## 809. *Cytidine Diphosphate Glycerol.*

By J. BADDILEY, J. G. BUCHANAN, A. P. MATHIAS, and A. R. SANDERSON.

One of the cytosine nucleotides isolated from *Lactobacillus arabinosus* has been identified as the cytidine diphosphate glycerol (II). On hydrolysis with dilute acid it yields cytidine-5′ phosphate (I) and α-glycerophosphate (III) with a trace of the β-isomer (IV). These were identified by paper chromatography, by the behaviour of the α-isomer towards periodate, and by enzymic dephosphorylation to glycerol.

When the nucleotide was heated with ammonia the cyclic glycerol phosphate (V) was formed, whereas the venom of *Crotalus atrox* hydrolysed it to cytidine, orthophosphate, and α-glycerophosphate. It is concluded that a pyrophosphate group links cytidine at position 5′ with glycerol in the α-position.*

RECENT improvements [1] in the isolation of the two cytosine nucleotides from extracts of *Lactobacillus arabinosus* [2] have enabled us to obtain purer preparations and consequently more reliable analyses of the ratio base : phosphorus in these substances, and it is clear now that they contain two phosphate residues per cytidine group. In the earlier work it was shown that both are derivatives of cytidine-5′ phosphate (I) and that substituents are probably attached to the phosphate group. We describe here evidence to show that the cytosine nucleotide which is eluted from the ion-exchange column last and which has the higher $R_F$ on paper chromatography in propan-1-ol–ammonia (previously called CPY) is the pyrophosphate (II).

It has become common practice in biochemical work to employ trivial descriptive names for compounds of this general type in which a nucleoside pyrophosphate is esterified

* A preliminary account was published by Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, **63**, 15P.

[1] Baddiley, Buchanan, Carss, Mathias, and Sanderson, *Biochem. J.*, 1956, in the press.
[2] Baddiley and Mathias, *J.*, 1954, 2723.

with some other compound. These names follow the pattern set by Leloir when he used the expression uridine diphosphate glucose (UDPG) for the coenzyme of galactowaldenase. On the same basis the nucleotide derivative (II) can be described as cytidine diphosphate glycerol (CDP glycerol). It must be emphasised, however, that this name, although convenient for many purposes, would not be readily adapted to possible synthetic analogues and that it is therefore important to retain the more systematic pyrophosphate nomenclature for chemical purposes. It is not easy to assign a chemical systematic name which would be applicable also to related compounds and, after consultation with the Editor, we suggest, for temporary use, the name $P^1$-cytidine-5′ $P^2$-glycerol-1 pyrophosphate; here the final numeral attached to the name of the polyol denotes the position of the ester group, as in the current nomenclature of nucleotides and the earlier (now abandoned) British nomenclature of carbohydrates.

As only a few milligrams of the compound have been available our evidence relies solely on paper chromatography for the identification of degradation products. The presence of impurities in the preparations could lead to ambiguity in some of the results and in order to overcome this all degradations were carried out on samples which had been run on paper and then eluted. A sample obtained in this way was homogeneous when examined by paper chromatography in several solvent systems. A single spot was observed which absorbed ultraviolet light and gave positive reactions for phosphate [3] and 1 : 2-glycols.[4]

Although the nucleotide is a diphosphate it has a higher $R_F$ in basic solvent systems than has cytidine-5′ phosphate and much higher than cytidine-5′ pyrophosphate (kindly supplied by Dr. J. L. Strominger). This suggests that it contains either fewer acidic groups than cytidine-5′ pyrophosphate or that an additional basic group is present. The latter is unlikely in view of the behaviour of the compound on the Dowex-2 (formate) column. Elution occurs at about the position expected for a cytidine diphosphate : [1] an additional basic group would advance the elution position very considerably.

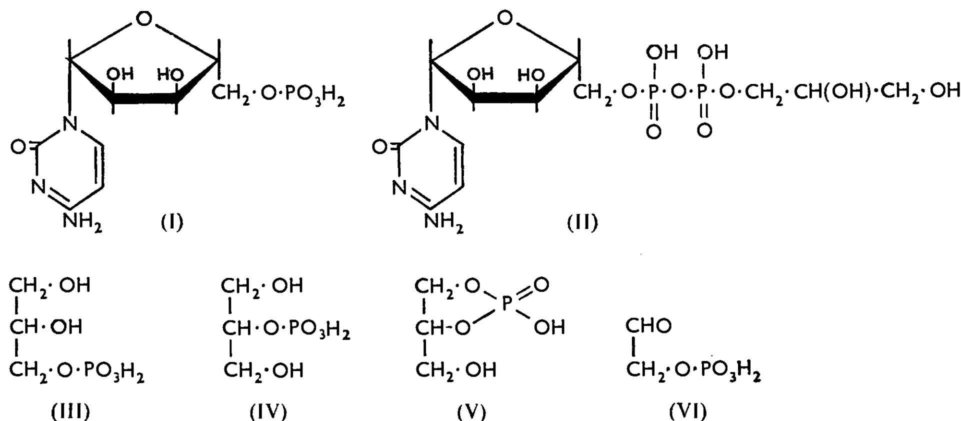
Hydrolysis with N-hydrochloric acid at 100° for 30 minutes and paper-chromatography in propan-1-ol–ammonia afforded two spots with the perchloric acid–molybdate spray reagent [3] for phosphoric esters. The slower-moving spot coincided with an area of absorption of ultraviolet light and corresponded to cytidine-5′ phosphate (I). Full identification of this nucelotide in acid hydrolysates was described earlier.[2] The other phosphoric ester in the hydrolysate could be separated from cytidine-5′ phosphate on a long paper. It did not absorb ultraviolet light but gave a strong reaction for a 1 : 2-glycol when sprayed with periodate followed by Schiff's reagent,[4] the spot developing within a few minutes. This behaviour has been observed with several acyclic glycols, but not with nucleosides and their derivatives which contain a ribofuranosyl structure : with ribofuranosides and other glycosides the colour develops slowly (several hours). The cytosine nucleotide itself rapidly gives a colour which intensifies and deepens somewhat during several hours under the conditions of the test. This suggests that the structure responsible for the rapid appearance of colour in the hydrolysis product is also present in the intact nucleotide. The intensification of colour which occurs later with this substance represents the contribution from the ribofuranose ring in the cytidine residue.

Although the nucleotide-free product of acid hydrolysis ran in propan-1-ol–ammonia as a single spot, in *tert.*-butyl alcohol–picric acid–water it was resolved into a main spot and a faint one with a higher $R_F$; only the former gave a positive glycol reaction. Further, when the mixture of these two phosphoric esters was eluted from a paper chromatogram, treated with periodate for two hours and then run on paper, the main spot had disappeared and was replaced by one which was indistinguishable from glycollaldehyde phosphate (VI). The faint spot was unchanged by this treatment. These results indicate that the main product from the acid hydrolysis contains the grouping $H_2O_3P{\cdot}O{\cdot}CH_2{\cdot}CH(OH){\cdot}CH(OH)$–. The faint spot would correspond with an isomeric structure $HO{\cdot}CH_2{\cdot}CH(O{\cdot}PO_3H_2){\cdot}CH(OH)$– which should be stable to periodate :

[3] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[4] Buchanan, Dekker, and Long, *J.*, 1950, 3162, cf. Baddiley, Buchanan, Handschumacher, and Prescott, *J.*, 1956, 2818.

this isomer may well have arisen through an acid-catalysed migration of the phosphate group during hydrolysis.

When the nucleotide was hydrolysed with ammonia at 100° for one hour it was partly converted into cytidine-5′ phosphate and a fast-running phosphate which gave no aldehyde with periodate. This new product must be a cyclic phosphate of the structure (V). It had a higher $R_F$ in propan-1-ol–ammonia, and a slower rate of migration on paper electrophoresis at pH 8·7, than had the phosphate formed in the acid hydrolysis. In addition, it was rapidly converted into a mixture of these acyclic phosphates on treatment with acid.




The lability towards acids suggested a 5-membered rather than a 6-membered cyclic phosphate.[5]

The simplest structure for the original nucleotide consistent with these observations is (II). Whereas esters of pyrophosphoric acid are usually stable towards dilute alkali, a hydroxyl group in the adjacent position reduces this stability through the possibility of cyclic phosphate formation. This accounts for the alkali-lability of uridine diphosphate glucose [6] and flavin-adenine dinucleotide.[7] It is interesting that, although the behaviour of the cytosine nucleotide is consistent with structure (II) since it yields a cyclic phosphate on treatment with ammonia, it is more stable in this respect than flavin-adenine dinucleotide or uridine diphosphate glucose. This increased stability is possibly a result of the adoption of a preferred *trans*-configuration of the β-hydroxyl group in the glyceryl residue. The formation of cyclic phosphates under alkaline conditions is probably an intramolecular reaction with steric requirements similar to those of the $S_N2$ type [8] and consequently would only operate over a narrow range of geometrical possibilities in which the reaction rate would decrease with increase in the angle between the pyrophosphate and the adjacent hydroxyl group. The more vigorous conditions required for acid-catalysed migration of the phosphate group in glycerol phosphates, compared with nucleoside-2′ and -3′ phosphates, may also be due to geometrical factors.

That structure (II) is correct for the nucleotide was shown by a study of the action of phosphatases on the intact substance and on its products of acid hydrolysis, as well as by direct comparison of the phosphates with authentic glycerol phosphates. An acid hydrolysate was run on paper in propan-1-ol–ammonia, and the area corresponding to the non-nucleotide phosphates was cut out, eluted, and hydrolysed by a mixed phosphatase preparation (Polidase-S). The products were identified by paper chromatography as orthophosphate and glycerol. Glycerol was identified by comparison with an authentic

[5] Brown, Magrath, and Todd, *J.*, 1952, 2708; Baddiley and Thain, *J.*, 1951, 3421; Baddiley, Buchanan, and Szabo, *J.*, 1954, 3826.
[6] Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426.
[7] Forrest and Todd, *J.*, 1950, 3295.
[8] Personal communication from Dr. D. M. Brown.

sample in several solvents. It was readily detected by the periodate–Schiff and periodate–benzidine [9] spray reagents and by its weak reaction with silver nitrate.[10]

When the nucleotide was hydrolysed by the phosphatases of *Crotalus atrox* venom the products were cytidine, orthophosphate, and α-glycerophosphate. In this experiment hydrolysis had occurred at the pyrophosphate linkage and also at the nucleoside–phosphate linkage. As no β-glycerophosphate was formed it was concluded that the original nucleotide was a derivative of α-glycerophosphoric acid.

The rapid formation of colour which the intact nucleotide showed in the periodate–Schiff test confirms the α-glycero-structure. In addition, the very small amount of β-glycerophosphate formed on short acid hydrolysis is consistent with an α-structure. The ratio, as estimated on a paper chromatogram, of α- to β-glycerophosphate produced in this way was higher than the equilibrium ratio for these esters which is attained after more vigorous acid treatment. However, the method of analysis is not accurate and these results give no real indication of the mechanism of hydrolysis. When the nucleotide was hydrolysed with sodium hydroxide the products were cytidine-5′ phosphate, β-glycerophosphate, and some α-glycerophosphate. The production of a relatively high proportion of a β-phosphate under these conditions is consistent with structure (II), since α-phosphatides give more β- than α-glycerophosphate under similar alkaline conditions.[11] It has not been established whether the glycerol α-residue has the D- or the L-configuration.

The cyclic phosphate (V) obtained by the action of ammonia on CDP-glycerol was indistinguishable from authentic glycerol 1 : 2-(hydrogen phosphate) [12] both on paper chromatography and on electrophoresis. It was shown that glycerol 1 : 2-(hydrogen phosphate) itself is unaffected by aqueous ammonia at 100° for 60 minutes.

The biochemical function of CDP-glycerol is not yet known. However, its general structure strongly suggests that it is a coenzyme involved in the metabolism of α-glycerophosphate. Since the latter substance is a component of phospholipids it may be that CDP-glycerol is concerned in the condensation of α-glycerophosphate with organic bases such as choline, ethanolamine, and serine, or with fatty acids. It may be significant that another natural derivative of cytidine-5′ pyrophosphate, namely, cytidine diphosphate choline, is concerned with the transfer of a phosphoryl choline molecule in phospholipid biosynthesis.[13]

## Experimental

The freeze-dried nucleotide was iolated from a 100 l. batch of bacterial culture by precipitation of nucleotides as mercury salts, ion-exchange chromatography on Dowex-2 (formate) resin, adsorption on charcoal, and elution with aqueous-alcoholic ammonia. Details of this method, and characterisation of the cytidine compound, have been described elsewhere.[1,2] Material obtained in this way was usually homogeneous when examined by paper chromatography but occasionally small amounts of an unidentified adenine nucleotide were detected. This could be removed by dissolving the freeze-dried material in water so that 1 ml. of the solution contained approx. 20 mg. of material and running a suitable quantity on paper in solvent system *B*, cutting out the appropriate area of paper and eluting it with water. In the following experiments the nucleotide was purified in this manner.

*Acid Hydrolysis*.—(*a*) A sample of nucleotide was hydrolysed for 30 min. at 100° with 6N-hydrochloric acid in a sealed tube. After evaporation in a desiccator *in vacuo*, the hydrolysate was examined by paper chromatography in solvent systems *A*, *B*, and *C* (see below). By comparison with authentic substances run on adjacent tracks the products were identified as cytidine-5′ phosphate, confirmed by its ultraviolet absorption spectrum, and a mixture of α- and β-glycerophosphate in which the α-form predominated. The glycerophosphates were separated from each other in solvent system A.

(*b*) Similar hydrolysis with N-hydrochloric acid effected complete hydrolysis of the pyrophosphate linkage but very little β-glycerophosphate was detected.

*Hydrolysis with Sodium Hydroxide*.—The nucleotide was hydrolysed in N-sodium hydroxide

[9] Cifonelli and Smith, *Analyt. Chem.*, 1954, **26**, 1132.
[10] Hough, *Nature*, 1950, **165**, 400.
[11] Baer and Kates, *J. Biol. Chem.*, 1948, **175**, 79; Long and Maguire, *Biochem. J.*, 1953, **54**, 612.
[12] Ukita, Bates, and Carter, *J. Biol. Chem.*, 1955, **216**, 867.
[13] Kennedy and Weiss, *J. Amer. Chem. Soc.*, 1955, **77**, 250.

at 100° in a sealed tube for 1 hr. Sodium ions were removed by treatment with a small amount of Dowex-50 ($H^+$ form) resin, and the solution was examined by paper chromatography in solvent systems *A*, *B*, and *C*. The products were cytidine-5′ phosphate and a mixture of α- and β-glycerophosphate in which the β-form predominated.

*Hydrolysis with Ammonia.*—The nucleotide was heated with ammonia (*d* 0·880) in a sealed tube at 100° for 1 hr. Hydrolysis was incomplete but the products were identified in solvent system *B* as cytidine-5′ phosphate and glycerol 1 : 2-(hydrogen phosphate). Paper electrophoresis at 200 v in 0·5M-ammonium acetate buffer (pH 8·7) of the phosphate which had been eluted from a paper chromatogram showed its identity with the synthetic material. It moved towards the anode much more slowly than did α- or β-glycerophosphate. The last two substances were not separated from each other.

After hydrolysis of the cyclic phosphate in N-hydrochloric acid for 7 min. at 100° paper chromatography in solvent systems *A* and *B* showed that it was converted into a mixture of glycerophosphates in which the α-form predominated.

*Oxidation with Periodate.*—The mixture of non-nucleotide phosphates (glycerol phosphates) obtained from an acid hydrolysis of CDP-glycerol was eluted from a paper chromatogram and treated with a slight excess (150 μg.) of sodium metaperiodate in 1·5% solution for 2 hr. at room temperature. Sodium ions were removed by treatment of the solution with a little Dowex-50 ($H^+$ form) resin, and the products were run on paper in solvent system *A*. The spot corresponding originally to α-glycerophosphate was no longer visible but one which was indistinguishable from glycollaldehyde phosphate was present. The spot corresponding to β-glycerophosphate was unchanged by periodate.

*Action of Phosphatases on Glycerophosphates.*—Glycerophosphates obtained as described above were treated at pH 7 with an equal volume of a 3% solution of Polidase-S (Schwarz Laboratories, Inc.). This enzyme preparation, which had been dialysed against distilled water at 4° for 4 days, contains several phosphatases including a highly active phosphomonoesterase. After 12 hr. at 37° the mixture was examined by paper chromatography in solvent systems *D*, *E*, and *F*. The products were identified by chromatography as inorganic phosphate and glycerol.

*Action of* Crotalus atrox *Venom.*—A solution of the nucleotide was treated with a 1% solution of the venom in a glycine–ammonia buffer at pH 9·0 for 12 hr. at 37°. The products were identified by chromatography in solvent system *A* as cytidine, α-glycerophosphate, and inorganic phosphate.

*Paper Chromatography.*—Unless otherwise stated ascending-front chromatography was carried out on Whatman No. 4 paper which had been washed with dilute acetic acid and water. The following solvent systems were used: *A*, *tert.*-butyl alcohol-picric acid-water (80 ml. : 2 g. : 20 ml.); *B*, propan-1-ol–ammonia–water (6 : 3 : 1); *C*, *iso*butyric acid–ammonia (10 : 6 ml. of 0·5N); *D*, butan-1-ol–acetic acid–water (organic layer) (4 : 1 : 5), *E*, butan-1-ol–ethanol–water (organic layer) (4 : 1 : 5); and *F*, butan-1-ol–benzene–pyridine–water (organic layer) (5 : 1 : 3 : 3). Results are tabulated.

| | $R_F$ in solvents | | | | | | $IO_4^-$–Schiff reaction |
|---|---|---|---|---|---|---|---|
| | *A* * | *B* | *C* | *D* | *E* | *F* | |
| Cytidine | — | 0·61 | — | — | — | — | S |
| CDP-glycerol | 0·65 | 0·31 | — | — | — | — | R |
| α-Glycerophosphate | 0·61 | 0·35 | 0·37 | — | — | — | R |
| β-Glycerophosphate | 0·67 | 0·37 | 0·39 | — | — | — | O |
| Cytidine-5′ phosphate | 0·38 | 0·18 | — | — | — | — | S |
| Glycerol 1 : 2-(hydrogen phosphate) | — | 0·60 | — | — | — | — | O |
| Glycollaldehyde phosphate | 0·42 | — | — | — | — | — | S |
| Glycerol | — | 0·74 | — | 0·54 | 0·52 | 0·63 | R |

S = Slow, R = rapid, O = no reaction; * = descending-front chromatography.

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King's College, University of Durham,
Newcastle upon Tyne.

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