectively, in 1 N HCl at 100°.

[b] On rechromatography.

[c] At pH 2 unless otherwise stated. Ratios used (VOLKIN AND COHN[18]) were: C 5′p (2.10, 1.55), C 3′p (2.00, 1.43), M (3.15, 3.4), T (0.72, 0.23), MT (1.40, 1.02), CT (1.27, 0.77), C_2T (1.51, 1.00), C_3T (1.65, 1.14), CT_2 (1.06, 0.56), CT_3 (0.96, 0.47).

[d] Monoester P, from bone phosphatase, as percentage of total P.

[e] Extinction coefficient at 260 mμ, pH 2, based on P analysis.

[f] Formulation based on evidence tabulated. Formulations based on less complete or unsatisfactory results are given in parentheses. M = 5-methyldeoxycytidine, C = deoxycytidine, T = thymidine, p = phosphate, Py = pyrimidine nucleoside residue.

[g] Base analysis in 4-hour analog: C/T = 1/1.

[h] Base analysis in 4-hour analog: C/T = 1/2.

[i] Base analysis in 4-hour analog: T only.

[j] Base analysis in 4-hour analog: C/T = 1/2.

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monoester phosphate but no more inorganic phosphate. If this is the case, then the initial breakdown of the apurinic acid chain may be ascribed almost exclusively to elimination of the deoxyribose residues formerly attached to purines.

Such a concept makes scission of the polynucleotide chain in a given region of the chain secondary to the elimination of the purines in that region rather than simultaneous with it; the latter may have been inferred from physicochemical studies of mild acid hydrolysis (THOMAS AND DOTY[20]). The ability of a cation-exchange resin to form apurinic acid (LALAND[21]) indicates that these two processes can be separated in time with respect to chains of at least the sizes found in apurinic acids. If no elimination or phosphate hydrolysis took place under these conditions, it should be possible to demonstrate single chains of molecular weight equivalent to those existing in the original double helix (WATSON AND CRICK[22,23]). However, a rather large lowering of chain length is indicated by the 15,000 molecular weight of apurinic acids produced by soluble acids (TAMM AND CHARGAFF[24]). A lowering of molecular weight in the absence of phosphate hydrolysis might be evidence for intrachain bonds of a special type, or for their absence (DEKKER AND SCHACHMAN[25]).

It seems reasonable to ascribe the bulk of inorganic phosphate produced in the first hour to the interpurine nucleotide positions. The figure of 33% observed is not unreasonable, for it may be regarded as the sum of the interpurine phosphate augmented by phosphate equal in amount to the Py_nP_n found. BURTON[26] finds a 25% release of phosphate as inorganic phosphate when the DISCHE[27,28] diphenylamine reaction is carried out at 30° rather than at 100°; this is increased to 30–31% by pretreatment of the DNA with dilute trichloracetic acid at 90°. Further support for the allocation of 25% of the total phosphate to interpurine linkages comes from the finding that 25% of the phosphate remains organically bound after bone phosphatase treatment of the acid digests. This phosphate lies between pyrimidine nucleoside residues and should equal in amount the interpurine phosphate. The secondary hydrolysis of Py_nP_{n+1} fragments must be measured by direct assay, or else be eliminated by shorter hydrolysis periods, before the contribution of mechanisms of hydrolysis other than simple elimination can properly be weighed.

If the interpurine and interpyrimidine phosphates are equivalent and together comprise 50% of the total phosphate of the original DNA, then the remaining 50% must lie between purine and pyrimidine residues and appear, in the acid digest, equally distributed between the 3′ and 5′ positions of the end deoxyribose moieties. The increment in the total inorganic phosphate produced by continued hydrolysis, which reaches a plateau at 50% in 4 hours, may be caused by the preferential hydrolysis of those phosphates located in 3′ positions, leaving the more acid-stable 5′ phosphates still attached.

It is quite clear, in conformance with previous conclusions (BROWN AND TODD[5,6]; KENT *et al.*[29]; JONES *et al.*[8,9]; BURTON[26]; SINSHEIMER[30]), that every variety of pyrimidine sequence must exist within the original DNA molecule (*i.e.*, C, T, CC, TT, CCC, TTT, and the sequence isomers of such varieties as CT, CTT, CCT). In view of the evidence for base pairing in DNA (WATSON AND CRICK[22,23]), the existence of analogous sequences involving adenine and guanine must also be presumed. It may be that different DNA's will show different "profiles" in a chromatographic analysis like that in Fig. 3 and thus display chemical differences based on a statistical distribution of sequence types. It is equally possible that molecular heterogeneity within isolated

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samples will give rise to the same, or similar, distributions regardless of source, thus obscuring any differences between intramolecular patterns.

SUMMARY

The acid hydrolysis of calf thymus DNA by normal HCl at 100° results primarily in nucleotides containing one more phosphate than pyrimidine, a number of which have been isolated and identified by ion-exchange chromatography. This is consistent with an elimination mechanism that leaves on the pyrimidine residue those phosphates formerly lying between purine and pyrimidine residues. It also indicates that the loss of purine residues is a necessary prerequisite to the rupture of the polynucleotide chain by mild acidic hydrolysis.

The presence of nucleotides containing equal amounts of pyrimidine and phosphate may be caused by the hydrolysis of phosphate from the initial products. About 33% of the phosphate appears as inorganic phosphate after 1 hour of hydrolysis; the bulk of this appears to be phosphate originally lying between purine residues in the original DNA. There is a slow rise to a new plateau of 50% at 3 to 4 hours. The sum of inorganic plus monoesterified phosphate remains constant from 1 hour to 4 hours, indicating a slow hydrolysis of monoester phosphate, presumably from the 3′ positions.

The presence of all varieties of mono-, di-, and trinucleotide sequences that are possible from cytidylic and thymidylic acids indicates that all possible sequences of pyrimidine and of purine nucleotides exist in thymus DNA.

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Received November 15th, 1956