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Thymidine Diphosphate Mannose and Thymidine Diphosphate Rhamnose in *Streptomyces griseus*

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Streptomyces griseus is grown in large amounts for the commercial production of streptomycin. Our interest in the mechanism of biosynthesis of the antibiotic led to a study of nucleotides in this organism, as it seemed possible that the carbohydrate residues in streptomycin, *N*-methyl-L-glucosamine and streptose, would occur in combination with nucleotides at some stage in the biosynthetic route.

Although nucleotides containing streptose or *N*-methyl-L-glucosamine have not yet been found in the complex mixture obtained from the micro-organism, several new nucleotides were detected. Two of these have been identified as thymidine diphosphate mannose and thymidine diphosphate rhamnose. The isolation and structure of these compounds is described, and their possible function as streptomycin precursors is discussed. A mechanism for the biosynthesis of rhamnose through

thymidine derivatives is suggested. A preliminary account of some of this work has been published (Baddiley & Blumsom, 1960).

MATERIALS AND METHODS

Abbreviations. TDP-mannose, thymidine diphosphate mannose; TDP-rhamnose, thymidine diphosphate rhamnose; TDP-glucose, thymidine diphosphate glucose.

Thymidine was purchased from the California Corp. for Biochemical Research, Los Angeles 63. Thymidine 5'-phosphate was kindly supplied by Dr A. M. Michelson. Uridine diphosphate glucose was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. Guanosine diphosphate mannose and uridine diphosphate *N*-acetylglucosamine were kindly supplied by Dr E. Cabib. DL-Glyceric acid was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. D-Glyceric acid was obtained by hydrolysis of its 3-phosphate with a prostate-phosphatase preparation. DPNH was purchased from C. F. Boehringer,

Mannheim. Rabbit-muscle enzymes were prepared by Racker's (1947) method. Crude yeast hexokinase was a gift from Sigma Chemical Co., St Louis, Mo.

Quantitative analysis. Phosphate was determined by the method of Fiske & Subbarow (1925), and reducing sugars were determined by the method of Park & Johnson (1949). Rhamnose was also determined according to Dische & Shettles (1948). Quantitative determination of thymine, thymidine and thymidine 5'-phosphate was based on molar extinction values at the appropriate wavelength of maximum absorption at known pH values.

Paper chromatography. Whatman no. 1 paper, previously washed with 2*N*-acetic acid and then water, was used with ascending or descending solvents.

Sugars were examined in butan-1-ol-pyridine-water (6:4:3) (Jeanes, Wise & Dimler, 1951) or in phenol-water (9:1) (Partridge, 1948). Spots were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) or with the phloroglucinol-hydrochloric acid reagent (Borenfreund & Dische, 1957). Polyols were converted in part into anhydro compounds and examined in propan-1-ol-ammonia (sp.gr. 0.88)-water (6:3:1) with the periodate-Schiff reagents (Baddiley, Buchanan & Cars, 1957b).

Pyrimidines and nucleotides were examined in butan-1-ol-water (Markham & Smith, 1949), propan-2-ol-ammonia (sp.gr. 0.88)-water (85:1:3:15) (Hershey, Dixon & Chase, 1953), propan-2-ol-conc. hydrochloric acid (171:41) (Wyatt, 1951), or two ethanol-ammonium acetate mixtures (Paladini & Leloir, 1952). The position of these compounds was demonstrated by observation in ultraviolet light. Nucleotides were also located with perchloric acid-molybdate reagent (Hanes & Isherwood, 1949).

Ionophoresis on paper was carried out with apparatus similar to that described by Markham & Smith (1952). Whatman no. 1 paper moistened with 0.05*M*-sodium borate solution (pH 9.2) was used for sugars (Consden & Stanier, 1952), a potential of 7.5 v/cm. being applied for 6 hr. The sugars were detected with alkaline silver nitrate by the modified technique of Frahn & Mills (1959).

Isolation of nucleotides

An ethanolic extract of *Streptomyces griseus* was kindly supplied by Dr I. L. S. Mitchell of Glaxo Laboratories Ltd. It had been prepared in the following manner.

Cells were harvested and treated immediately with an equal volume of ethanol. The suspension was boiled with stirring and kept at the boil for a further 2 min., then cooled and filtered. The residue was heated with two lots of ethanol as before and the extracts were combined and adjusted to pH 7.0.

The ethanol extract (5 l.) was passed through a column (50 cm. \times 4 cm.) of Dowex 1X2 resin (Cl⁻ form). The column was washed with water, then with 0.005*N*-hydrochloric acid until the extinction of the washings at 260 m μ was negligible. Nucleotides were eluted with a solution that was 0.01*N* with respect to hydrochloric acid and 0.18*M* with respect to lithium chloride. The eluate was neutralized with lithium hydroxide, evaporated *in vacuo* to about 70 ml. and poured with stirring into acetone-ethanol (4:1) (2 l.). The light-brown precipitate was collected, washed with acetone-ethanol (4:1) to remove traces of lithium chloride, dried *in vacuo* and stored at -20°.

A solution of the nucleotide mixture (about 1700 μ moles,

calculated as uridine from its absorption at 260 m μ) in water (1 l.) was passed through a column (100 cm. \times 2 cm.) of Dowex 1X2 (200-400 mesh) resin (Cl⁻ form). Elution was achieved with a linearly increasing concentration of lithium chloride at constant acid concentration in an apparatus similar to that described by Pontis & Blumsom (1958). The reservoir contained 4 l. of a solution which was 0.16*M* with respect to lithium chloride and 0.01*N* with respect to hydrochloric acid; the mixing chamber contained 0.01*N*-hydrochloric acid (4 l.). A flow rate of about 1.5 ml./min. was maintained and 10-ml. fractions were collected automatically. Compounds that absorbed ultraviolet light were detected, and their approximate amount was recorded automatically as they emerged from the column, by a combined ultraviolet light source, photocell, amplifier and recorder (Gilson Medical Electronics, Madison, Wis.). Elution was continued until the concentration of lithium chloride in the mixing chamber rose to 0.14*M*. At this point the reservoir was replaced by one containing 2 l. of a solution which was 0.18*M* with respect to lithium chloride and 0.01*N* with respect to hydrochloric acid, and the solution in the mixing chamber was changed to 2 l. of a solution which was 0.14*M* with respect to lithium chloride and 0.01*N* with respect to hydrochloric acid.

Fractions corresponding to ultraviolet-absorption peaks were combined, adjusted to pH 7.0 with lithium hydroxide solution and freeze-dried. Lithium chloride was extracted with an acetone-ethanol mixture as before and the dried preparations were kept at -20°.

Preparation of hydrolysates

Hexoses. The following procedure was adopted for the preparation of hexoses liberated by acid hydrolysis of the pyrimidine nucleotides from the organism. Appropriate nucleotide fractions were hydrolysed at 100° in 0.01*N*-sulphuric acid for 20 min. The resulting solutions were passed through small columns of Dowex 50 (H⁺) and Amberlite IR-4B(OH⁻) resins and the eluates were evaporated *in vacuo*. The residues were examined by paper chromatography.

Bases. Thymine was obtained from appropriate fractions in the following manner. The material was heated at 100° in 6*N*-hydrochloric acid for 8 hr. Most of the acid was removed by repeated evaporation with water, and an aqueous solution was then passed through columns of resins as for hexoses. The residue was examined spectroscopically and by paper chromatography.

Synthesis of thymidine diphosphate mannose

Calcium thymidine 5'-phosphate (300 mg.) was converted into the ammonium salt by passing its solution through a column of Dowex 50 (H⁺) resin, neutralizing with ammonia and evaporating the resulting solution to dryness *in vacuo*. To the residue was added formamide (7.5 ml.), 2*N*-ammonia solution (2.0 ml.) and a solution of dicyclohexylcarbodi-imide (900 mg.) in *tert*-butyl alcohol (6 ml.). The mixture was heated for 10 hr. at 80° and dicyclohexylurea was removed by filtration. The *tert*-butyl alcohol was removed from the filtrate under reduced pressure and the aqueous formamide solution was shaken with four lots of ether. Solvent was evaporated (oil pump), acetone (55 ml.) and ether (180 ml.) were added, and the mixture was kept in a refrigerator overnight. Solvent was

decanted from the semi-solid precipitate, which was dried *in vacuo*, then dissolved in water (25 ml.) and freeze-dried.

The phosphoramidate was homogeneous on paper chromatography in the propan-1-ol-ammonia solvent and had R_{TMP} 1.86. It was detected by observation in ultraviolet light and with the chlorine-benzidine reagents (Reindel & Hoppe, 1954). The yield, calculated from the absorption of a sample at 268 $m\mu$, was 580 μ moles.

Barium mannose 1-phosphate (Colowick, 1938) was converted into the tri-*n*-octylammonium salt by a procedure similar to that described by Michelson & Todd (1956) for glucose 1-phosphate. A solution of the tri-*n*-octylammonium salt (2.5 m-moles) in dry pyridine (25 ml.) was added to a solution of the phosphoramidate (580 μ -moles) in dry pyridine (10 ml.), and the mixture was shaken under anhydrous conditions for 5 days at 28°. Paper chromatography (ethanol-ammonium acetate, pH 3.8) indicated that the mannose nucleotide was formed in about 65% yield.

Most of the pyridine was evaporated *in vacuo*, water (25 ml.) was added, and the solution was shaken with three lots of ether. After aeration, products were adsorbed on a column (100 cm. \times 1 cm.) of Dowex 1X2 (200-400 mesh; Cl⁻ form). Elution was carried out with a linear gradient, with in the mixing flask a solution (2 l.) which was 0.1M with respect to lithium chloride and 0.01N with respect to hydrochloric acid, and in the reservoir a solution (2 l.) which was 0.18M with respect to lithium chloride and 0.01N with respect to hydrochloric acid. Fractions (10 ml.) were collected automatically at a flow rate of 1.5 ml./min. The mannose nucleotide emerged after about 2 l. of solution had been passed through the column. Appropriate

fractions were combined, the pH was adjusted to 7 with lithium hydroxide solution and water was removed by evaporation *in vacuo* to a final volume of 5 ml. A mixture (1 l.) of acetone-ethanol (4:1) was added and, after keeping at -14° overnight, the precipitated *lithium salt of TDP-mannose* was collected, washed with the acetone-ethanol mixture until free from chloride ions, dried (phosphorus pentoxide) and kept under anhydrous conditions at 0° (Found: P, 9.2; thymine, 17.9. $C_{18}H_{24}O_{16}N_2P_2Li_2 \cdot 4H_2O$ requires P, 9.5; thymine, 17.7%).

RESULTS

Several of the nucleotides corresponding to absorption peaks in the ion-exchange fractionation of the mixture from the organism were tentatively identified from their position in the elution pattern, ultraviolet absorption and behaviour on paper chromatography. Thus, the compound corresponding to peak VII is probably uridine diphosphate glucose and that corresponding to peak IX is uridine 5'-pyrophosphate. The intermediate peak VIII represented thymidine derivatives and corresponds to the material used in most of the work described here. R_F values for these nucleotides and several known compounds are given in Table 1.

Material corresponding to peak VIII was usually light yellow. The coloured impurity was removed by chromatography in the ethanol-ammonium acetate (pH 3.8) solvent. Complete separation of

Table 1. Paper chromatography of nucleotides from *Streptomyces griseus* and standard compounds

	$R_{adenosine}$ in ethanol-ammonium acetate-water at	
	pH 3.8	pH 7.5
Uridine 5'-phosphate	0.72	0.33
Uridine 5'-pyrophosphate	0.38	0.18
Guanosine diphosphate mannose	0.23	0.30
Uridine diphosphate glucose	0.40	0.41
Uridine diphosphate <i>N</i> -acetylglucosamine	0.54	0.53
Thymidine 5'-phosphate	0.77	0.50
Compound corresponding to peak VII	0.40	0.39
Compounds corresponding to peak VIII	0.53, 0.55	0.50, 0.35
Compound corresponding to peak IX	0.38	0.18
Nucleotide after treatment of VIII with 0.1N-HCl at 100° for 15 min.	0.76	0.50

Table 2. Paper chromatography and ionophoresis of hexoses from thymidine derivatives and standard compounds

	R_{ribose} in		Migration (cm.) on ionophoresis
	Butanol-pyridine-water	Phenol-water	
Fructose	0.85	0.65	1.15
Glucose	0.77	0.47	1.30
Mannose	0.85	0.55	0.89
Galactose	0.70	0.56	1.18
Rhamnose	1.09	1.05	0.66
Sugars from thymidine compounds	0.85, 1.07	0.54, 1.04	0.89, 0.65

the two nucleotide components in this fraction was only achieved after prolonged (40 hr.) paper chromatography.

Identification of hexoses

The hexoses liberated on mild acid hydrolysis of the thymidine compounds were examined on paper with butanol-pyridine-water or phenol-water solvent systems and by ionophoresis. From results given in Table 2 it was concluded that the hexose components were rhamnose and mannose.

The sugar mixture was reduced with sodium borohydride during 24 hr. at room temperature (Abdel-Akher, Hamilton & Smith, 1951). The salt-free products were heated for 2 hr. at 100° in 5*N*-hydrochloric acid, whereupon a mixture of hexitols and anhydro compounds was obtained. After evaporation of acid these were examined by paper chromatography in the propan-1-ol-ammonia solvent and detected with the periodate-Schiff reagents.

The ratio mannose:rhamnose in the mixture was calculated from the value for total reducing sugar (Park & Johnson, 1949) and the value for rhamnose (Dische & Shettles, 1948). The former method is equally sensitive to both sugars, whereas the latter method is insensitive to mannose. The ratio observed was 4:1.

Identification of base

The ultraviolet spectra at pH 7 and 12 of the nucleotide corresponding to peak VIII suggested that the base residue was entirely thymine. This was confirmed by chromatographic examination of the products of hydrolysis in 6*N*-hydrochloric acid. Thymine was identified by comparison with authentic bases in several solvent systems (Table 3).

Less vigorous hydrolysis (0.1*N*-hydrochloric acid for 15 min. at 100°) gave one nucleotide. This was examined by evaporation of acid, neutralization with ammonia and chromatography in the ethanol-ammonium acetate solvents (Table 1). The nucleotide was indistinguishable from authentic thymidine 5'-phosphate.

Quantitative analyses

The ratio thymidine:total phosphate:labile phosphate:hexose was 1.04:2.0:0.96:1.06 for the mixture of the two nucleotides. Total phosphate was taken as 2.0 and labile phosphate was determined by hydrolysis for 15 min. at 100° in *N*-sulphuric acid.

Identification of thymidine and its 5'-phosphate

Hydrolysis of the thymine nucleotide mixture in 0.1*N*-hydrochloric acid at 100° for 15 min. gave a nucleotide (see Table 1) indistinguishable from thymidine 5'-phosphate. It was isolated by absorption on a small column of Amberlite IR-4B (OH⁻) resin, elution with 5*N*-ammonia solution and evaporation to dryness.

The location of the phosphate residue in this nucleotide was confirmed by studies with *Crotalus adamanteus* venom. A solution of the nucleotide in water (0.2 ml.) and 0.1*M*-barbitone buffer, pH 9.0 (0.4 ml.), was mixed with a solution (0.3 ml.) of the venom (5 mg./ml.) and kept for 16 hr. at 37°. After removal of protein with trichloroacetic acid (final concentration, 3%) followed by centrifuging, most of the buffer was precipitated by evaporation *in vacuo*. Paper chromatography in *isopropanol*-ammonia and rechromatography in butan-1-ol-water yielded thymidine, identical with authentic material (see Table 3). The nucleoside did not react with the periodate-Schiff reagents (Buchanan, Dekker & Long, 1950; Baddiley, Buchanan, Handschumacher & Prescott, 1956) but gave a positive reaction for deoxy-sugars with the cysteine-sulphuric acid reagent (Buchanan, 1951).

Analysis of a sample of the thymidine from a paper chromatogram by the cysteine-sulphuric acid method for deoxy-pentoses (Stumpf, 1947; Manson & Lampen, 1951) gave a ratio thymine:deoxyribose as 1.03:1.0 (deoxyribose taken as 1.0).

Stability of mannose and rhamnose nucleotides towards ammonia

Samples of pure TDP-rhamnose, TDP-mannose, uridine diphosphate glucose and guanosine diphos-

Table 3. Paper chromatography of base and nucleoside compared with authentic compounds

	<i>R</i> _{adenine} in		
	Butan-1-ol-water	Propan-2-ol-ammonia	Propan-2-ol-HCl
Guanine	0.33	0.41	0.63
Uracil	0.78	0.97	1.88
Thymine	1.19	1.33	2.17
Uridine	0.38	0.79	1.83
Thymidine	1.12	1.46	2.31
Base from VIII	1.19	1.32	2.17
Nucleoside from VIII	1.12	1.44	2.29

phate mannose were dissolved in *N*-ammonia solution and kept for 18 hr. at 27°. The samples were then examined by paper chromatography in the ethanol-ammonium acetate (pH 3.8) solvent mixture, products being detected by examination in ultraviolet light and with the perchloric acid-molybdate reagent. Semi-quantitative information was obtained spectroscopically.

About 98% of the uridine diphosphate glucose and all the TDP-rhamnose had been converted into hexose cyclic phosphate and nucleoside monophosphates under the conditions employed, whereas guanosine diphosphate mannose and TDP-mannose were unaffected. The rhamnose cyclic phosphate (R_{TMP} 0.84) was not studied further.

Oxidation of thymidine diphosphate mannose to D-glyceric acid

Uridine diphosphate glucose and TDP-mannose were oxidized with periodate, then bromine, and glyceric acid was separated from the hydrolysed products. The following procedure and quantitative determinations are based on studies with the mannose-containing nucleotide; the procedure for the glucose compound was closely comparable. Glyceric acid 3-phosphate arising from the ribose phosphate residue in uridine diphosphate glucose would not have been hydrolysed under the acidic conditions used at one stage in this method, and thus would not contribute to the value found for *D*-glyceric acid.

The nucleotide (2.5 μ moles) was mixed with 0.13 *M*-sodium periodate (0.2 ml.) and, after 48 hr. at room temperature in the dark, excess of periodate was destroyed by the addition of ethylene glycol (0.01 ml.). The solution was kept for a further 48 hr. at room temperature then mixed with an excess of bromine in a buffer consisting of barium hydroxide (189 mg.) and benzoic acid (146 mg.) in water (35 ml.) at pH 5.5. After 60 hr. at room temperature in the dark, excess of bromine was removed by aeration and the solution was concentrated to 0.5 ml. *in vacuo*. Precipitated benzoic acid was removed by centrifuging and the solution was passed through a column (5 cm. \times 0.3 cm.) of Amberlite IR-120 (H^+) resin to remove barium ions, then evaporated to dryness at room temperature. The residue was hydrolysed with 5 *N*-hydrochloric acid for 5 hr. at 100°. Acid was removed by repeated evaporation with water and the pH was adjusted to a value above 11 with sodium hydroxide. This ensured hydrolysis of the bimolecular lactone of glyceric acid, which could be demonstrated chromatographically in the acid hydrolysate. Glyceric acid was separated by paper chromatography in propan-1-ol-ammonia and eluted from the paper with water. The purple colour it gave with the periodate-Schiff reagents

developed rapidly. The eluate, and an eluate from a corresponding control area of paper, were evaporated to dryness and the residues dissolved in water (0.1 ml.).

A sample (5 μ l.) of the glyceric acid solution was used for total glyceric acid determination. This was carried out by periodate oxidation (Dixon & Lipkin, 1954) during 60 min. at room temperature. Under these conditions 1 mol.prop. of periodate was consumed for each mol.prop. of glyceric acid. Further consumption of periodate was very slow. The concentration of glyceric acid determined by this method was 2.0 μ moles/ml.

Enzymic determination of *D*-glyceric acid was carried out according to the procedure described by Archibald, Baddiley & Buchanan (1961). Phosphorylation and reduction were combined in a single operation by mixing a sample (0.08 ml.) of the glyceric acid solution with a solution containing the following: 0.01 *M*-phosphate buffer, pH 7.3 (3.0 ml.); 0.1 *M*-magnesium sulphate (0.05 ml.); 0.2 *M*-adenosine triphosphate (0.05 ml.); rabbit-muscle enzymes (0.05 ml. of solution containing 40 mg./ml.); hexokinase (0.05 ml. of solution containing 80 mg./ml.), and reduced diphosphopyridine nucleotide (0.07 ml. of solution containing 20 mg./ml.). Oxidation of the pyridine nucleotide was observed spectrophotometrically at 37° during 60 min. The concentration of *D*-glyceric acid was 2.1 μ moles/ml., in close agreement with the value found by the chemical method. *L*-Glyceric acid was not reduced in this system.

DISCUSSION

Particular attention was directed towards a fraction which was eluted between uridine diphosphate glucose and uridine diphosphate, and which had an absorption spectrum closely similar to that of thymidine and its derivatives. Although this fraction contained two nucleotides, much of the preliminary degradative work was carried out with this rather than with the individual nucleotides. The mixture was hydrolysed at pH 1 for 15 min. at 100°. These hydrolysis conditions would be expected to release sugars from nucleoside diphosphate sugar compounds. Two sugars with R_{ribose} values corresponding to mannose and rhamnose were detected, and their identification was confirmed by paper electrophoresis in a borate buffer (pH 9.2). An alternative method for the identification of small amounts of sugars was applied successfully to this mixture. The sugars were reduced with sodium borohydride to the corresponding sugar alcohols, and these were readily identified by heating with mineral acid and examining the reaction mixture on paper chromatograms. It is known (Baddiley, Buchanan & Cars, 1957b) that

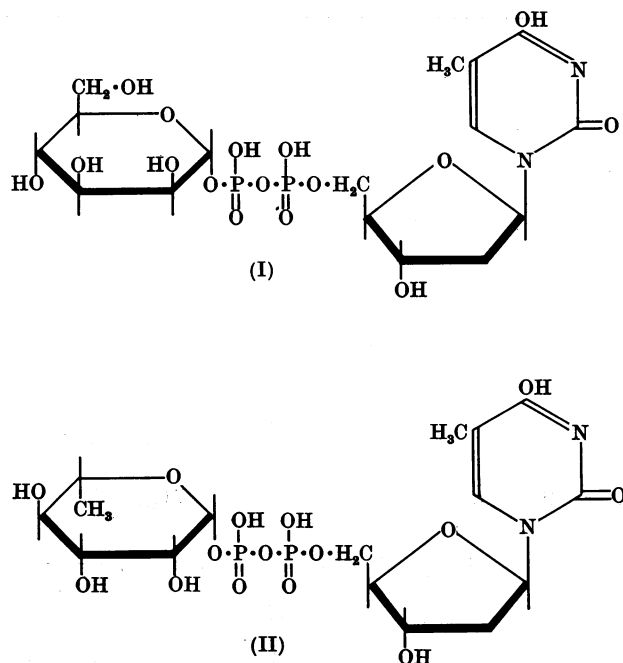
pentitols and hexitols treated in this way give anhydropolyols or mixtures of anhydropolyols that can be identified chromatographically. The position, colour and relative intensity of the spots corresponding to anhydropolyols obtained from the sugar alcohol mixture were identical with that given by a mixture (4:1) of mannitol and rhamnitol which had been treated similarly. It was shown from studies on the separated nucleotides that rhamnose was associated with the faster-moving nucleotide and mannose with the slower.

Although the ultraviolet-absorption spectrum of the nucleotide mixture suggested that both components were thymidine derivatives, the close similarity between the spectra of thymidine phosphate and uridine phosphate made it necessary to use other methods for the identification of the base. When the mixture was hydrolysed for 8 hr. at 100° in 6N-hydrochloric acid the only base formed was thymine. Moreover, hydrolysis at pH 1, followed by treatment with *Crotalus adamanteus* venom at pH 9, liberated thymidine. It gave the typical reaction for deoxynucleosides with the cysteine-sulphuric acid spray reagents, and the negative periodate-Schiff reaction on paper indicated the absence of an α -glycol system.

These observations and the quantitative analyses indicate that in the two nucleotides thymidine is joined at the 5'-position through a pyrophosphate residue to either mannose or rhamnose as shown in structures (I) and (II) respectively. The ready liberation of thymidine by the action of *C. ada-*

manteus venom on an acid hydrolysate indicates that a pyrophosphate residue is at the 5'-position, since it is known (Heppel & Hilmoe, 1951) that this venom contains a powerful 5'-nucleotidase, but has little action on nucleoside 3'-phosphates. The absence of reducing properties before hydrolysis, and the ready liberation of mannose and rhamnose by gentle acid hydrolysis, support the normal structure for this class of nucleotides in which the pyrophosphate group is attached to the 1-position of the hexose.

The stereochemical configuration of the mannose in TDP-mannose was established by a technique developed in connexion with other work (Archibald *et al.* 1961) and not previously applied to sugar phosphates. The nucleotide was first oxidized with periodate and the resulting dialdehyde was then oxidized with bromine water to a dicarboxylic acid (see Fig. 1). Acid hydrolysis gave glyceric acid together with glyoxylic acid and thymidine 5'-phosphate. The stereochemical configuration of the glyceric acid was established by enzymic phosphorylation followed by quantitative determination of D-glyceric acid 3-phosphate through reduction with reduced diphosphopyridine nucleotide in the presence of enzymes from rabbit muscle (cf. Baddiley, Buchanan & Carss, 1957*a*). All the glyceric acid formed in this way from TDP-mannose had the D-configuration, and it follows that the hexose in this nucleotide is D-mannose. Although this procedure can be applied to quite small samples of sugar phosphates or nucleotides,



the yield of glyceric acid is rather low. This probably arises through the complexity of the final oxidation mixture and the difficulty experienced in removing ions and nucleotide fragments before determining the glyceric acid. The acidic conditions that follow the oxidation sometimes cause partial conversion of the resulting glyceric acid into a dimeric lactone. This compound, which gives an amide with an R_f slightly higher than that of glyceric acid in propan-1-ol-ammonia, is readily converted into glyceric acid in alkali. It is reasonable to assume that the TDP-rhamnose is a deriva-

tive of L-rhamnose, this being the only natural form of rhamnose observed so far.

The configuration and conformation of the substituent group at the 1-position in the mannose and rhamnose residues of these nucleotides has been determined by studying their behaviour towards dilute ammonia in comparison with that of uridine diphosphate glucose. It is known that nucleoside diphosphate sugar coenzymes, like phosphodiester, are unstable towards dilute alkali if they possess a hydroxyl group in suitable stereochemical proximity to the pyrophosphate group. Hydro-

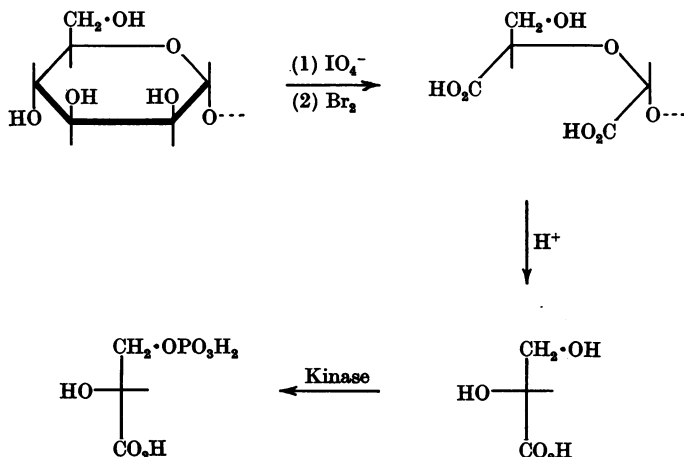


Fig. 1. Degradation of TDP-mannose to D-glyceric acid.

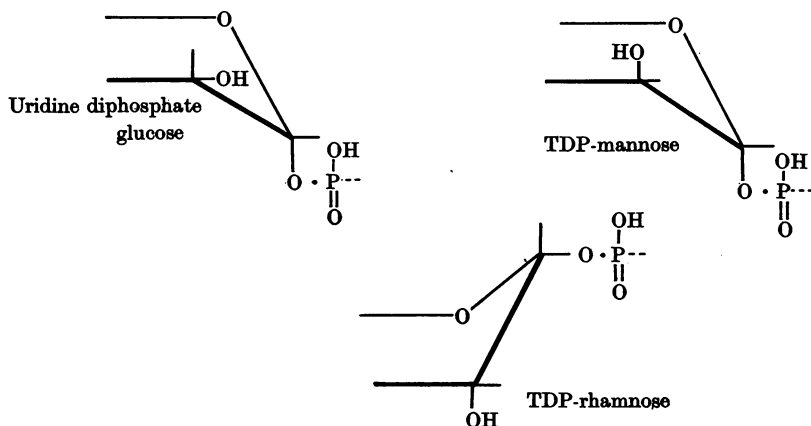


Fig. 2. Conformation of functional groups about $C_{(1)}$ and $C_{(2)}$ in different nucleotides.

	Configuration of glycoside	Relationship of substituents at $C_{(1)}$ $C_{(2)}$	Conformation of substituent at	
			$C_{(1)}$	$C_{(2)}$
Uridine diphosphate glucose	α	<i>cis</i>	Axial	Equatorial
TDP-mannose	α	<i>trans</i>	Axial	Axial
TDP-rhamnose	β	<i>cis</i>	Equatorial	Axial

lysis proceeds through intermediate formation of cyclic phosphates, and under suitable conditions the cyclic phosphate may be a principal hydrolysis product. Thus, the ready decomposition of uridine diphosphate glucose to glucose 1:2-cyclic phosphate in the presence of dilute ammonia at room temperature (Paladini & Leloir, 1952) supports the conclusion from other evidence that this nucleotide is a derivative of α -glucose 1-phosphate. In this case the *cis* relationship of the functional groups at the 1- and 2-positions greatly facilitates cyclic phosphate formation (Fig. 2).

Guanosine diphosphate mannose differs in that it is quite stable towards dilute ammonia (Cabib & Leloir, 1954), because of the *trans* relationship of the functional groups at the 1- and 2-positions in α -D-mannose 1-phosphate (Fig. 2) (Buchanan, Lynch, Benson, Bradley & Calvin, 1953). These groups in derivatives of α -D-mannose will both adopt the axial conformation and cyclization would be extremely difficult. Under conditions which ensured complete decomposition of uridine diphosphate glucose, both TDP-mannose and guanosine diphosphate mannose were unaffected; it follows that TDP-mannose must be an α -D-mannose derivative with the conformation illustrated in Fig. 2. Corresponding β -D-mannose 1-phosphate derivatives would have a *cis* arrangement of groups at the 1- and 2-positions, and these groups would have an equatorial-axial relationship. This situation would be very similar but not identical with that in uridine diphosphate glucose, and the nucleotides would resemble each other in their lability towards ammonia.

TDP-rhamnose was much less stable towards ammonia than was the mannose nucleotide. Although quantitative information was not obtained, paper-chromatographic evidence suggested that the rhamnose nucleotide was similar to uridine diphosphate glucose in its lability towards dilute ammonia. It follows that TDP-rhamnose is a β -rhamnose derivative, in which substituents at positions 1 and 2 are in the *cis* relationship, the phosphate being equatorial and the hydroxyl at the 2-position being axial (see Fig. 2). The relationship of substituents in this case is identical with that of β -mannosides.

Confirmation of the structure assigned to TDP-mannose was obtained by synthesis. Thymidine 5'-phosphoramidate was prepared from thymidine 5'-phosphate and ammonia in the presence of dicyclohexylcarbodi-imide (cf. Moffatt & Khorana, 1958; Chambers & Moffatt, 1958). Reaction between the phosphoramidate and the tri-*n*-octylamine salt of α -D-mannose 1-phosphate occurred readily in pyridine, and thymidine diphosphate mannose was isolated from the mixture of products by ion-exchange chromatography. The syn-

thetic and natural nucleotides were indistinguishable on paper and in their behaviour towards hydrolysis.

Thymidine diphosphate sugars are uncommon. Only recently have communications appeared describing a thymidine diphosphate rhamnose in *Lactobacillus acidophilus* R-26 and *Escherichia coli* 15 T⁻ (Okazaki, 1960), and thymidine diphosphate deoxy sugars in other organisms (Strominger & Scott, 1959). Although it is likely that the rhamnose nucleotide from the *Lactobacillus* is identical with that from *Streptomyces griseus*, no direct comparison has been made and the stereochemistry of the 1-position has not been determined for the former. TDP-mannose has not been described before and, in fact, the only nucleotide containing mannose which has been observed hitherto is guanosine diphosphate mannose.

Experiments on the biochemical significance of TDP-mannose and TDP-rhamnose are in progress, but preliminary results indicate that they are related to each other enzymically. It has been shown recently that an enzyme is present in *Streptococcus faecalis* and in *Pseudomonas aeruginosa* which synthesises TDP-glucose from thymidine triphosphate and α -D-glucose 1-phosphate. Other enzymes in these organisms are able to convert the TDP-glucose into TDP-rhamnose by an unidentified route (Kornfeld & Glaser, 1960; Pazur & Shuey, 1960). We find that an enzyme preparation from an acetone powder of *Streptomyces griseus* is able to convert TDP-mannose into TDP-glucose. Synthetic TDP-glucose was converted by this extract into TDP-mannose, but a simultaneous accumulation of TDP-rhamnose was not observed. However, considerable amounts of a new thymine nucleotide containing an unidentified sugar were formed from both TDP-glucose and TDP-mannose. The mechanism whereby such changes occur has not yet been established experimentally, but the overall process is similar to that in the conversion of guanosine diphosphate mannose into guanosine diphosphate L-fucose (Ginsburg, 1960).

A possible mechanism for the conversion of TDP-glucose into TDP-rhamnose is given in Fig. 3. Inversion and reduction at the 6-position could be achieved by dehydration to a glycoseen (III), followed by prototropic rearrangement to a 4-carbonyl intermediate (IV). The subsequent rearrangement of (IV) to the 3-carbonyl derivative (V) proceeds through a normal enediol intermediate. A spontaneous rearrangement of this type occurs during recrystallization of 2-oxoglucosides (Assarsson & Theander, 1958). Finally, reduction of (V) would give TDP-rhamnose. This last stage is analogous to the reduction of a carbonyl intermediate in the enzymic intercon-

version of uridine diphosphate glucose and uridine diphosphate galactose (Maxwell, 1956; Anderson, Landel & Diedrich, 1956; Kowalsky & Koshland, 1956; Kalckar & Maxwell, 1956).

An interesting feature of this scheme is that one dehydration and one reduction effect three stereochemical inversions. TDP-mannose is not an intermediate in this scheme but could be related to TDP-glucose by oxidation and reduction at the 2-position.

Interconversion of the thymidine derivatives would not necessarily involve change at the 1-

position. If the stereochemistry at this position remains unchanged in the transformation, then the α -D-mannose or α -D-glucose compounds would yield a β -L-rhamnose derivative. This stereochemical relationship is the one actually found in the natural nucleotides, and retention of stereochemical arrangement at position 1 supports the view that the interconversion of glucose, mannose and rhamnose occurs whilst they are in combination with nucleotides. The interconversion of guanosine diphosphate mannose and guanosine diphosphate fucose could occur along similar

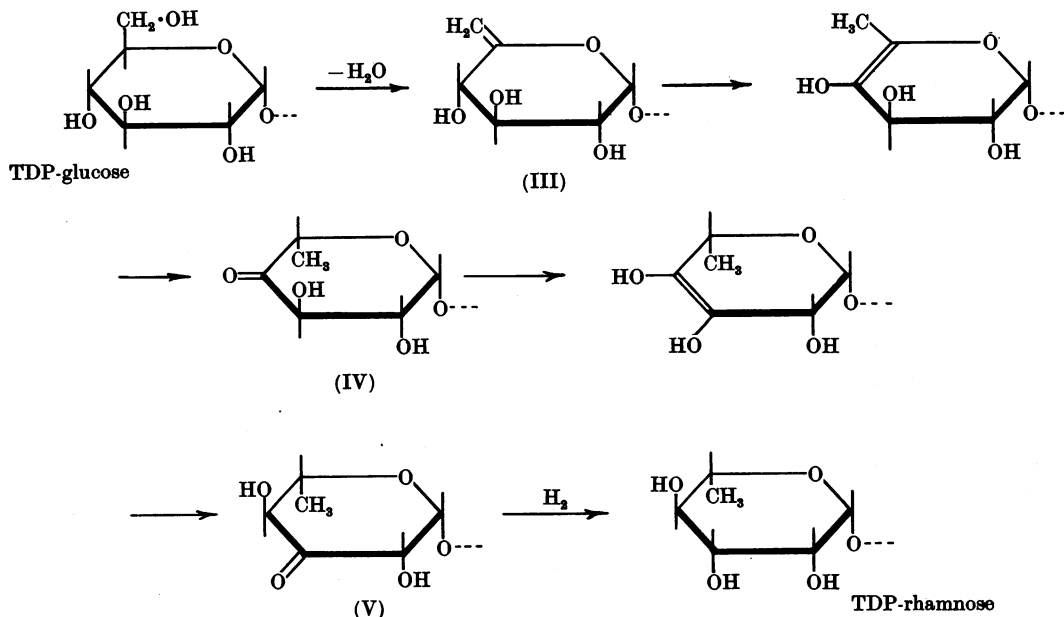


Fig. 3. Suggested mechanism for the conversion of TDP-glucose into TDP-rhamnose.

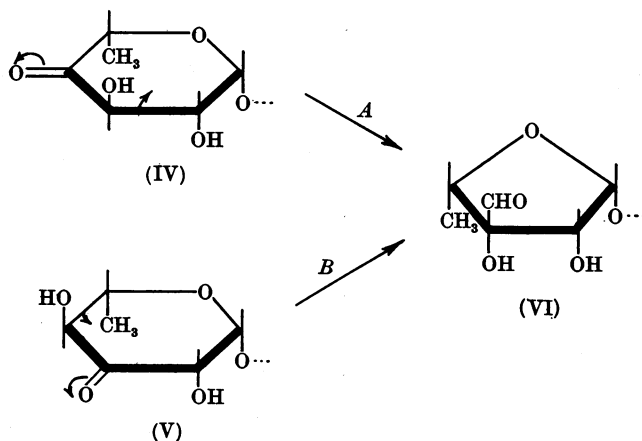


Fig. 4. Possible relationship between rhamnose and streptose derivatives.

lines. We are grateful to Dr J. G. Buchanan for valuable suggestions and discussion of this scheme.

Neither Kornfeld & Glaser nor Pazur & Shuey demonstrated the natural occurrence of TDP-glucose in extracts of the organisms which they studied. However, one of the fractions obtained by us from the ion-exchange chromatography of the nucleotides from *Streptomyces griseus* is a mixture of thymidine and uridine derivatives, which on acid hydrolysis yields glucose and probably fructose. Although this mixture has not yet been resolved it is likely that one of its components is TDP-glucose.

It is possible that both TDP-mannose and TDP-rhamnose are required in *Streptomyces griseus* for the synthesis of polysaccharides, since both mannose and rhamnose are present in the polysaccharide fraction from this organism (Kwapinski & Merkel, 1957). It is also possible that TDP-mannose would be concerned in the introduction of the D-mannosyl residue into streptomycin B (α -D-mannopyranosyl-streptomycin) (Fried & Titus, 1948; Staveley & Fried, 1949).

Another possible function of TDP-rhamnose or its precursors is in the biosynthesis of streptose, the unstable branched-chain sugar component of streptomycin. In Fig. 4 a simple rearrangement is given whereby a thymidine diphosphate streptose might be formed from TDP-rhamnose intermediates.

The carbonyl precursors (IV) and (V) could rearrange as shown to give the branched-chain sugar (VI), which correctly represents streptose (Fried, Walz & Wintersteiner, 1946; Kuehl, Clark, Bishop, Flynn & Folkers, 1949). In route *A* fission of the bond between C₍₃₎ and C₍₃₎ occurs, a new bond being formed between C₍₂₎ and C₍₄₎. In route *B* fission occurs between C₍₄₎ and C₍₅₎, the new bond being between C₍₃₎ and C₍₅₎. This rearrangement has some similarity to the pinacol-pinacolone transformation and to the glycol rearrangement postulated by Woodward (1957) for the biogenesis of a C-formyl group in the antibiotic magnamycin.

The biosynthetic scheme requires the existence of a thymidine diphosphate streptose. No compound of this structure has yet been characterized amongst the complex mixture of nucleotides in *Streptomyces griseus*, but it is known that unidentified thymidine compounds are present in this mixture. Moreover, a streptose nucleotide in which the streptose residue is in β -linkage with the pyrophosphate group would be particularly unstable, since the pyrophosphate in (VI) is in very close proximity to the *cis* hydroxyl at the 2-position. A comparable stereochemical arrangement at positions 1 and 2 occurs in ribose 5-phosphate 1-pyrophosphate.

SUMMARY

1. The nucleotide mixture obtained by alcoholic extraction of *Streptomyces griseus* contains several thymidine derivatives.

2. Thymidine diphosphate mannose and thymidine diphosphate rhamnose have been isolated from this source and their structures have been established. Products detected in their acid hydrolysates include thymine, inorganic phosphate and mannose or rhamnose. The two hexoses were identified chromatographically, and by reduction to the corresponding hexitols followed by treatment with acid. Hydrolysis with rattlesnake venom gave thymidine.

3. The mannose possesses the D-configuration, since the nucleotide, after oxidation with periodate then bromine water followed by acid hydrolysis, gave D-glyceric acid. The configuration of the glyceric acid was established by enzymic phosphorylation and determination of D-glyceric acid 3-phosphate.

4. The above method for determining the configuration of sugar phosphate residues is generally applicable.

5. The stability of the mannose nucleotide towards ammonia, and the instability of the rhamnose nucleotide under comparable conditions, indicate that the former is an α -mannose derivative and the latter is a β -rhamnose derivative. Conformational aspects of these nucleotide structures are discussed.

6. A chemical synthesis of thymidine diphosphate mannose is described.

7. The enzymic conversion of thymidine diphosphate glucose into thymidine diphosphate mannose has been observed and a possible mechanism for the biosynthesis of rhamnose is given.

8. Other likely biochemical functions for the thymidine derivatives are discussed. These include a suggested scheme for the biosynthesis of streptose.

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The Ribitol Teichoic Acid from *Lactobacillus arabinosus* Walls: Isolation and Structure of Ribitol Glucosides

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It has been known for some time that considerable amounts of a ribitol teichoic acid occur in the walls of *Lactobacillus arabinosus* 17-5 (Baddiley, Buchanan & Greenberg, 1957; Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958a; Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958b). Preliminary studies, carried out largely with the aid of paper chromatography,

suggested that this compound is a polymer in which ribitol units are joined together through phosphodiester linkages, and that glucosyl and alanine ester groups are also present (Armstrong *et al.* 1958b). It is clear that this teichoic acid resembles the ribitol teichoic acid in the walls of *Bacillus subtilis* (Armstrong, Baddiley & Buchanan, 1960, 1961), but several differences in the detailed structure of the two polymers were observed in the preliminary work. Hydrolysis with acid and alkali

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