Distribution of Pyrimidine Bases in Herring-Roe Deoxyribonucleic Acid

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Although considerable evidence is now available for the 3':5'-polynucleotidic structure of deoxyribonucleic acid (DNA), little is known of the sequence of the purine and pyrimidine groups. Partial degradation of DNA, achieved with deoxyribonuclease, results in a variety of products, including di- and tri-nucleotides (Smith & Markham, 1952*a, b*; Sinsheimer, 1954), some of which bear both purine and pyrimidine bases. The validity of such products as representing unaltered sequential fragments of the polynucleotide chain is, however, ambiguous, owing to the possibility of enzymic resynthesis or rearrangement. It is desirable therefore that chemical methods should be developed in order to study the problem.

It has been shown that in dilute aqueous acids purines alone are liberated from deoxyribonucleic acids, yielding strongly reducing products, which are still of large size and which have been called 'apurinic acids' (Tamm, Hodes & Chargaff, 1952). The sole nitrogenous constituents of these products are reported to be pyrimidines (cf. Lucy & Kent, 1953) present in the same ratio as in the parent DNA. Apurinic acids have, in general, much smaller molecular weights than the parent DNA, probably because of the hydrolysis of some of the internucleotidic linkages.

Unlike DNA, apurinic acids are degraded in mild alkaline solutions (Tamm, Shapiro, Lipshitz & Chargaff, 1953). The nature of the degradation is, however, uncertain, since several reactions may be involved. In addition to the cleavage of ester phosphate bonds of the purine-free sugar residues, random breakdown of the sugar itself cannot be discounted. The latter ambiguity may be circumvented and the purine-linked sugars 'marked' by making use of the earlier observation (Kent, 1950) that treatment of deoxyribonucleic acid with phenylmethanethiol in acidic conditions resulted in liberation of purines and the 'blocking', as mercaptals, of the reducing-sugar groups thus formed. The resulting sulphur-containing residue resembled the apurinic acids in being alkali-labile. In this case, however, D-2-deoxyribose dibenzylmercaptal was isolated as a crystalline derivative. A modification of the method has been successfully used (MacDonald & Knight, 1953) to identify the purine-

linked sugar in the ribonucleic acid of tobacco mosaic virus.

In the present work, herring-rope deoxyribonucleic acid was treated with ethanethiol in the presence of hydrochloric acid (Kent & Lucy, 1954) and the non-diffusible derivative, mercaptalated deoxyribonucleic acid (MDNA), degraded with alkali to a mixture of stable pyrimidine polynucleotides (PPN).

Our results, of which preliminary reports have already been published (Lucy & Kent, 1953; Kent & Lucy, 1954), have been criticized by Jones & Letham (1954) on the ground of their inability to prepare MDNA from DNA. Although the yields of MDNA (on a weight basis) are in general only about 10% of the initial DNA, we believe that the method furnishes a suitable procedure for the examination of sequences of adjacent pyrimidine nucleotides in DNA. Available evidence indicates that only purines are liberated in the mercaptalation reaction and that pyrimidine polynucleotides are stable under the conditions of mercaptalation. Jones & Letham (1954) claim to have obtained higher yields of mercaptalated derivative by the action of thioglycolic acid on DNA in the presence of anhydrous zinc chloride. Under these conditions, however, it is uncertain whether the mercapto acid behaves solely as a thiol or whether it forms esters with the nucleic acid or its derivatives.

METHODS AND MATERIALS

Elementary analyses for P, N and S were performed by Drs Strauss and Weiler, Oxford. Freeze-dried specimens of DNA, MDNA and PPN were analysed and the results corrected for the moisture content, which was determined separately (Lee & Peacocke, 1951). Phosphorus was also determined by the method of Allen (1940).

Ultraviolet-absorption measurements were made on aqueous solutions with Beckman and Hilger Uvispek spectrophotometers, with 1 cm. quartz cells.

Purine and pyrimidine analyses were performed by the hydrolytic and the paper-chromatographic procedures described by Wyatt (1951).

Dische extinction coefficients were determined by the method of Allerton, Overend & Stacey (1952).

Paper-electrophoretic separations were carried out on Whatman no. 1 paper in 0.2M sodium acetate buffer (containing 0.05M sodium chloride) (pH 5) or in 0.2M sodium

borate buffer (pH 8), (Pasternak & Kent, 1952), with potential gradient 12v/cm.

Commercial deoxyribonucleic acid (from herring roe) was supplied by L. Light and Co. Ltd., Colnbrook, Bucks.

Fibrous herring-roo deoxyribonucleic acid was prepared by the method of Daly, Allfrey & Mirsky (1950).

Paper partition chromatography. Chromatograms were prepared on Whatman no. 1 paper by downward development in the following solvent systems: (a) *n*-butanol-ethanol-water (4:1:5 by vol.); (b) phenol saturated with water; (c) isopropanol (138 ml.)-HCl (sp.gr. 1.18, 31.7 ml.)-water (30.5 ml.); (d) water-poor phase of ethyl acetate (100 ml.)-pyridine (45 ml.)-water (10 ml.). Spraying reagents used were aniline hydrogen phthalate (Partridge, 1949) for reducing sugars, perchloric acid and ammonium molybdate (Hanes & Isherwood, 1949) for phosphate esters, and I_2 vapour (Greenway, Kent & Whitehouse, 1953) for mercaptals. Ultraviolet photography was used to detect purines, pyrimidines and their derivatives (Markham & Smith, 1949).

EXPERIMENTAL

Mercaptalation of DNA. (i) Ethanethiol (15 ml.) was added to fibrous DNA (0.49 g.) in a glass-stoppered flask. Hydrochloric acid (sp.gr. 1.18, 0.5 ml.) was then added and the mixture was shaken for 14 hr. at 18°. The reaction mixture was diluted with water (45 ml.) and the unchanged thiol removed by extraction with ether. The aqueous layer was then brought to pH 7 by addition of $NaHCO_3$ and was dialysed in cellophane against distilled water until chloride-free. During dialysis a white solid separated within the sac and was found to be chiefly a mixture of crystalline adenine and guanine. Freeze-drying of the contents of the sac furnished 45 mg. of a white amorphous solid, MDNA (1), containing: N, 9.90; P, 9.62; S, 7.8%.

(ii) MDNA (2) was prepared from commercial herring-roo DNA (6 g.). Ethanethiol (45 ml.) and HCl (sp.gr. 1.18, 12 ml.) were employed and the product was isolated as in the preparation of MDNA (1). The product contained: N, 8.43; P, 7.46; S, 5.9%.

Attempted mercaptalation of apurinic acid. Apurinic acid (0.7 g.) prepared from herring-roo DNA by the method of Tamm *et al.* (1952) was suspended in ethanethiol (30 ml.)

saturated with dry HCl. After 1.5 hr. at 0, when solid had dissolved, ether (25 ml.) was added and sufficient dilute $NaHCO_3$ solution to bring the pH to 7. The aqueous layer was re-extracted with ether, then dialysed at 0° in cellophane for 94 hr. against distilled water. Freeze-drying of the dialysed solution yielded a partially mercaptalated derivative (47 mg. Found: N, 4.59; P, 8.01; S, 4.8%).

Diffusible products arising during mercaptalation. In the course of preparation of MDNA (1), the material was dialysed in cellophane against distilled water (3.72 l. for 92 hr.). The external solution was concentrated under reduced pressure below 30° to 10 ml. The resulting concentrate was examined by paper chromatography. The results are summarized in Table 1.

Properties of MDNA (1). Paper-chromatographic examination of this material in butanol-ethanol-water revealed the presence of a major constituent (R_F 0.0-1) and two minor substances (R_F 0.26 and 0.32). The substance (R_F 0.26) contained P and S, and when eluted in 0.1N-HCl was found to have λ_{max} 275 m μ . The second minor constituent (R_F 0.32) also contained P and had an absorption maximum at λ_{max} 275 m μ . but was sulphur-free.

Fig. 1. Alkaline degradation of mercaptalated deoxyribonucleic acid (MDNA). ---, Deoxyribonucleic acid; —, MDNA (2).

Table 1. Diffusible products formed during preparation of MDNA (1)

Solvent	Detection of components			Authentic compounds
	Ultraviolet absorption	Iodine vapour	Perchloric acid-molybdate	
isoPropanol (138 ml.)-hydrochloric acid (sp.gr. 1.18, 31.7 ml.)-water (30.5 ml.)	0.33	—	—	0.31 Guanine
	0.49	—	—	0.43 Adenine
	0.71	—	—	0.90 Deoxycytidine-5' phosphate
	0.88	—	0.90	—
	1.0	—	0.97	0.97 Thymidine-3':5' diphosphate
<i>n</i> -Butanol-acetic acid-water (4:1:5 v/v)	0.0-11	0.09-0.11	—	—
	0.27	0.19†	—	—
	0.36	—	—	—
	0.45	—	—	—
	0.61	0.88*	—	0.9 Deoxyribose diethylmercaptal (?)

* ++ Dische test, P-free.

† ++ Dische test, contained P.

Alkaline degradation of mercaptalated deoxyribonucleic acid (MDNA). (i) In a typical experiment, the rate of degradation was followed spectroscopically. MDNA (2) (0.9 mg.) was dissolved in 0.5N-NaOH (20 ml.) and solution maintained at 33.5°. The change in light absorption at 268 m μ . was measured at intervals (Fig. 1). A similar quantity of fibrous DNA was treated simultaneously under identical conditions.

(ii) Mercaptalated nucleic acid, MDNA (2) (92 mg.), was treated with 0.01N-NaOH (6 ml.) for 237 hr. at 18°, and dialysed twice in cellophan against distilled water (90 ml., each time for 48 hr.). The solution (19.3 ml.), adjusted to neutrality, when freeze-dried yielded a white amorphous product (PPN). (Found: N, 6.91; P, 8.31; S, 3.3%.)

Diffusible products formed during the alkaline degradation of MDNA. Examination of the external solutions from the dialysis of PPN in (ii) above failed to reveal any inorganic phosphate. The two solutions contained phosphoric esters (23.5 and 7 μ g. P/ml. respectively) corresponding to 48% of the combined P of the MDNA. A portion of the first external solution (25 ml.), neutralized by being shaken with the acidic form of Amberlite IR 100 (H) (Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.), was concentrated to 1 ml. and examined by paper chromatography in ethyl acetate-pyridine-water. Two components (R_F 0.03 and 0.13) were detectable in ultraviolet light, on treatment with iodine vapour and by spraying with perchloric acid and ammonium

(Mozingo, 1941) for 5 hr. After removal of the catalyst the solution was concentrated at 40° under reduced pressure. A solid (60 mg.) was obtained which contained S, 1.63%. The initial MDNA contained S, 5.9%. The product was methylated under the conditions described by Anderson, Barker, Gulland & Lock (1952), absolute methanol (15 ml.), methyl iodide (2 ml.) and dry Ag₂O (2 g.) being used. The product was found to have an apparent methoxyl content (Zeisel) of 13.7%. The methylated product was treated with 0.1N-NaOH (3 ml.) at 100° for 4 hr. After neutralization with 0.1N-HCl, the solution was concentrated to dryness. The product had OMe 6.35% (corrected for the presence of NaCl) and was free from traces of organically bound sulphur.

RESULTS

The mercaptalated nucleic acid (MDNA) was an amorphous solid, highly soluble in water, and it gave a negative Fehling's reaction. The ratio of contained thymine to cytosine was little different from that of the parent nucleic acid. The analytical composition of DNA and MDNA (1) is shown in Table 2.

In a model experiment each of the four bases was treated with ethanethiol and hydrochloric acid under the same conditions as those prevailing in

Table 2. *Composition of fibrous-herring-roe DNA, MDNA (1), MDNA (2) and PPN*

Bases* (Moles/4 atoms of P)	DNA	MDNA (1)	MDNA (2)	PPN
Adenine	1.00	0.03	0.0	0.0
Guanine	0.77	0.11	0.16	0.0
Cytosine	0.70	1.44	1.93	1.19
Thymine	1.01	2.02	2.73	1.90
Thymine/cytosine (mol.prop.)	1.45	1.40	1.45	1.60
Nitrogen (atoms/4 atoms of P)	12.92	9.10	10.0	7.36
Sulphur (atoms/4 atoms of P)	0.0	3.17	3.07	1.56
Dische value (per atom of P)	1850	940	1140	990
(ϵ_p) (λ_{max} . 259 m μ .)	6476	—	—	—
(ϵ_p) (λ_{max} . 270 m μ .)	—	4555	4630	5032

* 5-Methylcytosine was not determined in these preparations.

molybdate (Bandurski & Axelrod, 1951). The ultraviolet-absorbing character of these components was shown, by hydrolysis in 70% (w/w) HClO₄ and paper chromatography, to be due to cytosine and thymine residues. A further component, R_F 0.31, which contained no N or P but which on elution gave a positive Dische reaction and positive tests for S, was present.

Properties of PPN. The substance was chromatographically immobile in butanol-ethanol-water. When examined by paper electrophoresis at pH 7.93 (0.2M sodium borate buffer containing 0.05M-NaCl) and at pH 5 (0.2M sodium acetate buffer containing 0.05M-NaCl), under a potential gradient of 12v/cm., the substance moved as a single component.

Retreatment of isolated PPN with ethanethiol and HCl under the conditions of preparation of MDNA was without action on PPN.

Attempted desulphuration and methylation of MDNA. MDNA (117 mg.), dissolved in 50% (v/v) aqueous methanol (30 ml.), was warmed at 60° with 1 g. of Raney nickel

the preparation of MDNA. The bases were recovered, as indicated by R_F values and by ultraviolet-absorption characteristics.

Towards the Dische diphenylamine reagent (Deriaz, Overend, Stacey, Teece & Wiggins, 1949), MDNA (1) gave less coloration than an equivalent amount of the parent DNA, the respective values being 940 and 1850 (Spekker units/atom P) (Allerton *et al.* 1952). This change is consistent with the replacement of the readily hydrolysed purine groups by $(-S.C_2H_5)_2$ in MDNA. This is further emphasized by the changes in spectral properties. Whereas fibrous DNA has an extinction of 6476 (ϵ_p extinction/atom P) at λ_{max} . 259 m μ ., MDNA (1) gives a value of 4555 (ϵ_p) at λ_{max} . 270 m μ .

In a preliminary examination in the Svedberg ultracentrifuge it was found that the sedimentation

constant of MDNA (1) is considerably less than that of the parent DNA. Paper electrophoresis of MDNA (1) suggested the presence of at least two ultraviolet-absorbing components.

MDNA (2) was readily degraded in alkaline conditions and the degradation was accompanied by a marked increase in optical density at 268 m μ . and liberation of at least two diffusible products having the properties of deoxycytidine phosphates. Inorganic phosphate was detected in only trace amounts and no substance having reducing-sugar properties was liberated. Model experiments with D-arabinose diethyl mercaptal showed that such derivatives were stable under the alkaline conditions employed in these investigations. Under the conditions used by Brown, Magrath & Todd (1952) for the alkaline degradation of ribonucleotides, it was found that the ϵ_p values of MDNA increased more rapidly than those of a specimen of DNA similarly treated (Fig. 1). In this reaction 48 % of the phosphorus was liberated as ester phosphate, the non-diffusible product (PPN) having the composition shown in Table 2. Both in chromatographic and paper-electrophoretic studies, PPN behaved as a single component.

DISCUSSION

The experimental results indicate that mercaptanalysis of deoxyribonucleic acid is achieved through the purine-linked sugars and that the reaction takes the course shown in Fig. 2.

nucleic acid 2 atoms of sulphur enter. The analytical composition (Table 2) shows that in mercaptalation 1.62 moles of adenine+guanine (per 4 atoms of P) have been removed and that 3.17 atoms of sulphur are present in the product, i.e. 1.58 sugar residues have been converted into diethylmercaptals. The ratio is therefore 1:2:1. No evidence has yet been found of interaction in any other way between deoxyribonucleic acid components and ethanethiol.

Mercaptalation, under the conditions described, undoubtedly results in products of considerably decreased molecular weight. The reaction is accompanied by liberation of purines, some diffusible nucleotides and deoxyribose derivatives. These appear to include thymidine-3':5' diphosphate and deoxycytidine-5' phosphate, and will be described in a later communication. Pyrimidines and inorganic phosphate are, however, released in not more than trace amounts.

The effect of mercaptalation is to convert every formerly purine-linked sugar into stable acyclic derivatives. Every such sugar residue is endowed with an additional hydroxyl group at C₍₄₎, i.e. that which was formerly held in the ring structure. This hydroxyl group is in the α position to the phosphate esters at C₍₅₎ and C₍₃₎, and a situation similar to that in ribose nucleic acids exists. It has been pointed out (Brown & Todd, 1952) that such α -hydroxy secondary phosphate esters are alkali-labile through the agency of an intermediate cyclic phosphate-ester form, cleavage of which leads to

Fig. 2. Mercaptalation of purine deoxyribonucleotidic residues.

This is supported by the following arguments: (a) The diminished Dische coloration shown by MDNA compared with DNA. (b) DNA has ϵ_p 6476 at λ_{max} 259 m μ ., whereas the derived MDNA has ϵ_p 4555 at λ_{max} 270 m μ . [cf. ϵ_p 4578 at λ_{max} 270 m μ . for herring-roe apurinic acid and ϵ_p 4600 at λ_{max} 268 m μ . for calf-thymus apurinic acid (Tamm *et al.* 1952)]. (c) The reaction (Fig. 2) requires that for each purine displaced from the

two isomeric primary phosphates. In the case of MDNA, cyclic phosphates may be formed between the 4':5'- and 3':4'-positions, yielding on subsequent hydrolysis the 3':4'- or 5'-phosphate esters.

The experimental results show that MDNA is indeed easily degraded by dilute alkali with elimination of 48 % of its phosphorus as esters, but without the liberation of inorganic phosphate. The non-diffusible residue (PPN) shows properties

consistent with the loss during hydrolysis of pyrimidine-free constituents by (a) increase in ϵ_p from 4630 to 5032 without shift in $\lambda_{\max}$, 270 m μ ., (b) decrease in sulphur content of the product PPN, and (c) increase in the N/P ratio.

No free pyrimidines are formed in the reaction. Examination of the diffusible products by chromatography (after removal of the interfering inorganic substances by means of an ion-exchange resin) gave evidence of the presence of both thymine and cytidine nucleotides.

The presence of sugar hydroxyl groups in MDNA is further demonstrated by the fact that, after partial reductive desulphuration with Raney nickel (Mozingo, 1941), the material can be methylated under the same conditions as ribose nucleic acid and its derivatives. The apparent methoxyl content (13.7%) is unlikely to be an accurate value, however, owing to the presence of residual thioethyl groups which interfere in the Zeisel determination. It is of interest, however, that this value is decreased to 6.35% by alkaline hydrolysis, a means by which fortuitous methyl phosphoric esters are hydrolysed and the remaining sulphur is expelled. The resulting methylated derivative, unlike MDNA, is stable and does not undergo the 'elimination' reaction in alkaline conditions to form analogues of PPN.

It will be noticed that alkaline degradation of MDNA does not result in complete expulsion of the mercaptalated deoxyribose residues. The retention of sulphur (1.56 atoms/4 atoms of P) is, however, not inconsistent with the 'elimination' mechanism of alkaline breakdown of nucleotides, since products may result, e.g. 4'-phosphate, in which migration has resulted in the blocking of the hydroxyl group requisite for cyclic phosphate-ester formation. Such a product should be stable to alkaline reagents, the retained mercaptalated residue constituting an end-group. Until this has been further investigated, ambiguity persists in the interpretation of the results. It can, however, be concluded that herring-roe deoxyribonucleic acid is not entirely composed of alternating purine and pyrimidine nucleotides and that regions of adjacent pyrimidine nucleotides occur.

In the minimal arrangement, PPN can be regarded as a trinucleotide bearing a mercaptalated-sugar end-unit. In such a substance two deoxycytosine nucleotides (C) are adjacent to one thymidine nucleotide (T) in the form TCT or CCT. Throughout this investigation, bases in DNA have been expressed for convenience in terms of 4 atoms of phosphorus, though neither in this nor in the majority of other reported results are 4 bases present for 4 atoms of phosphorus. In PPN, however, the correspondence is close, 3.87 moles of 1'-substituents (thymine + cytosine + $\frac{1}{2}$ atomic sul-

phur content) being present for every 4 atoms of phosphorus.

The results indicate that mercaptalation provides a route for the separation of consecutive pyrimidine nucleotides from DNA without possible ambiguities of enzymic rearrangements. These pyrimidine polynucleotides will be discussed further in a subsequent publication.

SUMMARY

1. Herring-roe deoxyribonucleic acid has been partly degraded to pyrimidine polynucleotides by non-enzymic methods.

2. The purines were selectively removed and the liberated reducing sugars were blocked in the acyclic form as ethylmercaptals. The thymine/cytosine ratio of the product differed little from that of the original DNA.

3. The mercaptalated product was hydrolysed by dilute alkali to yield diffusible organo-phosphorus compounds, and a non-diffusible purine-free substance containing thymine and cytosine.

4. The degradation is consistent with the 'elimination' mechanism of nucleotide breakdown.

5. The results suggest that there are regions in DNA where at least three pyrimidine nucleotides are linked together.

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The N-Terminal and Sulphur-containing Residues of Bacitracin A

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The results of electrometric titrations and of reactions with 1-fluoro-2:4-dinitrobenzene (FDNB) led Newton & Abraham (1953a, b) to conclude that bacitracin A contained a free δ -amino group which was part of an ornithine residue and a free α -amino group which was part of a leucine or isoleucine residue. Other workers failed to demonstrate the presence of an *N*-terminal amino acid in the molecule (Ingram, 1953; Porath, 1953). Lockhart, Newton & Abraham (1954) confirmed the results of earlier experiments and reported that the dinitrophenyl (DNP) derivative of bacitracin A yielded DNP-isoleucine on hydrolysis. However, they stated that the yield of DNP-isoleucine was much less than that to be expected from one *N*-terminal residue per molecule. Some uncertainty remained, therefore, about the nature of the structure carrying the postulated free α -amino group.

Analysis of the amino acids formed on hydrolysis with acid first suggested that bacitracin A contained only two residues of isoleucine (Barry, Gregory & Craig, 1948), but studies of the amino acid sequence showed that isoleucine residues occurred at three positions in the peptide chain (Craig, Hausmann & Weisiger, 1954; Lockhart & Abraham, 1954a; Hausmann, Weisiger & Craig, 1955a; Weisiger, Hausmann & Craig, 1955). One of the isoleucine residues appeared partly as *allo*-isoleucine in acid hydrolysates (Piez, 1954). In the light of earlier experiments, the results of partial hydrolysis could best be accommodated by assuming that bacitracin A contained an *N*-terminal isoleucine residue that was linked through its carboxyl group to a potential cysteine residue. Nevertheless, the complete picture appeared to be more complicated. Both Lockhart & Abraham

(1954a) and Hausmann *et al.* (1955a) suggested that the amino group of this isoleucine was engaged in cross-linkage with a phenylalanine residue, or interacted with the latter during the hydrolytic breakdown of the molecule. This suggestion was made because several peptides containing the sequence Phe.Ileu appeared to be present in partial hydrolysates. The homogeneity of some, at least, of the peptides obtained by Lockhart & Abraham (1954a) was open to question, but the bis-DNP derivative of a peptide Phe.Ileu.CySH.Leu, obtained by Hausmann *et al.* (1955a), appears to have been well characterized.

The sulphur atom in bacitracin A is present as a masked thiol group which appears as part of a cysteine residue on hydrolysis. Newton & Abraham (1953b) suggested that bacitracin A contained a thiazoline ring:

This suggestion was made to account for the ready liberation of a thiol group in hot acid solution and for the behaviour of bacitracin A on hydrogenolysis. After treatment with Raney nickel an alanine residue was present whose α -amino group was free, and two new substances were detected among the products of hydrolysis. One of these substances was thought to be an amino alcohol, because hydrolysates in which it was present yielded ammonia and a volatile aldehyde on treatment with periodate. The changes occurring on hydrogenolysis could be interpreted in terms of the removal of the sulphur atom, followed by the hydrolysis and reduction of a resulting $-\text{CH}=\text{N}-$ grouping.

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