$\epsilon_{max.}$. If, however, a similar compound is obtained from a reaction mixture containing the $C_{20}H_{26}O$ compound its properties suggest $C_{25}H_{34}O$, i.e. it derives from the $C_{20}H_{26}O$ compound.

SUMMARY

1. Oppenauer oxidation of vitamin A using diethyl ketone as hydrogen acceptor results in a complex mixture from which a compound $C_{20}H_{26}O$ (isomeric with retinene$_2$) can be isolated. It gives an oxime, m.p. 175–177°, isolated by previous workers.

2. The $C_{20}H_{26}O$ compound, is reducible *in vitro* and *in vivo*, but the product differs from vitamin A_2.

3. The Oppenauer oxidation seems to proceed via retinene$_1$, $C_{20}H_{28}O$, which can either be further dehydrogenated to $C_{20}H_{26}O$ or can condense with diethyl ketone, to a ketone $C_{25}H_{36}O$. Condensation of the $C_{20}H_{26}O$ compound with diethyl ketone to yield $C_{25}H_{34}O$ is not excluded.

4. Vitamin A acetate is not dehydrogenated directly to vitamin A_2.

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The Structure of Ribonucleic Acids

1. CYCLIC NUCLEOTIDES PRODUCED BY RIBONUCLEASE AND BY ALKALINE HYDROLYSIS

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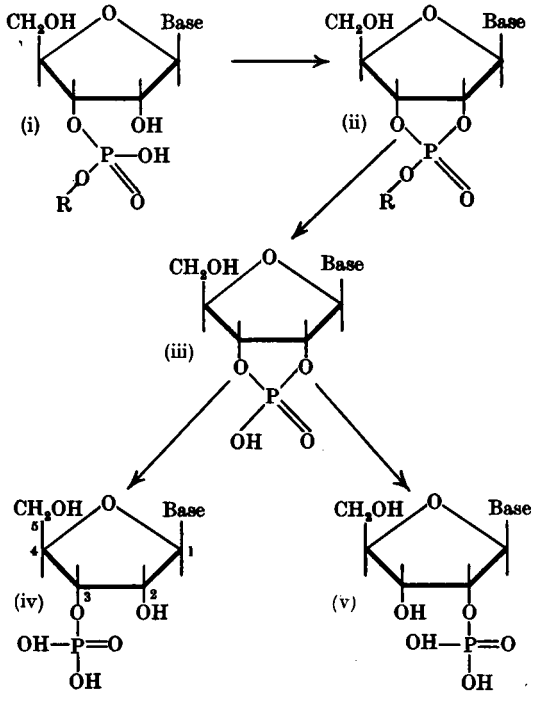
One of the most striking chemical differences between the two main types of nucleic acid is that N-sodium hydroxide or potassium hydroxide at room temperature hydrolyses ribonucleic acids (RNA) rapidly and completely to nucleotides, but does not hydrolyse deoxyribonucleic acids in this way. The nucleotides produced on alkaline hydrolysis of RNA were thought for many years to be nucleoside 3′-phosphates (Levene & Harris, 1932), but recently Carter (1950), Cohn (1950) and also Loring & Luthy (1951) have demonstrated that all four ribonucleotides exist in the alkaline hydrolysates as pairs of isomers. These pairs, which have been called '*a*' and '*b*' nucleotides, differ in their chromatographic behaviour and are quite distinct from the nucleoside 5′-phosphates. They are stable to alkali, but may be interconverted by a short treatment with dilute acid (Brown, Haynes & Todd, 1950), and evidence has been given by Brown & Todd (1952*a*) that the '*a*' and '*b*' nucleotides are the nucleoside 2′- and 3′-phosphates, though not necessarily in that order, rather than α- and β-isomers (Doherty, 1950). The '*a*' and '*b*' nucleotides differ considerably in several properties such as solubility, and it is of interest to note that only the '*b*' forms of the pyrimidine nucleotides are liberated from RNA by ribonuclease, while at pH 8 a crude phosphatase from potato acts much more rapidly on the '*b*' form of adenylic acid than on the '*a*' form (Cohn, 1951; Kornberg & Pricer, 1950).

Working on the ribonuclease digestion of RNA (Markham & Smith, 1951*b*), we found that our digests contained a third type of nucleotide which belongs neither to the '*a*' or '*b*' series, nor to the group of nucleoside 5′-phosphates, but which could be converted to the former type by further treatment with ribonuclease. By a fortunate coincidence at about this time we had a discussion with Prof. A. R. Todd and several of his colleagues, and they were kind enough to describe their theory of the origin of the '*a*' and '*b*' nucleotides of Carter and Cohn to us (Brown & Todd, 1952*b*). This theory requires that the first step in the hydrolysis of a polynucleotide chain by alkali should be the formation of a tertiary phosphate ester (i → ii). This takes place by the esterification of the hydroxyl group on the sugar rings at carbon atom 2′ or 3′, whichever is free, by the primary phosphoric acid (—OH) group which is present in each internucleotide link.



Thus each phosphoric acid residue is in ester linkage with the hydroxyl groups at 2′ and 3′ of one nucleotide plus a hydroxyl group of the adjacent nucleotide in the chain. This structure is unstable and breaks down, leaving a series of 2′:3′-monohydrogen phosphate esters of nucleosides which, for convenience, we shall call 'cyclic' phosphates or 'cyclic' nucleotides (iii) in conformity with Brown & Todd. These substances are themselves unstable to alkali and break down, giving a mixture of the nucleoside 3′- and 2′-phosphates (iv and v). This theory, which is based upon the known properties of other phosphate esters, also explains the interconvertibility of the nucleoside 2′- and 3′-phosphates by acid, the alkali stability of deoxyribonucleic acids (which have no hydroxyl group at C—2′ and consequently cannot form a cyclic intermediate) and also suggests that the sugars which can form nucleic acids of the RNA type must have *cis* 2- and 3-hydroxyl groups.

Now it was quite evident from the data in our possession that cyclic nucleotides like (iii) would behave very much as did our nucleotides, and we were able to compare the synthetic and natural materials and demonstrate their identity. We have also managed to prepare all the four cyclic mononucleotides from RNA by careful alkaline hydrolysis and have thus been able to show that the scheme of Brown & Todd accurately represents the main steps in the alkaline hydrolysis of RNA.

EXPERIMENTAL

Materials

Commercial yeast RNA was reprecipitated and deproteinized (Smith & Markham, 1950). For the preparation of yeast RNA baker's yeast was treated with ethanol and ethanol-ether and dried. The dry yeast was then boiled at pH 8–8·4 in water for 20 min. An equal volume of ethanol was then added to the yeast suspension when cold and the precipitate was spun off. The supernatant liquid was acidified to pH 4·5 by addition of a small amount of acetic acid. The impure nucleic acid which precipitated was deproteinized (Sevag, Lackman & Smolens, 1938), precipitated with 50% (v/v) ethanol at pH 4·5 and dried.

Turnip yellow mosaic virus RNA was prepared as described previously (Markham & Smith, 1951*a*).

The 2′:3′-monohydrogen phosphates of cytidine, uridine and adenosine were given to us by Mr D. Magrath, who prepared them by a method to be published (Brown, Magrath & Todd, 1952).

Methods

Hydrolysis in presence of barium carbonate

RNA suspended in water was boiled under reflux in the presence of a small quantity of $BaCO_3$ for periods of up to 4 hr. (A useful time is 1 hr. at 100°.) Small amounts may be heated on a water bath in a stoppered tube. Barium carbonate, though very sparingly soluble in water, dissociates slightly in hot water to give a small amount of $Ba(OH)_2$, and this permits an easily controlled and mild alkaline hydrolysis. Solutions of NH_3 were also used at 50°, but this treatment tends to cause the production of nucleosides. In either case the yield of the cyclic phosphates is small.

Ribonuclease digestion

Solutions of crystalline ribonuclease were added to solutions of RNA at pH 7·5 and 20° in cellophan bags suspended in a large volume of water; the bags were agitated mechanically. The dialysate was collected at intervals and concentrated *in vacuo*. The dialysate contained large quantities of cyclic pyrimidine nucleotides, but only traces

of cyclic adenylic and guanylic acids, and then only from undegraded RNA. The reasons for this will be discussed in a later paper.

In preparing the cyclic nucleotides by this method, it is essential to keep the enzyme concentration low, so as to allow the nucleotides to escape further digestion by diffusing out of the bag. A suitable quantity of enzyme is 100 μg. in 10 ml. of a solution containing about 500 mg. of RNA.

Chromatographic solvents

Solvent 1. $(NH_4)_2SO_4$ sat. in water, 80 parts; 0·5 M-sodium acetate, 18 parts; *iso*propanol, 2 parts (v/v/v). *Solvent* 2. *iso*Propanol, 70 parts; water, 30 parts (v/v). *Solvent* 3. As solvent 2, with 0·35 ml. NH_3 solution (0·880 sp.gr.) added for each 1 l. of gas space in the tank and poured into the bottom of the tank. *Solvent* 4. *sec.*-Butanol sat. with water, 98%; glacial acetic acid 2%, (v/v).

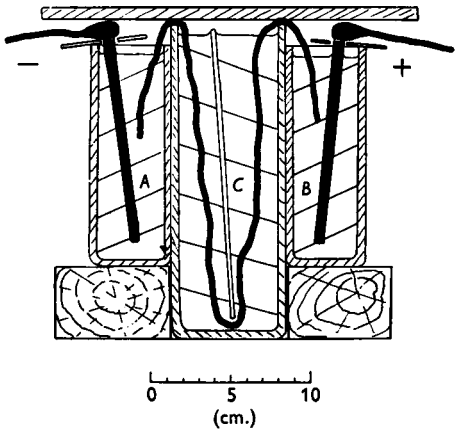


Fig. 1. The electrophoresis apparatus. Three standard museum jars (Baird and Tatlock, Ltd.: *A* and *B*, 6 × 4 × 2 in., *C*, 8·25 × 4·63 × 2·5 in.) were set up as shown, *A* and *B* containing buffer solution and *C* containing CCl_4 to cool the paper. The carbon electrodes were insulated at the top with tape after attaching the high-tension leads. The plate-glass lid covers the electrodes for safety. The spacer in jar *C* is celluloid, as are the two plates at the top of the electrodes.

Electrophoretic separation

The apparatus, which has been described briefly (Markham & Smith, 1951*c*), is shown in Fig. 1. The paper, a strip of Whatman no. 3 paper, 56 × 8 cm., is soaked in 0·05 M-ammonium acetate or formate buffer, and blotted to remove surplus moisture. The mixture to be run is put on a suitable place, usually about 12 cm. from the negative end, in a line at right angles to the long axis of the paper. About 0·1–0·2 ml. of solution is used. The paper is then immersed in CCl_4 in vessel *C* and the ends are allowed to dip into vessels *A* and *B* which contain 0·05 M-buffer. CCl_4 is essential as it prevents heating and evaporation and so the rates of movement of the bands are uniform and reproducible. The CCl_4 also prevents liquid siphoning over into the central part and waterlogging the paper. It is also transparent to the ultraviolet light used to detect the bands, and being non-polar has no tendency to dissolve out the nucleotides.

A potential of 1000 V. is applied to the ends of the paper, and a suitable power supply is shown in Fig. 2. The current used is 8–10 ma. with the buffers mentioned and an average run takes 2 hr. If phosphate buffers are used the potential should be dropped to 700 V. to prevent overheating. It is perhaps unnecessary to mention that the power supply is dangerous.

We use ammonium acetate and formate buffers as the components travel as spots on chromatograms. It is therefore unnecessary to desalt material removed from the electrophoresis strips before chromatography.

Location of the nucleotides. The nucleotides are located on the chromatogram and electrophoresis strips by ultraviolet photography (Markham & Smith, 1949, 1951*a*). When Whatman no. 3 paper is used the photographic exposure is 3–5 min.

Quantitative analysis. The purine and pyrimidine content of preparations was determined by a chromatographic method (Markham & Smith, 1951*a*). The phosphorus analyses were made by the method of Allen (1940).

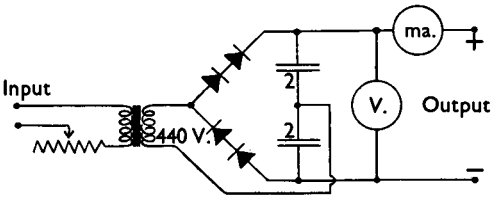


Fig. 2. The 1000 V. power supply for the electrophoresis apparatus. The transformer output must be at least 440 V. r.m.s., at 60 ma. or more (a 250–0–250 V. transformer is suitable). The metal rectifiers are in pairs of 350 V., 60 ma., in series. The condensers are 2 μF., 1000 V. working. The series resistance in the transformer primary is 1000–5000 Ω rated at 200 ma. and may be replaced with advantage by a variable autotransformer.

Preparation of the cyclic nucleotides

A ribonuclease digest of RNA was put on to the top of a piece of Whatman no. 3 paper in a line 25 cm. long. The quantity used was 10–20 mg. of digest in 0·5 ml. This was then run in solvent 3 for such a time that yeast adenylic acid would have run about 15 cm. (the solvent being allowed to run off the notched end of the paper). It was found that there were several bands of substances on the paper. The lowest of these (band 6), which has an R_F value of about 1·7 times that of yeast adenylic acid, contained all the cyclic nucleotides excepting that of guanine, which was found above (band 5*a*) with an R_F about 1·35 times that of yeast adenylic acid. Cyclic adenylic acid and guanylic acids will only be found in digests of good RNA and then only in minute amounts. The other bands contained substances which will be described in the following paper.

Cyclic pyrimidine nucleotides. These may be obtained in quantity from band 6 and may then be resolved from each other by: (*a*) paper chromatography in solvent 2, in which the cyclic uridylic acid has a higher R_F value than the cyclic cytidylic acid or (*b*) chromatography in solvent 4 which gives smaller R_F values but the same separation. Solvent 4 can, in fact, resolve the cyclic pyrimidine nucleotides from digests of commercial RNA directly, the other components having negligible R_F values. The cyclic pyrimidine nucleo-

tides can also be separated by electrophoresis for 2 hr. at 1000 V. and pH 3·5 when the uridylic acid will move about 16 cm. and the cytidylic acid about 6·5 cm. towards the positive electrode.

Cyclic purine nucleotides. Cyclic purine nucleotides comprise only about 2% of the total material in a yeast RNA digest and are not easy to demonstrate in such digests. Commercial yeast RNA which we have used yielded no cyclic purine nucleotide on ribonuclease digestion but after $BaCO_3$ hydrolysis these could be isolated in small yield. The $BaCO_3$ hydrolysate is best further digested with ribonuclease to remove the cyclic pyrimidine nucleotides and then separated on paper in solvent 1. Band 5*a* is almost pure cyclic guanylic acid and band 6 cyclic adenylic acid. These may be further purified by electrophoresis, when the contaminating nucleosides are left behind. The adenylic acid will move about 8 cm. and the guanylic acid about 14 cm. in 2 hr. at 1000 V. and pH 3·5.

RESULTS

Cyclic pyrimidine nucleotides

Cyclic uridylic acid has been analysed for phosphorus and uracil, which are present in the molar ratio 0·95:1 (theory 1:1). The absorption spectrum

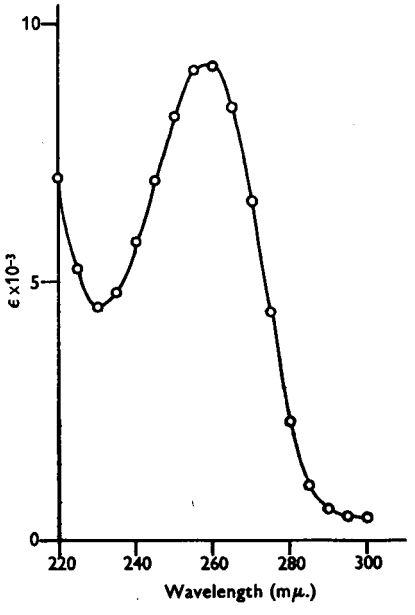


Fig. 3. The absorption spectrum of cyclic uridylic acid at pH 7·3.

is shown in Fig. 3. Cyclic cytidylic acid has a molar ratio of cytosine/P of 0·93:1 (theory 1:1). The absorption spectrum is shown in Fig. 4. The chromatographic behaviour is identical with that of the synthetic material (which was contaminated with the non-cyclic form of the nucleotides) in all solvent systems used (solvents 1–4 and several others).

Perhaps the most striking proof of the identity of the natural and synthetic material is the fact that both are hydrolysed slowly to the free nucleotides by crystalline ribonuclease. In this hydrolysis only the '*b*' isomer is formed (Brown, Dekker & Todd, 1952).

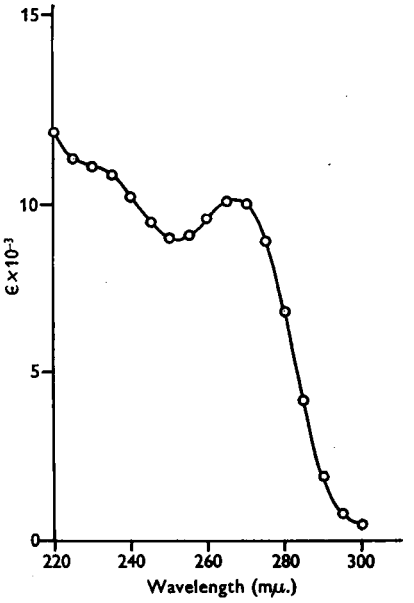


Fig. 4. The absorption spectrum of cyclic cytidylic acid at pH 7·3.

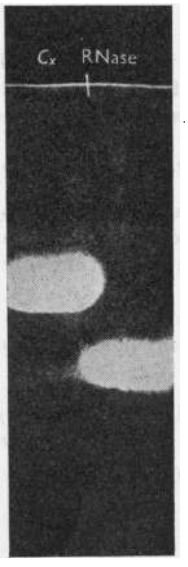


Fig. 5. The electrophoretic separation of the cyclic cytidylic acid and ordinary cytidylic acid obtained from the former by ribonuclease digestion. Strip run for 1 hr. in phosphate buffer, pH 7·4, at 20 V./cm. The cyclic form having one less OH group moves more slowly. The white line shows where the spots were put on.

After hydrolysis it is possible to demonstrate the presence of an additional acidic group dissociating at a pH between 3·5 and 7, by electrophoresis of the material at pH 7·4 (Fig. 5). This group is, of course, a secondary phosphoric acid —OH, which is not to be found in the cyclic forms (see formulae iii–v).

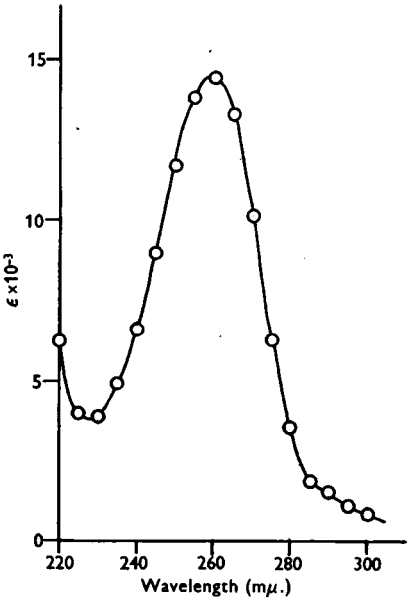


Fig. 6. The absorption spectrum of cyclic adenylic acid at pH 7·3.

At pH 3·5 the two forms have the same mobility. The properties of the cyclic and non-cyclic pyrimidine nucleotides are summarized in Table 1.

Cyclic purine nucleotides

The molar ratio phosphorus/adenine in the cyclic adenylic acid has been found to be 1·00:1 (theory 1:1). The absorption spectrum is shown in Fig. 6.

The molar ratio phosphorus/guanine in the cyclic guanylic acid has been found to be 0·93:1 (theory 1:1). The absorption spectrum is given in Fig. 7.

The cyclic adenylic acid has been compared with synthetic material (which was only about 80% pure), but the guanylic acid derivative has only been identified by analogy, i.e. general electrophoretic and chromatographic properties.

The general properties of the cyclic purine nucleotides are similar to those of the corresponding pyrimidine nucleotides with the important exception that they are *completely resistant to ribonuclease*. On hydrolysis with cold N-hydrochloric acid or N-sodium hydroxide, they give rise to

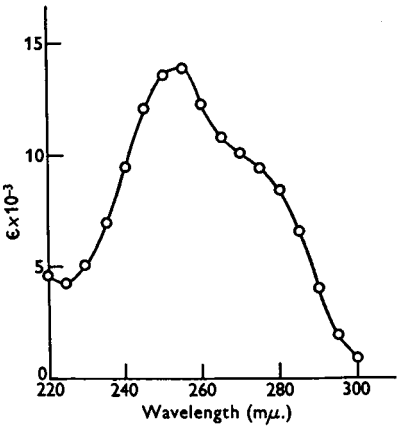


Fig. 7. The absorption spectrum of cyclic guanylic acid at pH 7·3.

mixtures of the '*a*' and '*b*' nucleotides, which may be demonstrated by running in solvent 1. Table 1 also includes a summary of the properties of these nucleotides.

DISCUSSION

From the data presented in this paper there can be little doubt that the scheme of Brown & Todd (1952*b*) for the alkaline hydrolysis of RNA is essentially correct. The isomeric nucleotides of Cohn (1950) and Carter (1950) must therefore be

Table 1. *The properties of the cyclic nucleotides contrasted with those of the analogous nucleoside 2′- and 3′-phosphates*

Cyclic nucleotides	Nucleoside 2′- and 3′-phosphates
Greater R_F value in solvent 3	Smaller R_F value in solvent 3
Smaller R_F value in solvent 1	Larger R_F value in solvent 1
Same electrophoretic mobility at pH 3·5	Same electrophoretic mobility at pH 3·5
Smaller relative electrophoretic mobility at pH 7·4	Larger relative electrophoretic mobility at pH 7·4
Pyrimidine derivatives hydrolysed slowly by ribonuclease	Unaffected by ribonuclease
Hydrolysed exceedingly slowly by prostate phosphomonoesterase	Dephosphorylated rapidly by phosphomonoesterase
Hydrolysed by cold N-NaOH to give a mixture of the nucleoside 2′- and 3′-phosphates	Unaffected by cold N-NaOH

regarded as artifacts of hydrolysis. The question still remains as to which of the two types of nucleotide, the 3′ or the 2′ nucleotide, is the natural form. By analogy with deoxyribonucleic acid, the 3′ type is the natural choice, but a definite solution of this problem will probably demand some new approach, and, of course, it is by no means certain that all the nucleotides will have the same configuration, though this would seem likely.

From the evidence which we have presented here, it might be thought that our 'cyclic' nucleotides might be dinucleotides or pyrophosphates. As we shall describe the isolation and properties of both cyclic and non-cyclic dinucleotides in the next paper, it may suffice here to mention that dinucleotides have smaller R_F values in all solvent systems, a higher electrophoretic mobility at all pH values, and, of course, are only half dephosphorylated by phosphomonoesterase.

The most striking point emerging from these experiments is the evident analogy between the action of ribonuclease and that of alkali on RNA, and the discrimination of the former between purine and pyrimidine nucleotides. This is in keeping with the specificity of ribonuclease, which we shall describe in detail later. At this point it may be pertinent to remark that, as we believe that the mechanism of action of ribonuclease on the cyclic pyrimidine nucleotides is the same as that of the enzyme on intact RNA, the cyclic nucleotides, which may be synthesized or isolated in adequate amounts, are obvious substrates for kinetic and other studies on ribonuclease, particularly as the reaction is of a simple type while, as the rate of reaction is relatively slow, it may be followed without difficulty.

SUMMARY

1. Cyclic nucleotides (2′:3′-monohydrogen phosphate esters of nucleosides) have been obtained from ribonucleic acids by ribonuclease action and by careful alkaline hydrolysis.

2. The properties of these substances have been described and they have been compared with synthetic specimens.

3. The cyclic pyrimidine nucleotides are digested by ribonuclease to give ordinary nucleotides while the analogous purine derivatives are not.

4. A method and apparatus for separating these substances by paper strip electrophoresis is described.

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