refluxed with acetic acid (3 ml.) and acetic anhydride (1.5 ml.) for 2 hr., after which the product was again extracted by procedure A to yield an oil (150 mg.). This oil in benzene was chromatographed on Al_2O_3 (3 g.), when the diphenyl was eluted with benzene. Ether eluted an oil (60 mg.) which was dissolved in CHCl_3 (1.0 ml.) with acetic acid (2.0 ml.) and treated with CrO_3 (40 mg.) in water (0.4 ml.)-acetic acid (1.5 ml.) for 0.75 hr. in a water bath maintained at 50°. After cooling and the addition of methanol (0.5 ml.) and water (5 ml.), the product was isolated by procedure A and hydrolysed by refluxing (0.5 hr.) with ethanolic KOH (3 ml.). This hydrolysate was filtered, the filtrate acidified with aqueous HCl and the precipitated acid removed by filtration. After drying, the acid was recrystallized twice from ethyl acetate to yield crystals (7 mg.) of 3 α :7 α :12 α -trihydroxy-25-L-coprostanic acid, m.p. 194–196°, mixed m.p. with the Japanese ' β ' acid (above) 194–196°.

SUMMARY

1. The Kolbe electrolytic cross-coupling synthesis has been employed for the synthesis of homocholic, bishomocholic, 3 α :7 α :12 α -trihydroxy-25-L-coprostanic and the two (25-L and 25-D)-3 α :7 α :12 α -trihydroxyhomocoprostanic acids, the latter two compounds being thus related to L- and D- β -methylglutaric acids and hence to L- and D-glyceraldehyde.

2. The two (25-L and 25-D)-3 α :7 α :12 α -trihydroxyhomocoprostanic acids were degraded by the Wieland-Barbier method into the two corresponding (25-L and 25-D)-3 α :7 α :12 α -trihydroxycoprostanic acids. These latter two acids were identical with the two naturally occurring 3 α :7 α :12 α -trihydroxycoprostan-26-oic acids (Japanese ' α ' and ' β ' trihydroxycoprostanic acids respectively), which have been isolated from the biles of various species.

3. The formation of a new asymmetric centre by the oxidation *in vivo* of a methyl group in cholesterol to give bile alcohols or bile acids may be considered to be non-specific, in the sense that both optical isomers are obtained.

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The Isolation of Cytidine Diphosphate Glycerol, Cytidine Diphosphate Ribitol and Mannitol 1-Phosphate from *Lactobacillus arabinosus*

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It was shown earlier (Baddiley & Mathias, 1954) that hot aqueous ethanolic extracts of *Lactobacillus arabinosus* contain, in addition to many of the known nucleotide coenzymes, two hitherto undescribed cytosine nucleotides. When the work was started the only known natural cytosine nucleotides were those obtained by chemical or enzymic hydrolysis of nucleic acids. More recently the

presence of cytidine 5'-mono-, -di- and -tri-phosphates has been demonstrated in various animal tissue extracts (Hurlbert, Schmitz, Brumm & Potter, 1954). The natural occurrence of these simple cytosine nucleotides suggests that derivatives of cytidine 5'-pyrophosphate with co-enzyme function might also be found. This is supported by the recent discovery that cytidine

diphosphate choline is a coenzyme in the synthesis of lecithin from phosphorylcholine (Kennedy & Weiss, 1955).

Preliminary analysis of the impure cytosine nucleotides from *L. arabinosus* suggested that they were monophosphates derived from cytidine 5'-phosphate and bearing substituents on the phosphate groups. We have now repeated the isolation, purification and analysis of these bacterial nucleotides.

Our earlier isolation technique was laborious and difficult, since it involved ion-exchange chromatography followed by preparative-scale paper chromatography in order to separate the two cytosine nucleotides from each other and from inorganic phosphate. A much more satisfactory method is described here and includes gradient elution on Dowex-2 resin (formate form), followed by adsorption on charcoal and elution with ammonia.

D-Mannitol 1-phosphate has also been isolated in appreciable amounts from this micro-organism.

EXPERIMENTAL

L. arabinosus 17-5 (strain supplied by Dr D. E. Hughes) was grown on a hydrolysed casein medium supplemented with yeast extract and trace elements as described previously (Baddiley & Mathias, 1954), but growth was allowed to take place at 28° for 24 hr. rather than at the higher temperature used before. In the later stages of the work the organism was grown in 100 l. batches by Mr F. C. Belton, Ministry of Supply, Microbiological Research Department, to whom we are greatly indebted. Cells were harvested and extracted; the nucleotides were precipitated as their mercury salts and then converted into ammonium salts as described previously.

Ion-exchange chromatography

Measurements of ultraviolet absorption were made by means of a Unicam ultraviolet spectrophotometer, with a 1.0 cm. silica cell.

The conditions required to give a satisfactory separation of the two cytidine nucleotides by gradient elution on Dowex-2 resin (formate form) were determined in a series of small-scale analytical runs. The most satisfactory procedure was scaled up to allow the separation of nucleotides from a 100 l. batch of culture as follows:

Dowex-2 resin (chloride form) was converted into the formate form (Hurlbert *et al.* 1954) and the extremes of fine and coarse particles were removed by repeated settling of the resin, followed by decantation. A column of resin 50 cm. long was formed in a glass tube (52 cm. $\times$ 2.5 cm.) containing a glass-wool plug at the lower end. The resin was added in five portions and was allowed to settle under a pressure of approx. 10 lb./in.². Finally the column was washed with 1 l. of water containing a few drops of CHCl₃ for sterilization purposes. The rate of flow was conveniently adjusted by applying pressure.

The ammonium salts of the nucleotide fraction from a 100 l. culture (approximately 12 g. based on freeze-dried weight) in 1.5 l. of water adjusted to pH 7.0 were run on the column at a rate not exceeding 4.5 ml./min. Nucleosides

and lightly adsorbed materials were washed off the resin with approx. 2 l. of water, which reduced the optical density at 260 m μ . of the eluate to less than 0.1.

Gradient elution of the nucleotides from the column was performed with increasing concentration of formic acid (Hurlbert *et al.* 1954), and fractions of 14.2 ml. were collected with an automatic fraction collector. The optical density at 260 m μ . of each fraction was measured, and the nature of the purine or pyrimidine base present in each peak was established from the characteristic ratio of optical densities at 275 and 260 m μ . (Hurlbert *et al.* 1954). Fig. 1 shows a typical elution diagram obtained under the conditions described.

The mixing flask initially contained water (1400 ml.) and the reservoir N formic acid. At point A the acid in the reservoir was changed to 2N and at point B to 4N. The gradient was approximately linear (Cherkin, Martinez & Dunn, 1953). Peak IV corresponds to the cytosine nucleotide previously called CPX (Baddiley & Mathias, 1954), and peak V to the other cytosine nucleotide (CPY). These have now been identified as cytidine diphosphate ribitol and cytidine diphosphate glycerol respectively. The other peaks have been tentatively identified as follows. Peak I: diphosphopyridine nucleotide with some cytidine 5'-phosphate. The latter was shown to be present by rechromatography of peak I on a small Dowex-2 (formate) column, by gradient elution with ammonium formate solution. Peak II: adenosine 5'-phosphate. Peak III: an adenine derivative. This peak emerges with peak IV if the 2N formic acid stage is omitted in the elution. In a small-scale run the eluate corresponding to these combined peaks was freeze-dried and the resulting solid was hydrolysed with 72% (w/v) HClO₄ at 100° for 1 hr. The hydrolysate was chromatographed on Whatman no. 4 paper in propan-2-ol-water-HCl (Smith & Wyatt, 1951), with a descending front. On examination of the dried paper in ultraviolet light three spots were observed. (*R_F* values are not recorded because the solvent was allowed to drip from the paper.) The spots were cut out and eluted, together with 'blank' spots, and measurement of the spectra of the eluted materials showed them to be adenine, cytosine and cytidine. The last arose from incomplete hydrolysis of CPX. (The HClO₄ used was an

Fig. 1. Chromatography of extract of *L. arabinosus* on Dowex-2.

old sample and had apparently deteriorated.) Peak VI: mainly guanosine 5'-phosphate. The elution was discontinued beyond fraction number 290.

Charcoal purification of nucleotides

Norit grade A charcoal was washed several times alternately with 2*N* formic acid and a solution containing ethanol, water and aqueous NH_3 (sp.gr. 0.880) (5:4:1 by vol.). The charcoal was finally washed with water until neutral. The fractions corresponding to each cytidine peak were bulked and treated separately as follows:

To the bulked fractions (approx. 250 ml. of solution) was added 5 g. of washed charcoal. After vigorous stirring for 5 min., followed by standing for 30 min., the suspension was filtered through a bed of washed Supercel (Johns-Manville Co. Ltd.) silica formed in a small glass column (diam. 4 cm.) plugged at the lower end with glass wool. The small charcoal column thus formed was washed with 100 ml. of 2*N* formic acid and the washings were added to the filtrate. The charcoal was washed with water until the washings were neutral and the nucleotide was then eluted during a period of 1 hr. by passing through the column 100 ml. of a mixture of ethanol (25 vol.), water (24 vol.) and aqueous NH_3 (sp.gr. 0.880; 1 vol.). Without delay, the ammoniacal solution was evaporated under reduced pressure below 20° to about 2 ml., and the ammonium salt of the nucleotide was finally obtained by freeze-drying this solution.

The contaminating non-nucleotide phosphoric esters were present in the formic acid filtrate and washings from the charcoal column; the esters were recovered by evaporating the solution under reduced pressure below 25° to small bulk and subsequently freeze-drying.

In a typical experiment, starting with 100 l. of medium, the net weight of harvested cells was 298 g. From this the yield of CPX (CDP-ribitol) was 25 mg., calculated as cytidine, and the yield of CPY (CDP-glycerol) was 13 mg., also calculated as cytidine from its optical density at 280 μ . The recovery from the charcoal in both cases was about 80%.

Paper chromatography of cytosine nucleotides and related compounds

Ascending-front chromatography was carried out on Whatman no. 4 paper, previously washed with dilute acetic acid, then with water. Spots were examined under ultraviolet light and phosphoric esters were detected by the perchloric acid-molybdate spray reagent of Hanes & Isherwood (1949). Substances containing an α -glycol grouping were detected by the periodate-Schiff spray reagents (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956). The diphosphopyridine nucleotide spot gave the blue fluorescence in ultraviolet light characteristic of a quaternary pyridinium compound after exposure for 1 hr. to the vapour of a mixture of ethyl methyl ketone (1 vol.) and aqueous NH_3 (sp.gr. 0.880; 1 vol.) as described by Kodicek & Reddi (1951).

The solvent systems used were as follows: *A*, *n*-propanol-aqueous NH_3 (sp.gr. 0.880)-water, 6:3:1 by vol. (Hanes & Isherwood, 1949); *B*, *n*-butanol-acetic acid-water, 4:1:5 by vol., the organic phase being used (Partridge & Westall, 1948); *C*, isobutyric acid-ammonium isobutyrate (10:6 by vol. of isobutyric acid and 0.5*N*- NH_3 solution; Chargaff, Doniger, Elson, Magasanik & Vischer, 1950).

Identification of D-mannitol 1-phosphate

The non-nucleotide phosphoric ester which was separated from CDP-ribitol by the charcoal treatment appeared to be homogeneous when run on paper in several solvent systems. In the *n*-propanol- NH_3 -water solvent system the ester had an R_F of 0.30. D-Mannitol 1-phosphate was isolated from this eluate in the following manner. Ammonium formate was added slightly in excess of that required to neutralize the primary phosphoryl dissociation. Water and formic acid were removed by concentration *in vacuo* to a small volume, then freeze-drying. The residue obtained in this way from three 100 l. batches of bacterial culture was dissolved in water and the solution was passed through a column (30 cm. $\times$ 1.5 cm.) of Amberlite IR-120 (H^+ ion form) resin. The eluate was adjusted to pH 8 with $\text{Ba}(\text{OH})_2$ solution clarified by centrifuging, and evaporated *in vacuo* to 5 ml. Ethanol (15 ml.) was added and the precipitated barium salt was collected by centrifuging. The barium salt was dissolved in water (5 ml.) and reprecipitated with ethanol (15 ml.), then washed with acetone and dried in a desiccator over P_2O_5 . The yield was 940 mg. This was homogeneous as determined by paper chromatography. (Found: P, 6.9; Ba, 34.3. Calc. for $\text{C}_6\text{H}_{13}\text{O}_6\text{PBa}_0.5\text{H}_2\text{O}$: P, 7.6; Ba, 34.3%.)

A sample (approx. 50 μ g.) of the phosphate (obtained by running the ammonium salt on a paper chromatogram in solvent system *A* and eluting) in water (approx. 10 μ l.) was mixed with NaIO_4 (20 μ l. of a solution containing 40 mg./ml.) and the solution was kept at room temperature for 3 hr. A small amount of Dowex-50 (H^+ ion form) resin was added and the solution evaporated to 10 μ l. *in vacuo*. The residue was run on a paper chromatogram. A similar experiment was carried out on α -glycerophosphate (10 μ l. of a solution containing 15 mg. of the phosphate/ml.) in order to produce a standard preparation of glycolaldehyde phosphate.

In *tert*-butanol-picric acid-water (80 ml.:2 g.:20 ml.) both products had R_F 0.5 (descending-front method); in solvent system *C* both had R_F 0.33 (ascending front). The substances were detected by spraying with the molybdate reagent (Hanes & Isherwood, 1949) and with periodate followed by Schiff's reagent (Buchanan *et al.* 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956). The blue colour in the latter method developed slowly.

D-Mannitol was prepared from the phosphate by the action of phosphatases. The barium salt of the phosphate (940 mg.) was dissolved in water (100 ml.) and ammonium acetate buffer (25 ml., 0.3*M* to pH 7.5) was added, followed by a 1% solution of Polidase-S (Schwarz Laboratories Inc.) (25 ml.) which had been dialysed against water, and a few drops of toluene. The solution was incubated at 37° and

Table 1. R_F values of substances from ion-exchange column

Whatman no. 4 filter paper was used. Details of the solvent systems are given in the text.

Solvent system	...	...	A	B	C
Cytidine 5'-phosphate			0.26	—	0.53
Adenosine 5'-phosphate			0.32	0.11	0.61
Diphosphopyridine nucleotide			0.24	0.03	0.60
CDP-ribitol			0.35	—	0.34
CDP-glycerol			0.38	—	0.39
Mannitol 1-phosphate			—	—	0.36

after 42 hr. a sample was withdrawn and examined by paper chromatography. About half of the phosphate had been liberated as inorganic orthophosphate. Barium phosphate was removed by centrifuging, the pH was adjusted to 5.5 with acetic acid, and 2 ml. of a prostate phosphate solution, obtained from Dr G. Schmidt, was added. After 16 hr. at 37° hydrolysis was complete. The solution was centrifuged, then passed through a column (1.8 cm. $\times$ 10 cm.) of Dowex-50 (H^+ form) resin to remove Ba^{2+} and NH_4^+ ions. Water was removed by evaporation *in vacuo*, whereupon the residue of mannitol crystallized. Recrystallized from ethanol it formed needles (231 mg.), m.p. 166°, undepressed in admixture with an authentic sample, m.p. 166°. Its hexa-*O*-acetyl derivative had m.p. 125°, $[\alpha]_D^{25} + 16.9^\circ$ (c, 3.4 in benzene), also undepressed when mixed with an authentic sample, m.p. 125°, $[\alpha]_D^{25} + 17.0^\circ$ (c, 3.4 in benzene). The polyol and authentic mannitol were identical in their behaviour on paper chromatography. In solvent system *A* both had R_F 0.58 and in pyridine-ethyl acetate-water (2:7:1 by vol.) they had R_F 0.12.

Charcoal appeared to separate two non-nucleotide phosphoric esters from CDP-glycerol. These had R_F values in the *n*-propanol- NH_3 -water solvent system of 0.26 and 0.30 respectively. The substance with the latter R_F value is probably D-mannitol 1-phosphate.

Determination of base:phosphorus ratios in cytosine nucleotides

Total phosphorus determinations were carried out on chromatographically pure samples of nucleotides by the method of Allen (1940). The 'cytidine' content was determined from the optical density at 280 $m\mu$. in 0.01N-HCl, assuming a value of 12 800 for ϵ . The following ratios of base:P were found: CDP-ribitol, 1:1.85; CDP-glycerol, 1:2.03. In each case the inorganic phosphate present was less than 5% of the total phosphate.

RESULTS AND DISCUSSION

In the earlier ion-exchange separations on Dowex-1 (chloride form) the two cytosine nucleotides were obtained as a single fraction which contained a large amount of inorganic phosphate. When Dowex-2 (formate form) was used with a continuously increasing concentration of formic acid during the elution, a clean separation of the two substances was achieved (see Fig. 1). These were examined by paper chromatography in *n*-propanol-ammonia-water. No cross-contamination was observed, but occasionally an adenine nucleotide impurity was detected in both substances. No inorganic phosphate could be detected in any of the fractions up to the point at which guanosine 5'-phosphate had been eluted.

The first cytosine nucleotide to be eluted from the column (previously called CPX, Baddiley & Mathias, 1954) had a lower R_F in *n*-propanol-ammonia-water than the second cytosine nucleotide (previously called CPY) (see Table 1 for R_F values). Both nucleotides were contaminated with non-nucleotide phosphoric esters. These could not be

separated by ion-exchange chromatography and had R_F values similar to the cytidine compounds in *n*-propanol-ammonia-water and in *isobutyric acid*-ammonia-water. Good separation of these phosphates from the nucleotides was achieved by adsorption of the latter on charcoal, followed by elution with aqueous ethanolic ammonia.

The two cytidine compounds when purified in the above manner usually moved on paper as single spots, the first nucleotide from the column moving more slowly than the second. When base:phosphorus ratios were determined earlier on impure samples a value of about 1:1 was obtained in each case. However, the large blank values for orthophosphate in these determinations were disturbing and these ratios have been redetermined. It is now found from the absorption of light at 280 $m\mu$. and from total phosphorus values of highly purified samples that both nucleotides contain two phosphate groups to one cytidine residue.

The nucleotide with the higher R_F in *n*-propanol-ammonia has been identified as cytidine diphosphate glycerol (CDP-glycerol) (I), in which the pyrophosphate group joins cytidine at position 5' with glycerol in the α -position. This structure has been deduced from a study of the products of hydrolysis of CDP-glycerol with acid, alkali and enzymes. A short account of this evidence has been presented already (Baddiley, Buchanan, Carss, Mathias & Sanderson, 1956), and full details will be published elsewhere.

It is not yet possible to assign a definite function to this nucleotide, but its structure strongly suggests that it may be a coenzyme for some enzymic

reaction which involves glycerophosphate. By analogy with CDP-choline, it is possible that it might participate in phospholipid synthesis in which it could act as a donor of the α -glycerophosphate residue to bases or fatty acids.

The other cytosine nucleotide has been identified in a somewhat similar manner as cytidine diphosphateribitol (CDP-ribitol) (II) (Baddiley, Buchanan, Carss & Mathias, 1956). The configuration of the ribitol 1-phosphate residue in this nucleotide is not yet settled, and so it is not possible to relate it biochemically to D-ribose 5-phosphate (or D-ribulose 5-phosphate) on the one hand or to D-ribose 1-phosphate (or D-ribulose 1-phosphate) on the other.

The function of the ribitol nucleotide is unknown. It would seem likely that it acts as a coenzyme in some reaction involving pentitol 1-phosphates. Although pentitols, including ribitol (adonitol) have been found to occur naturally, and are utilized by some micro-organisms, the mechanism of their metabolism is unknown and pentitol phosphates have not been detected hitherto.

The presence of these nucleotides in *L. arabinosus* suggests the existence of an active polyol metabolism in this organism. This is further supported by the identification of one of the non-nucleotide phosphoric esters in extracts of *L. arabinosus* as D-mannitol 1-phosphate (III). Identification was achieved by enzymic dephosphorylation to D-mannitol, characterized as the free polyol and as its hexa-O-acetate. The phosphate must occupy position 1, since treatment with periodate gave glycollaldehyde phosphate (identified chromatographically), which proves the presence of the grouping $\text{H}_2\text{O}_5\text{P} \cdot \text{O} \cdot \text{CH}_2 \cdot \text{CH}(\text{OH}) \cdot \text{CH}(\text{OH})-$. Positions 1 and 6 in mannitol are identical.

Until recently hexitol phosphates had not been detected in Nature. It is now known, however, that fructose 6-phosphate is reduced to mannitol 1-phosphate in the presence of DPN and an enzyme which occurs in certain bacteria (Wolff & Kaplan, 1956). *L. arabinosus* contains particularly large amounts of this enzyme.

SUMMARY

1. Cytidine diphosphate glycerol, cytidine diphosphate ribitol and D-mannitol 1-phosphate have been isolated from *Lactobacillus arabinosus*.

2. The method of isolation includes gradient-elution chromatography on Dowex-2 resin and adsorption on charcoal.

3. The base:phosphate ratio in both nucleotides was 1:2, indicating that they are derivatives of cytidine diphosphate.

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