A Method for the Determination of Nucleotide Sequence in Polyribonucleotides

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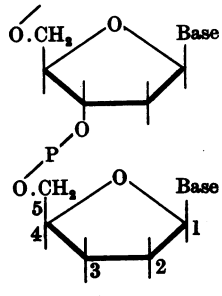
Our knowledge of the structure of ribonucleic acids (RNA) has advanced considerably in recent years. In the first place, the refinement of chromatographic techniques has made possible an accurate estimation of the purine and pyrimidine composition (Vischer & Chargaff, 1948; Markham & Smith, 1949; Carter, 1950; Markham & Smith, 1951). Secondly, with the aid of both chemical and enzymic methods it has been shown that the constituent nucleosides in RNA are linked together as in I,



I

through phosphodiester bridges betwen the '*b*' position and $C_{(5)}$ of adjacent ribose groups (Cohn & Volkin, 1951; Brown & Todd, 1953; Heppel, Markham & Hilmoe, 1953; Whitfeld & Markham, 1953). Conclusive evidence that $C_{(3')}$, and not $C_{(2')}$, is involved in the link has only recently been obtained by Khym, Doherty, Volkin & Cohn (1953) and by Brown, Fasman, Magrath, Todd, Cochran & Woolfson (1953), who, independently, have identified the *b* isomer of adenylic acid as adenosine 3′-phosphate. Thirdly, it has been possible to identify those nucleotides occupying the terminal positions in small polyribonucleotide chains (Cohn, Doherty & Volkin, 1952). Except in the case of trinucleotides this procedure, however, does not indicate the sequence of non-terminal nucleotides and, as no other method has provided this information, the advance of our knowledge in this respect has been limited.

Recently, Whitfeld & Markham (1953) indicated that the method, by which they determined the nature of the purine internucleotide link in di-nucleotides from RNA, might well be applied to the determination of the nucleotide sequence in polyribonucleotides. This work has now been extended and the stepwise degradation of many di- and trinucleotides carried out successfully. The method is applicable in principle to larger polyribonucleotides.

The method consists of the dephosphorylation of the terminal nucleotide of a polyribonucleotide with phosphomonoesterase and then oxidation by periodate of the exposed adjacent hydroxyl groups. Subsequent incubation at pH 10 splits off the oxidized nucleoside residue to leave a polyribonucleotide containing one less nucleotide than the original compound. By a repetition of the process, nucleotides can be removed, singly until only one remains.

While this work was being carried out, Brown, Fried & Todd (1953) suggested the possibility of a degradation procedure, such as the above, for the determination of nucleotide sequence. The authors based their assumption upon the observed alkalilability of the phosphate group of the periodate-oxidation product from adenosine 5′-benzylphosphate.

MATERIALS AND METHODS

Ribonucleic acids. RNA, prepared from turnip yellow mosaic virus (Markham & Smith, 1952*b*), and reprecipitated commercial yeast RNA were used as sources of di- and trinucleotides.

Chromatographic solvents

Solvent 1. *iso*Propanol–water (70 : 30, v/v), with NH_3 in the vapour phase (Markham & Smith, 1952*a*). Solvent 2. Saturated $(NH_4)_2SO_4$–M sodium acetate–*iso*propanol (80:18:2, v/v/v) (Markham & Smith, 1952*a*). Solvent 3. *iso*Propanol, 170 ml.; 10 N-HCl, 44 ml.; water to 250 ml. (Wyatt, 1951).

The positions of the spots on the chromatograms were detected by ultraviolet photography (Markham & Smith, 1949).

Hydrolysis methods

(1) *Alkaline hydrolysis.* N-NaOH at 20° for 18 hr. (Markham & Smith, 1951) breaks phosphodiester internucleotide links of RNA proximal to $C_{(5')}$. The products of hydrolysis may be identified, after neutralization of excess alkali, by chromatography in solvents 1, 2 and 3.

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(2) HCl *hydrolysis.* N-HCl at 100° for 1 hr. (Smith & Markham, 1950) releases pyrimidine nucleotides and purine bases from RNA. Chromatography of the hydrolysate in solvent 3 permits identification of the products.

(3) $HClO_4$ *hydrolysis.* 72% $HClO_4$ at 100° for 2 hr. (Marshak & Vogel, 1951) yields purine and pyrimidine bases from RNA. The hydrolysate is diluted with an equal volume of water before being chromatographed in solvent 3.

Preparation of polyribonucleotides

The dinucleoside monophosphate, cytidylyl-cytidine, was obtained by enzymic synthesis from cytidine 2′:3′-phosphate and cytidine (Heppel, Whitfeld & Markham, 1954).

All other polyribonucleotides used in these experiments were prepared by one or other of the following methods.

Paper chromatography and paper electrophoresis. In the initial experiments, turnip yellow mosaic virus RNA (0·2 g. in 10 ml. water) was digested to completion (pH 7·0, 24 hr., 55°) with 1·7 mg. pancreatic ribonuclease and then dialysed against three changes of 100 ml. of distilled water. The combined dialysate was concentrated *in vacuo* and the method of Markham & Smith (1952*b*) was followed to separate out ribonuclease-resistant dinucleotides. This involved chromatography in solvent 1 and elution of the dinucleotide-containing bands, which were then submitted to electrophoresis at pH 3·5 on filter-paper strips. By this method adenylyl-cytidylic acid, adenylyl-uridylic acid, guanylyl-cytidylic acid and guanylyl-uridylic acid were isolated in small quantities (2–4 mg.).

Ion-exchange chromatography. In later experiments the ion-exchange chromatographic method of Cohn *et al.* (1952) was followed for the isolation of trinucleotides and larger amounts of dinucleotides. Commercial RNA (1·0 g.), in 10 ml. of water, was digested to completion (pH 7·0, 40 hr., 55°) with 4·3 mg. ribonuclease and the solution adjusted to pH 2 with 0·5 N-HCl. The insoluble material was removed and the supernatant liquid was adjusted to pH 9 with N-NH_3 before being adsorbed on to a column of Dowex-1 resin (2% cross-linked, 200–400 mesh, Cl^- form, 18 cm. × 4·9 sq.cm.). HCl–NaCl mixtures were used as eluting solvents and 16 ml. fractions were collected at a flow rate of 80 ml./hr. The optical density of every fourth fraction was read at two wavelengths (260 and 280 mμ.); in the vicinity of a peak the optical density of each fraction was read. All the fractions constituting a peak were combined and concentrated. This was done by diluting the combined eluate to reduce the Cl^- concentration to less than 0·01 M, adjusting to pH 9, readsorbing on to a small Dowex-1 column (1·5 cm. × 4·1 sq.cm.) and eluting with a minimal amount of 0·05 N-HCl. This eluate was then deacidified by extraction with a 5% (v/v) solution of tri-*n*-octylamine in $CHCl_3$ (Hughes & Williamson, 1951) and finally concentrated *in vacuo*. Each product was tested for homogeneity by paper chromatography in solvent 1 and by paper electrophoresis, and the ratio of the bases present was determined by hydrolysis with N-HCl followed by chromatography.

The elution pattern obtained was similar to that published by Cohn *et al.* (1952). However, one of the apparently homogeneous trinucleotide peaks was shown, by chromatography in solvent 1, to have two components. The major component (75%) was the trinucleotide guanylyl-adenylyl-cytidylic acid and the minor component (25%) was the dinucleotide adenylyl-guanylic acid. The presence of such a relatively large quantity of this particular dinucleotide in a ribonuclease digest of RNA is an indication of the degraded condition of the commercial RNA.

RESULTS

The determination of the nucleotide sequence in a polyribonucleotide chain is dependent upon (*a*) the identification and (*b*) the removal, one at a time, of the terminal nucleotide residues; the former may be carried out either before or after the latter. The simplest method for the identification of the terminal residue is to isolate it, after it has been split off, and then to identify its base, and this is the method described below. Another method, which is almost as satisfactory, is to hydrolyse the dephosphorylated polyribonucleotide with alkali and identify the nucleoside which is liberated and which is derived from the terminal group containing a free α-glycol system.

As an example of the procedure, its application to the degradation of a dinucleotide and a trinucleotide will be described in detail. For the sake of convenience, the method is divided into three steps which together remove one nucleotide residue from the polyribonucleotide. In Table 1 are listed those polyribonucleotides which have been subjected to the stepwise degradation procedure. Except in the case of cytidylyl-cytidine, in which step 1 was not necessary, as there is no terminal phosphate in a dinucleoside monophosphate, all the polynucleotides mentioned were treated according to one or the other of the procedures described below.

Degradation of a dinucleotide

Step 1. A dinucleotide of cytidylic acid and adenylic acid was incubated for 90 min. at 37° with 0·2 ml. of a prostate phosphomonoesterase preparation (Markham & Smith, 1952*b*), a quantity found by experiment to be more than adequate for the purpose. This treatment removed the free phosphoryl group but was too short to permit hydrolysis of the internucleotide link by the slight contaminating phosphodiesterase activity. The dephosphorylated product was isolated by chromato-

Table 1. *Polyribonucleotides which have been degraded by the procedure described in this paper*

A = adenylic acid, C = cytidylic acid, G = guanylic acid, U = uridylic acid; the nucleotide unit written on the right has a phosphomonoester group on $C_{(3')}$.

Dinucleoside monophosphate	Dinucleotides	Trinucleotides
C-cytidine	AC	AAC
	AU	AGC
	GC	GAC
	GU	
	AG	

graphy in solvent 1; removal of the terminal phosphate approximately doubles the R_F value in this solvent (Fig. 1*a*, cols. 2 and 3). Alkaline hydrolysis of a sample of the eluted product showed the presence of adenylic acid and cytidine in the molar ratio 1:1·07.

Step 2. This dinucleoside monophosphate was oxidized with an excess of 0·1 M-$NaIO_4$ (30 min., 20°) and rechromatographed in solvent 1. In this solvent the oxidation product had an R_F value slightly less than that of adenylic acid (Fig. 1*a*, col. 4). As both iodate and periodate absorb ultraviolet light, they also are detected when the chromatogram is examined. Iodate tends to trail but the major portion moves in a position just ahead of adenylic acid, whereas the excess periodate does not move from the origin in this solvent (Fig. 1*a*, col. 4). The removal of excess periodate by chromatography in solvent 1 is not a satisfactory method when the oxidation product has a very small R_F value. In such cases the same result can be achieved by the addition of glucose, ethylene glycol or glycerol to the reaction mixture. Also, if the polyribonucleotide in question were sufficiently large, it would be possible to dialyse away the excess periodate.

Step 3. The oxidation product was eluted and the eluate made 0·05 M with respect to glycine buffer (pH 10). If necessary, the final adjustment to pH 10 was effected with N-NH_3. A drop of $CHCl_3$ was added to prevent bacterial growth, and the mixture kept at 37° for 18 hr.; under these conditions normal internucleotide links are quite stable. Chromatography in solvent 1 showed two products, one with an R_F value the same as that of adenylic acid, and the other with an R_F value similar to that of a nucleoside (Fig. 1*a*, col. 5). Hydrolysis of the latter product with $HClO_4$ yielded one base only, cytosine. The former product was identified as adenylic acid by its mobility in two solvents and its absorption spectrum, and, by chromatography in solvent 2, it was shown to be adenosine 3′-phosphate.

This evidence shows that the dinucleotide was adenylyl-cytidylic acid.

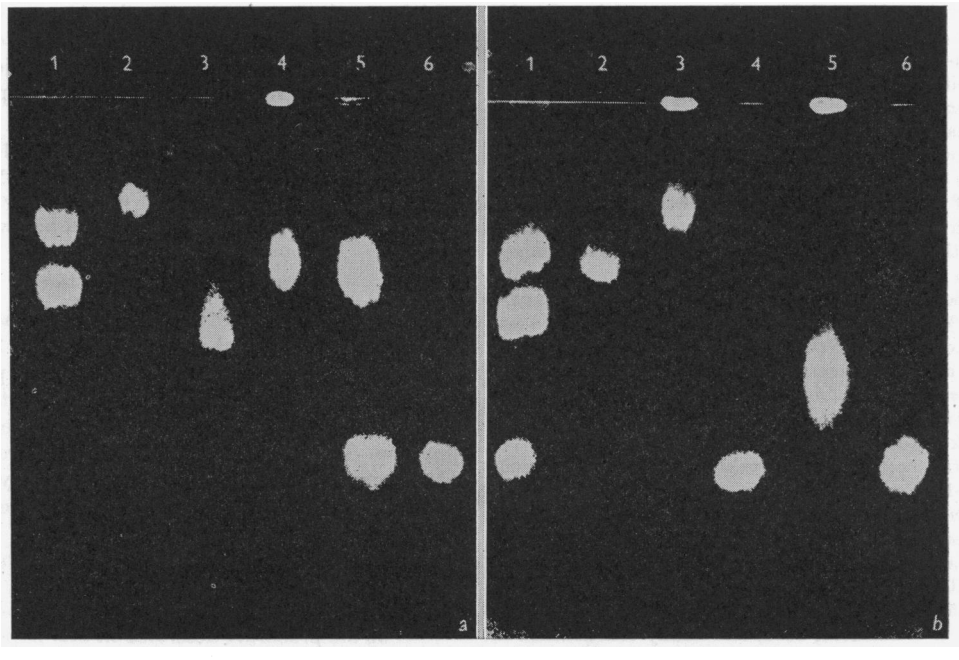


Fig. 1. (*a*) Descending chromatogram run in solvent 1, showing the stepwise degradation of adenylyl-cytidylic acid. Col. 1: reference spots, guanylic acid and adenylic acid (descending order); col. 2: adenylyl-cytidylic acid; col. 3: dephosphorylated adenylyl-cytidylic acid; col. 4: periodate oxidation product of (3); excess periodate at the origin; col. 5: adenylic acid and cytosine-containing fragment (descending order), derived from (4) by treatment at pH 10; col. 6: degradation product obtained from cytidine by periodate oxidation and treatment at pH 10. (*b*) Descending chromatogram run in solvent 1, showing the degradation of cytidine 5′-phosphate and cytidine. Col. 1: reference spots, guanylic acid, adenylic acid and cytidine (descending order); col. 2: cytidine 5′-phosphate; col. 3: periodate oxidation product of cytidine 5′-phosphate; col. 4: product derived from (3) by treatment at pH 10; col. 5: periodate oxidation product of cytidine; col. 6: product derived from (5) by treatment at pH 10. Excess periodate can be seen at the origin in cols. 3 and 5.

Degradation of a trinucleotide

A trinucleotide containing cytosine, adenine and guanine was examined. Dephosphorylation with phosphomonoesterase and chromatography in solvent 1 (step 1) produced a compound which had an R_F value 0·7 that of guanylic acid, and which, on alkaline hydrolysis, yielded adenylic acid, guanylic acid and cytidine in approximately equivalent amounts (1:0·95:0·95). Oxidation of this trinucleoside diphosphate with $NaIO_4$ (step 2) gave a product which had an R_F value in solvent 1 approximately 0·5 that of guanylic acid. This oxidation product, after treatment at pH 10 (step 3), showed, by chromatography in solvent 1, the presence of two substances. The component formed in the greater amount, (*a*), had an R_F value similar to that of a guanine-containing dinucleotide ($0·6 \times R_F$ value of guanylic acid), and the smaller component (*b*) had an R_F value similar to that of a nucleoside. Alkaline hydrolysis of a sample of (*a*) produced adenylic acid and guanylic acid in the molar ratio 1:1·03, and $HClO_4$ hydrolysis of (*b*) showed the presence of cytosine only. The remainder of the dinucleotide (*a*) was then treated as in steps 1–3, to produce a guanine-containing fragment and a mononucleotide which was shown to be adenosine 3′-phosphate. From these observations the trinucleotide in question was designated adenylyl-guanylyl-cytidylic acid.

Note. Acid hydrolysates of nucleotide derivatives eluted from the adenylic acid region of chromatograms run in solvent 1 sometimes failed to show the expected free base. This apparent disappearance of the base was attributed to the presence of iodate, which was formed during the periodate oxidation (step 2), and which was eluted off the chromatogram along with the oxidized nucleoside. Guanine, cytosine and uracil, when hydrolysed in acid with iodate, lose their ultraviolet-absorbing properties, due, probably, to the destruction of the ring structure by oxidation. This phenomenon could be avoided by removal of the iodate with sulphur dioxide before hydrolysis.

Nature of the purine internucleotide link in RNA

The terminal monoribonucleotide, which was obtained by stepwise degradation of the polynucleotides listed in Table 1, was shown, in each case, to be a nucleoside 3′-phosphate. As the conditions under which the experiment was carried out would not permit the transfer of the phosphoryl group from $C_{(2')}$ to $C_{(3')}$, the above result could be regarded as the first unequivocal evidence that the purine nucleosides are joined through $C_{(3')}$ of the ribose ring by phosphodiester bridges to other purine and pyrimidine nucleotides (Whitfeld & Markham, 1953). Although the $C_{(3')}$–$C_{(5')}$ linking has only been demonstrated in these isolated di- and tri-nucleotides, it would seem justifiable to assume that it occurs in intact RNA, as the model compounds used were derived from a ribonuclease digest of RNA.

Confirmation of this result has been supplied by Heppel *et al.* (1953), who carried out studies with an enzyme fraction from spleen which splits internucleotide links without the intermediate formation of a nucleoside 2′:3′-phosphate. Digestion of RNA 'core' and dinucleotides with this enzyme produced purine nucleoside 3′-phosphates only, and as the possibility of phosphoryl migration during the reaction had been eliminated, the authors concluded that the configuration of the purine internucleotide link in the original RNA was $C_{(3')}$–$C_{(5')}$. This observation has been recently supplemented (Brown, Heppel & Hilmoe, 1954) by the demonstration that the same spleen enzyme hydrolyses simple esters of the nucleoside 3′-phosphates only and does not attack esters of the nucleoside 2′-phosphates.

DISCUSSION

Any method which is designed for the determination of nucleotide sequence in a polyribonucleotide must have two properties. In the first place, the method must be able to differentiate the phosphodiester link adjacent to the terminal nucleotide from all other internucleotide links in the chain. Secondly, the conditions under which the reaction is carried out must be such that the remainder of the molecule is unaffected and ready for a repetition of the same process. The procedure which has been described in this paper fulfils both these conditions and is put forward as a general method for the determination of nucleotide sequence in any unbranched polyribonucleotide.

With regard to the first requirement, the reaction is specific for the internucleotide link adjacent to any nucleotide residue which either has a free α-glycol system, or can be dephosphorylated to expose such a system. For the sake of convenience these nucleotides will be referred to as susceptible nucleotides. If RNA is assumed to be an unbranched chain of nucleotide residues, linked together by $C_{(3')}$–$C_{(5')}$ phosphodiester bridges, then only one nucleotide in the chain has the required specifications, and that nucleotide is one or other of the terminal nucleotides. There is, however, one exception to this and that is the case in which the RNA has a nucleoside 2′:3′-phosphate as a terminal residue (Markham & Smith, 1952*c*). Such molecules would not contain a susceptible nucleotide and it would be necessary to open the cyclic phosphate end group before sequence determination would be possible. This might be done by treating the RNA with 0·1 N-hydrochloric acid at 18° for 4 hr., condi-

tions to which normal internucleotide links are stable. The nucleoside 2′:3′-phosphate would be converted into the open nucleotide, which could then be dephosphorylated to expose an α-glycol group.

With regard to the second requirement, the conditions under which the reaction is carried out involve a very mild oxidation with neutral periodate at room temperature and the subsequent incubation of the oxidation product at 37° at pH 10. Such conditions do not have any effect on any part of the polyribonucleotide except the susceptible terminal nucleotide, which is split off to leave a polyribonucleotide which contains one less nucleotide residue than the original and which is then ready for a repetition of the same process.

The possibility of simplifying the degradation procedure, as described in this paper, by combining the three steps of the cycle into one step, has been examined, but it would appear that little or nothing would be gained by such a modification, because of the extra precautions which would have to be taken against possible secondary reactions.

The method as it stands does not call for large amounts of material although, obviously, the larger the polyribonucleotide to start with, the more of it is necessary for a complete sequence determination. In the initial experiments larger amounts than necessary of the polynucleotides were used, as it was desirable to analyse the products of each step in the reaction, but now that the principle of the method has been established it should be possible to determine the nucleotide sequence in $(0{\cdot}5n–1{\cdot}0n)$ mg. of polyribonucleotide (where n is the number of nucleotide residues in the chain).

All the above considerations apply if RNA, or the polyribonucleotide in question, is an unbranched chain. If this is not the case (two possible types of branching have been postulated for RNA), then the effect of such branches upon the degradation procedure must be examined.

The first type of branching to be considered is that in which $C_{(2')}$ of a main-chain nucleotide residue is linked by a phosphate group to $C_{(3')}$ of the terminal nucleotide of a branch chain (Brown & Todd, 1952; Cohn *et al.* 1952). A polyribonucleotide having this structure would contain one susceptible nucleotide, at one end of the main chain, and so stepwise degradation could proceed along the main chain as far as the branch point. Here, however, the attachment of the branch chain to $C_{(2')}$ of the nucleoside would prevent the oxidation of the latter with periodate. Consequently, only the positions of those nucleotides which occur between the susceptible terminal nucleotide and the first branch point of the main chain could be determined.

The second possible type of branching is that which might arise from triesterification of a phosphate group (Fletcher, Gulland & Jordan, 1944; Cohn *et al.* 1952). Whether a triply esterified phosphate group would be stable to the conditions of the reaction is not certain but, if it were, then it would not be possible to determine, by this method, the nucleotide sequence in a polyribonucleotide containing such a group. Stepwise degradation could proceed along the main chain until the branch point was reached, but at this point the presence of the branch would prevent the exposure, by phosphomonoesterase treatment, of *cis*-hydroxyl groups. So, as with the first type of branching, the sequence of only those nucleotides which lie between the susceptible nucleotide and the first branch point of the main chain could be determined.

As yet, no conclusive evidence has been produced that RNA is a branched molecule and, until now, there has not been any method for providing such information. However, from the above discussion it is immediately apparent that an answer to this question might be obtained by the application of the degradation procedure. Any polyribonucleotide which, after one or more nucleotides have been split off, resists further degradation, will, in all probability, have a branched structure. If RNA is a branched molecule, then the method described in this paper will not be of great assistance in the determination of nucleotide sequence in such molecules unless the branch chains can be removed without affecting the structure of the main chain.

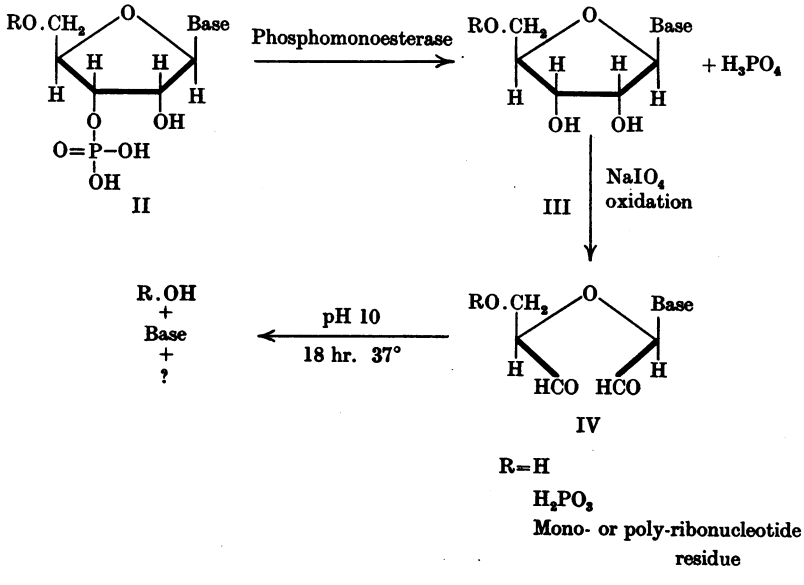
Moreover, even if RNA has an unbranched structure, it is perhaps still too early to assess the true value of the degradation method. Present-day knowledge indicates that RNA consists of a number of fairly short-chain molecules (Markham & Smith, 1952*c*), and little information would be gained from the application of the method to such a mixture. Should it be possible to resolve this mixture into fractions, each containing a number of identical chains, then the value of the degradation method as a means of comparing the nucleotide sequence in the various fractions becomes obvious.

A more immediate application of the method might be to an examination of the structure of RNA 'core'. The non-dialysable fraction of an RNA–ribonuclease digest, or 'core', appears to consist of polyribonucleotides with average chain length of 4–6 nucleotide residues (Markham & Smith, 1952*c*; Volkin & Cohn, 1953). The combination of Cohn's ion-exchange method for the separation of polyribonucleotides (Cohn *et al.* 1952) and the stepwise degradation procedure described in this paper might assist considerably in establishing the composition of the 'core'. For instance, if the 'core' were subjected to two cycles of the degradation procedure, the average chain length of the component polynucleotides would be reduced to 2–4 nucleotide residues. The shortened 'core'

polynucleotides could then be separated by ion-exchange chromatography and their composition and nucleotide sequence determined.

Mechanism of the reaction

The first two steps of the reaction, involving the removal of the free phosphate group from (II) with phosphomonoesterase and the oxidation of the exposed *cis*-hydroxyl groups in (III) with periodate to give a dialdehyde derivative (IV), are quite straightforward. The theory, however, behind the reaction which occurs when the oxidized residue is subjected to mild alkaline treatment (step 3) is by no means clear.



An insight into the possible mechanism of the latter reaction was afforded by an examination of the periodate oxidation products of cytidine and cytidine 5′-phosphate. It was first noticed that the cytosine-containing fragment which was obtained during the degradation of adenylyl-cytidylic acid did not have the same R_F value in solvent 1 as that of the periodate oxidation product of cytidine (Fig. 1*a*, col. 5; Fig. 1*b*, col. 5). If, however, the latter was treated at pH 10 it was converted into a product which appeared to be identical with that obtained from adenylyl-cytidylic acid (Fig. 1*a*, col. 6; Fig. 1*b*, col. 6). Furthermore, the periodate oxidation product of cytidine 5′-phosphate could also be converted into the same end-product by treatment at pH 10 (Fig. 1*b*, cols. 2–4).

These three cytosine-containing fragments, derived from adenylyl-cytidine, cytidine and cytidine 5′-phosphate by periodate oxidation and incubation at pH 10, were shown to be identical with each other and with the free base, cytosine. This identification was based on (1) the same R_F values in solvents 1–3, (2) the same ionophoretic mobilities at pH's 1·7, 3·5 and 7·0, and (3) identical absorption spectra in acid, in alkali and at pH 7·0.

One other observation which indicates the complicated nature of the reaction was made. When the dialdehyde derivative of cytidine was treated at pH 10 for a short time only (30 min.), two ultraviolet-absorbing products could sometimes be detected by chromatography in solvent 1. The slower moving component was the end-product of the reaction (cytosine), while the faster moving component was an intermediate product in the reaction. In all probability, other intermediates were also formed.

The experimental results support the suggestion of Brown *et al.* (1953) that the initial action of alkali on the periodate-oxidized dinucleoside monophosphates is an elimination process, since these substances are phosphate esters of β-hydroxyaldehydes. Thereafter, the liberated fragment undergoes one or more reactions of an unknown nature leading apparently to the free base as end-product.

SUMMARY

1. A method is described whereby the sequence of nucleotides in an unbranched polyribonucleotide can be determined.

2. This method has been applied successfully to five dinucleotides and three trinucleotides.

3. The possible mechanism of the reaction is discussed.

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The Occurrence of Isomaltose among the Products of heating Glucose in dilute Mineral Acid

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Since the identification by Emil Fischer (1890, 1895) of the disaccharide 'isomaltose' among the products of action of cold concentrated hydrochloric acid on glucose there have been numerous reports of such condensations in the presence of mineral acids, both under conditions likely to produce dehydration (cf. Levene & Upts, 1925; Pacsu & Mora, 1950; Kent, 1953), and also in aqueous solution (cf. Brown & Millar, 1899; Harrison, 1914; Berlin, 1926; Georg & Pictet, 1926; Moelwyn-Hughes, 1928). The characterization of the products was, however, hindered by the lack of suitable methods for their separation.

Later authors (Frahm, 1941; McDonald, 1950; Pirt & Whelan, 1951) have measured changes in reducing power and optical rotation in solutions of glucose and mineral acids under various conditions, but only Frahm considered condensation as a contributory cause. Täufel & Reiss (1951) used paper chromatography to estimate the changes in glucose content of a 10% solution in 3N hydrochloric acid heated at various temperatures; they found a 17% loss of glucose after 3 hr. at 100° and also noted that material with smaller R_F values appeared.

Whitby (1952), using glucose oxidase, found a loss of 4% of glucose from a 2·5% solution heated for 2·5 hr. at 100° in N hydrochloric acid.

This paper describes qualitative and quantitative observations made on the products of heating glucose in N aqueous acid (sometimes called 'reversion products') by the use of paper chromatography. The isolation of one major component of this mixture by charcoal–Celite chromatography is described and evidence is presented that this component is isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose).

The conception that the branched structures of glycogen and amylopectin arise through the presence of α-1:6-linkages has depended mainly upon the isolation of isomaltose derivatives from enzymic (Montgomery, Weakley & Hilbert, 1949) and acidic (Wolfrom, Lassettre & O'Neill, 1951; Wolfrom, Tyree, Galkowski & O'Neill, 1951; Thompson & Wolfrom, 1951) hydrolysates of the two polysaccharides. The discovery that mould-enzyme preparations can form the isomaltose type of linkage from maltose (Pan, Andreasen & Kolachov, 1950) has cast doubts on the reliability of evidence from enzymic digests, and it has been suggested that the isomaltose found in acid hydrolysates might have been formed by reversion from free glucose. Myrbäck (1941) produced evidence to show that condensation products were absent from his starch hydrolysates but the methods available to him would seem to have been inadequate to settle