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Guanosine Diphosphate Glucose and Guanosine Diphosphate Fructose from *Eremothecium ashbyii*

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Guanosine diphosphate mannose was first isolated from baker's yeast (Cabib & Leloir, 1954) and has been shown to be present also in chicken oviduct (Strominger, 1955) and moulds (Ballio, Casinovi & Serlupi-Crescenzi, 1956). During the course of an investigation of the water-soluble nucleotides in the mould *Eremothecium ashbyii* we have isolated guanosine diphosphate mannose and shown that it is accompanied by two hitherto undescribed nucleotides which appear to be guanosine diphosphate glucose and guanosine diphosphate fructose. A preliminary account of this work has been published (Pontis, James & Baddiley, 1959).

EXPERIMENTAL

Materials. The guanosine and uridine phosphates were purchased from Pabst Laboratories, Wis., U.S.A. Uridine diphosphate glucose, uridine diphosphate *N*-acetylglucosamine and guanosine diphosphate mannose (GDPM) were a generous gift from Dr E. Cabib. These were supplied as barium salts and, before chromatography, the barium was removed by double decomposition with sodium sulphate.

Methods. Extinctions were measured in a Unicam SP. 500 spectrophotometer. The following analytical procedures were used: that of Fiske & Subbarow (1925) for phosphates,

that of Park & Johnson (1949) for reducing power and the Roe (1934) or the indole- H_2SO_4 method (Colowick & Kaplan, 1957) for fructose. In both methods for fructose the total volume was reduced to 0.5 ml.

Paper chromatography of sugars was carried out with butanol-pyridine-water (3.0:2.0:1.5) (Jeanes, Wise & Dimler, 1951) or phenol-water (Partridge, 1948). The chromatograms were developed with the alkaline silver (Trevelyan, Procter & Harrison, 1950) or benzidine-trichloroacetic acid (Bacon & Edelman, 1951) reagents.

For the chromatography of nucleotides the $(\text{NH}_4)_2\text{SO}_4$ -propan-2-ol-sodium acetate solvent (Markham & Smith, 1951) was used, and also ethanol-ammonium acetate mixtures (Paladini & Leloir, 1952). The latter solvent (pH 3.8) was used also for the chromatography of sugar phosphates. The position of the ultraviolet-absorbing substances was ascertained with a Hanovia lamp and that of phosphates by spraying with the Hanes & Isherwood (1949) molybdate reagent.

Ionophoresis on paper was performed with an apparatus similar to that described by Markham & Smith (1952), with Whatman no. 1 paper soaked in 0.05 M-sodium borate, pH 9.2, for sugars (Consden & Stanier, 1952) and 0.02 M-sodium acetate-HCl buffer, pH 2.0, for bases. The sugars were revealed on paper electrophoresis with the alkaline silver reagent by the modified technique described by Cabib & Leloir (1958).

In chromatographic as well as in ionophoresis experiments the position of the substances on the paper was referred to the position of an appropriate standard (ribose for

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free sugars and adenosine for nucleotides and sugar phosphates), and the results were expressed as the ratio of the distance travelled by the unknown substance to the distance travelled by the standard substance.

Isolation of guanosine diphosphate mannose: preparation 1

Mould culture. *Eremothecium ashbyii* was obtained from the National Collection of Type Cultures and was cultivated as described by MacLaren (1952). Cultures were grown in 250 ml. Erlenmeyer flasks containing 50 ml. of medium at 28° under aerobic conditions (shaker) and were maintained by weekly transfer.

For the preparation of a large batch an inoculum (50 ml.) grown as described above was used to inoculate each of five 2 l. flasks containing 500 ml. of medium. After a week their contents were transferred to five 15 l. bottles each containing 10 l. of medium, where the mould was grown for a further week under conditions of forced aeration.

Preparation of the nucleotide extract. The mould culture was filtered through a 20 cm. Büchner funnel, washed with ice-water and pressed as much as possible. The mould cake (500 g. wet wt.) was cut into small pieces and mixed with

1 vol. of cold 10% trichloroacetic acid and homogenized in a Waring Blendor at full speed for 2 min. The heavy suspension was kept in the refrigerator for 1 hr. and then filtered, and the mould cake was extracted again as before three times. As soon as each acid filtrate was obtained it was extracted four times with an equal volume of ether. The acid extracts were combined, adjusted to pH 7.0 by addition of NaOH, and aerated.

Preparation of the extract for column chromatography. The acid extract (1.75 l. containing 5000 μ moles of nucleotides, calculated as uridine from *E* at 260 $m\mu$) was run through a Dowex-1 X2 (200–400 mesh) column (20 cm. $\times$ 3 cm.) in the chloride form. The column was washed with water until *E* at 260 $m\mu$ dropped below 0.1 and the nucleotides were eluted in a single step with 0.5N-CaCl₂ in 0.01N-HCl (720 ml.). The nucleotides eluted in this fraction amounted to 1800 μ moles (calculated as uridine). The solution was adjusted to pH 6.8 by addition of a saturated solution of Ca(OH)₂. Precipitation of part of the inorganic phosphate present in the extract occurred during the neutralization. The solution was filtered, concentrated *in vacuo* to a small volume, freeze-dried and the nucleotides were recovered as calcium salts (Pontis & Blumsom, 1958).

Ion-exchange chromatography. A sample of the pigment-free extract (100 ml. containing 1600 μ moles of nucleotides, calculated as uridine) was run through a Dowex-1 X2 (200–400 mesh) column (100 cm. $\times$ 2 cm.) in the chloride form. The elution procedure of Pontis & Blumsom (1958) was used (mixing chamber containing 20 l. of 0.1 mN-HCl; reservoir containing 12 l. of 0.15N-CaCl₂ in 0.01N-HCl; $A_2/A_1 = 0.6$). A flow rate of about 2 ml./min. was used and 38 ml. fractions were collected automatically. The relevant part of the elution diagram appears in Fig. 1. The fractions corresponding to each peak were concentrated, and nucleotides were recovered as calcium salts after freeze-drying and addition of ethanol-ether (1:1) mixture. The substances corresponding to the different peaks were tentatively identified by their ultraviolet-absorption spectra. Peaks IV, V and VI were further characterized by paper chromatography of the nucleotides in the ethanol-ammonium acetate solvents. Sugars liberated by acid hydrolysis were identified by paper chromatography.

Isolation of guanosine diphosphate mannose: preparation 2

Preparation of the ethanolic extract. Wet mould cells (4 kg.) were kindly supplied by Mr L. R. P. Meakin of The Distillers Co. Ltd. (Biochemicals), Liverpool. This was mixed with 4 l. of ethanol and the mixture was heated with stirring until it boiled. After standing overnight (5°) it was filtered through a 20 cm. Büchner funnel with filter-aid. The extract was adjusted to pH 7.0 with alkali before chromatography. If necessary it was stored at -15°.

Ion-exchange chromatography. An ethanolic extract (2 l., containing about 14 000 μ moles of nucleotides, calculated as uridine) was run through a Dowex-1 X2 (200–400 mesh) column (100 cm. $\times$ 3 cm.) in the chloride form. The elution procedure of Pontis, Cabib & Leloir (1957) was used with the difference that the column was washed with 0.01N-HCl (16.9 l.) until the pH of the effluent was 2.0, before the start of the gradient elution (mixing chamber containing 22 l. of 0.02N-NaCl in 0.01N-HCl; reservoir containing 0.1N-NaCl in 0.01N-HCl). Fractions of 90 ml. were collected. The results are given in Fig. 2. The

Fig. 1. Separation of mould nucleotides by ion exchange (see preparation 1 in the Experimental section). Peak I, uridine derivative; peaks II and III, flavin derivative; peak IV, uridine diphosphate *N*-acetylglucosamine, guanosine diphosphate glucose and guanosine diphosphate fructose; peak V, uridine diphosphate glucose and uridine diphosphate; peak VI, GDPM.

nucleotides corresponding to peak I amounted to 248 μ moles, expressed as guanosine from E at 260 $m\mu$ (this value is corrected for the absorption due to riboflavin, which was calculated from E at 450 $m\mu$). The nucleotides from this solution were adsorbed on to charcoal and then eluted with aqueous 50% ethanol. The flavins remained on the charcoal but the recovery of GDPM (80 μ moles) was poor.

Isolation of guanosine diphosphate mannose: preparation 3

Ion-exchange and paper chromatography. An ethanolic extract prepared as described for preparation 2 (1.5 l. containing 2400 μ moles, expressed as uridine) was run through a Dowex-1 X 2 (200–400 mesh) column (25 cm. $\times$ 5 cm.) in the chloride form. After washing the column with water, nucleotides were eluted with 0.01 N -HCl (10.9 l.) followed by 0.2 N -CaCl₂ in 0.01 N -HCl. The second eluent was used only after E at 260 $m\mu$ had dropped below 0.1. The last fraction (7 l. containing 4250 μ moles, expressed as uridine) was adjusted to pH 6.8 by addition of a saturated solution of Ca(OH)₂ and the volume reduced to 600 ml. by concentration *in vacuo* at 30–35°. The calcium salts were precipitated

by addition of 10 vol. of ethanol-ether (1:1) mixture and the solution was left overnight at -10° . The supernatant was decanted and the remaining suspension was centrifuged in the cold. The precipitate was washed five times with ethanol-ether mixture, twice with ether and finally dried in a desiccator. The yield was 800 mg. Before chromatography the calcium salts were suspended in water and converted into sodium salts by treatment with 0.1 N -sodium oxalate. The isolation of the mould GDPM was carried out from this solution by chromatography on Whatman no. 3 paper with ethanol-ammonium acetate (pH 3.8) for 60 hr. After chromatography the ultraviolet-absorbing zone corresponding to GDPM was cut off, the nucleotides were eluted with water and rechromatographed with the ethanol-ammonium acetate solvent (pH 7.5). After elution from the paper as before about 15 μ moles of GDPM was obtained.

Although this nucleotide preparation consisted mainly of GDPM, it contained the guanosine diphosphate glucose and guanosine diphosphate fructose. Material obtained by the methods described in preparations 2 and 3 was used in the subsequent studies on the composition of the new nucleotides. The mixture of nucleotides was chromatographically indistinguishable in both cases, and no solvent mixture was found which could resolve the three components.

Degradation products of guanine nucleotide mixture

Identification of sugars. For the quantitative tests the nucleotide mixture was hydrolysed with 0.01 N -H₂SO₄ for 20 min. at 100°. The mixture was neutralized with 0.05 N -Ba(OH)₂ and the nucleotides were precipitated by addition of 2 vol. of 0.18 N -ZnSO₄ and 1 vol. of 0.37 N -Ba(OH)₂. After centrifuging, reducing power was determined in a portion of the supernatant.

For the determination of fructose a similar technique was used, but the portions of the supernatant were evaporated to dryness *in vacuo*, the residues were dissolved in 0.1 ml. of water and then 0.01 ml. of 1% indole in ethanol and 0.4 ml. of 75% H₂SO₄ were added. The mixture was shaken and the colour which developed after 2 hr. at room temperature was measured at 480 $m\mu$. This colorimetric method is highly specific for fructose. Pure mannose gave an apparent fructose value of 0.6% of that given by a corresponding amount of fructose.

In order to obtain a solution of the sugars suitable for chromatography and ionophoresis, the nucleotide mixture was hydrolysed as before and the solution was treated successively with a cation-exchange and an anion-exchange resin (Dowex-50 and Amberlite IR-4B). The resulting solution was evaporated and examined by paper chromatography and ionophoresis (Table 2).

A sample of mannose was heated with HCl under conditions similar to those described above, and then the solution was neutralized with Ba(OH)₂ and subjected to the ZnSO₄ and ion-exchange procedures. Paper chromatography of the resulting solution demonstrated the absence of fructose and glucose. Both visual and photographic techniques were employed in the examination of the paper and it is considered that the method would have demonstrated the presence of these sugars in concentrations lower than 0.5% of the mannose.

For the determination of fructose with the resorcinol-HCl reagent a hydrolysate was subjected to ionophoresis and the appropriate area of the paper was cut out and

Fig. 2. Separation of the mould nucleotides by ion exchange (see preparation 2 in the Experimental section). Peak I, flavin derivative, GDPM, guanosine diphosphate glucose and guanosine diphosphate fructose; peak II, uridine diphosphate *N*-acetylglucosamine; peak III, uridine diphosphate glucose.

Table 1. *Paper chromatography of nucleotides obtained by acid hydrolysis of mould-nucleotide mixture*

	<i>R</i> _{adenosine} of u.v.-absorbing spots		
	Ethanol-ammonium acetate		Ammonium sulphate-propan-2-ol-sodium acetate
	pH 7.5	pH 3.8	
Yeast GDPM	0.13	0.11	3.47
Yeast GDPM + mould nucleotides	0.13	0.11	3.46
Mould nucleotides	0.13	0.11	3.44
Uridine diphosphate glucose	0.27	0.27	4.46
Uridine diphosphate <i>N</i> -acetylglucosamine	0.39	0.36	3.94
Guanosine diphosphate	0.03	0.05	3.42
Mould nucleotides heated for 15 min., pH 2.7 at 100°	0.03	0.06	3.43
Guanosine 5'-phosphate	0.10	0.32	3.20
Mould nucleotides heated for 3 hr., pH 2.7 at 100°	0.10	0.33	3.22
Guanosine 3'-phosphate	0.12	0.41	2.54

Table 2. *Paper chromatography and ionophoresis of sugars from mould nucleotides*

Ionophoresis was carried out on Whatman no. 1 paper, 70 cm. × 20 cm., with a potential of 600 v applied across the length of the paper during 6 hr.

	<i>R</i> _{ribose}		
	Paper chromatography		Ionophoresis with borate-buffered paper
	Butanol-pyridine-water*	Phenol-water	
Galactose	0.62	0.54	1.15
Glucose	0.70	0.48	1.32
Fructose	0.79	0.82	1.17
Mannose	0.83	0.61	0.94
Acetylglucosamine	0.96	1.34	0.37
Hydrolysed mould nucleotides	0.71, 0.83	0.46, 0.59, 0.81	0.94, 1.18, 1.33

* In this solvent mannose and fructose overlap.

Table 3. *Paper chromatography of the sugar phosphates obtained by the action of pyrophosphatase on mould nucleotides*

	<i>R</i> _{adenosine} in ethanol-ammonium acetate (pH 3.8)
Sugar phosphates from mould	0.59
GDPM	0.13
Glucose 1-phosphate	0.60
Glucose 6-phosphate	0.62
Fructose 1-phosphate	0.68
Fructose 6-phosphate	0.73
Fructose 1:6-diphosphate	0.29
Sucrose phosphate*	0.54

* From the data of Leloir & Cardini (1955).

eluted. Sodium borate was removed from the eluate by treatment with Dowex-50 (hydrogen form), followed by evaporation to dryness with methanol.

Hydrolysis to guanosine diphosphate and to guanosine 5'-phosphate. A sample of nucleotide mixture was hydrolysed at pH 2.7 during 15 min. at 100°. The acid was neutralized with dilute aq. NH₃ solution and the solution used directly for chromatography (Table 1).

For further hydrolysis to guanosine 5'-phosphate a sample was hydrolysed at pH 2.7 during 3 hr. at 100° and treated as described above before chromatography (Table 1). The conditions of hydrolysis were similar to

those used by Cabib & Leloir (1954) for the determination of the structure of GDPM from yeast.

Action of pyrophosphatase from snake venom. To the nucleotide mixture (2 μmoles) in 2 ml. of 0.2% barbiturate buffer, pH 8.0, was added *Crotalus atrox* venom (2 mg.) and the mixture was incubated at 35° for 24 hr. The solution was treated with charcoal (about 10 mg.), centrifuged and the supernatant after concentration was used directly for chromatography. The sample was divided into two equal portions which were run in adjacent tracks on the paper. After chromatography in the ethanol-ammonium acetate solvent (pH 3.8) one track was cut out and sprayed with the reagent for phosphates. In addition to inorganic phosphate (from the hydrolysis of guanosine 5'-phosphate) a spot with *R*_{adenosine} 0.59 was observed (Table 3). This was cut from the appropriate area of the second track and eluted. The eluted material was hydrolysed in *N*-HCl for 30 min. at 100° and the resulting solution was treated with Dowex-50 and Amberlite IR-4 B resins and then concentrated *in vacuo*. The sugars in this solution were identified by ionophoresis as described earlier. Three sugars with the *R*_{ribose} values of mannose, glucose and fructose were observed.

RESULTS

Isolation of the guanosine diphosphate mannose fraction. In the initial experiments the mould was extracted with cold 10% trichloroacetic acid,

followed by extraction with ether to remove the trichloroacetic acid. As *E. ashbyii* produces large amounts of riboflavin the extract contained much riboflavin and other pigments which interfered with the normal development of the ion-exchange chromatograms, either by tailing continuously during the elution or by diminishing the capacity of the column. This difficulty was overcome by passing the extract through a short ion-exchange (Dowex-1) column, which was then washed with water, and the nucleotides were eluted as a single fraction with 0.5 N-CaCl₂ solution (pH 2.0), followed by concentration and recovery of the nucleotides as calcium salts. The water removed the riboflavin and some pigments, and others remained adsorbed at the top of the column. The calcium salts were converted into sodium salts and chromatographed with gradient elution on a column of Dowex-1 X2 resin. Fig. 1 shows the elution pattern of the zone corresponding to the hexose-containing nucleotides.

The ultraviolet-absorption spectrum of peak IV was that of uridine. Paper chromatography in the ethanol-ammonium acetate solvents showed that this peak was mainly uridine diphosphate *N*-acetylglucosamine accompanied by traces of a slow-moving nucleotide with the same $R_{\text{adenosine}}$ as GDPM and possessing an absorption spectrum corresponding to that of guanosine. It is likely that this was GDPM, as this nucleotide is the main component of peak VI and a slight amount of cross-contamination would be expected owing to the large amount of GDPM present in these extracts (Fig. 1). However, paper chromatography of the products of mild acid hydrolysis of this minor component of peak IV revealed the presence of reducing sugars which behaved like glucose and fructose rather than mannose. Unfortunately, the amount available was insufficient for a detailed study. With the larger batches of mould the cells were extracted with ethanol and different procedures were used for the isolation of the GDPM fraction. However, it was not possible on the larger scale to separate the glucose- and fructose-containing nucleotides from GDPM.

Chromatography on Dowex-1 X2 resin in the chloride form with concave-gradient elution with CaCl₂, a procedure which gave good results in the initial experiments, was not successful in the large-scale experiments, possibly owing to the presence of large amounts of phosphorylated derivatives of riboflavin, which in the CaCl₂ system are eluted from the column in the same zone as the required nucleotides. Moreover, in the large-scale work the relative amounts of uridine diphosphate glucose and guanosine compounds in the trichloroacetic acid extract were reversed (Figs. 1 and 2), and this hindered the separation. On the other hand, gradient elution with NaCl gave a good resolution

of nucleotides but the flavin-containing peak overlapped the GDPM fraction (Fig. 2). During the subsequent concentration process on charcoal most of the flavin was removed, but this technique resulted in poor recovery of the guanine nucleotides.

In order to overcome these difficulties a simpler elution procedure was coupled with paper chromatography. The ethanolic extract was run through a short Dowex-1 column, which was then washed with water followed by 0.01 N-HCl, which eluted most of the monophosphates, and finally 0.2 N-CaCl₂ in 0.01 N-HCl, which eluted nucleoside diphosphates and nucleoside diphosphate sugar derivatives. After concentration the calcium salts from the last fraction were converted into sodium salts by treatment with Dowex-50 (sodium) resin or by double decomposition with 0.1 N-sodium oxalate and were examined by paper chromatography in the acidic ethanol-ammonium acetate solvent. The appropriate band was cut from the paper, nucleotides were eluted and rechromatographed in the neutral ethanol-ammonium acetate solvent. Alternatively, the sodium salts were run in the (NH₄)₂SO₄-propan-2-ol-sodium acetate solvent, in which flavins remain at the origin.

The amounts of guanosine diphosphate glucose and guanosine diphosphate fructose obtained from the mould in the small-scale experiments, where they were free of GDPM, indicated that they were present in the mould in a concentration of about 10 μ moles/kg.

Identification of the nucleotide moiety. The absorption spectrum of the GDPM fraction from the mould was indistinguishable from that of guanosine and showed the same changes with pH. Two phosphate groups were present per molecule of guanosine and one was liberated as inorganic phosphate by hydrolysis for 20 min. in N-HCl at 100°.

Hydrolysis at pH 2.7 for 15 min. at 100° yielded guanosine diphosphate, whereas more prolonged hydrolysis (3 hr.) gave guanosine 5'-phosphate. These were identified by comparison with suitable standards in three solvent systems (Table 1). Guanine was produced by hydrolysis in N-HCl for 120 min. at 100°. This was identified by paper chromatography in the propan-2-ol-HCl solvent (Wyatt, 1951), when it had R_f 0.22. Guanine was also identified by ionophoresis in 0.02 M-sodium acetate-HCl buffer, pH 2.0. The amount of material used in all these experiments was sufficiently large to enable the detection of other nucleotides and purines which may have been present in concentrations lower than 1% of the total.

Identification of the sugar moiety. Mild acid hydrolysis of the GDPM fraction liberated reducing sugars, which were submitted to paper chromatography in butanol-pyridine-water and phenol-

water, followed by development with alkaline silver or benzidine reagents. Three spots were observed with the same R_{fructose} values as glucose, fructose and mannose; similar results were obtained by ionophoresis on borate-buffered paper (Table 2). Fructose was further characterized by its reaction with resorcinol-HCl and with the indole- H_2SO_4 reagents. The absorption spectrum of the colour formed from fructose from mould nucleotides after treatment with resorcinol and concentrated acid is compared in Fig. 3 with the one obtained from pure fructose.

The reducing value of the hydrolysate was determined by Park & Johnson's (1949) method, in which the three sugars give the same colour intensity. This was used to calculate the ratio guanosine:total phosphate:reducing sugars, which was 1.04:2:1.05 (total phosphate was taken as 2). The individual sugars were determined quantitatively by eluting appropriate areas after paper ionophoresis and then estimating reducing power. The values obtained for glucose and fructose

Fig. 3. Absorption spectra of the fructose moiety of guanosine diphosphate fructose and pure fructose treated with resorcinol and hydrochloric acid. (a) Fructose; (b) hydrolysed GDPM.

Fig. 4. Liberation of fructose from the mould nucleotides (upper curve) heated at pH 2.0, 100°. The value obtained after 20 min. was taken as 100. The lower curve is for fructose 1-phosphate.

showed that together they comprised approximately 6% of the total hexose present in the GDPM from the mould.

The rate of liberation of reducing sugars from the mould nucleotide fraction is very similar to that of mannose liberation from yeast GDPM. However, small differences in the rate of hydrolysis owing to the presence of the glucose and fructose compounds would be difficult to observe on account of the large amount of mannose present. On the other hand, Fig. 4 shows specifically the rate of liberation of fructose on acid treatment. Fructose was estimated by the indole- H_2SO_4 method, which is very sensitive for this sugar even in the presence of glucose and mannose. It was shown that although the rate of liberation of fructose from the nucleotide is faster than that from fructose 1-phosphate it is somewhat slower than the liberation of mannose from GDPM, which is complete in about 5 min. at the same pH (Cabib & Leloir, 1954).

Further evidence of the nature of the linkage between the sugars and the nucleotide was obtained from the action of snake-venom pyrophosphatase. Chromatography of the products of this enzymic hydrolysis indicated the formation of a mixture of organic phosphates, which moved as a single spot in the solvent system used (Table 3). No attempt was made to isolate the guanine derivatives produced during the hydrolysis. These were removed from the reaction mixture by addition of charcoal in order to avoid interference with the chromatography of the sugar phosphates. The organic phosphates were eluted from the paper chromatogram and hydrolysed with acid, followed by paper ionophoresis on borate-buffered paper. After development with alkaline silver three spots with the same R_{fructose} values as mannose, glucose and fructose were observed.

DISCUSSION

The difficulty experienced in attempts to separate the glucose- and fructose-containing nucleotides from GDPM suggests that they possess marked structural similarities. Moreover, as all three give guanosine diphosphate or 5'-phosphate under identical conditions of hydrolysis it is concluded that both glucose and fructose are attached to guanosine through a 5'-pyrophosphate group. This is supported by the action of rattlesnake-venom pyrophosphatase, the formation of a mixture of sugar phosphates in this experiment confirming the conclusion that these were joined to the nucleoside through a pyrophosphate linkage.

Although the mixture of sugar phosphates formed by the action of the pyrophosphatase was not resolved, it is clear that this must have been a mixture of hexose monophosphates and could not

have contained a phosphate of sucrose or of any other disaccharide containing both glucose and fructose. This is apparent from a comparison of the R_f value of the sugar phosphates formed with those reported in similar solvents for phosphates of sucrose (Leloir & Cardini, 1955).

Moreover, the sugar phosphate mixture had R_f typical for hexose phosphates in 90 % formic acid-ether (2:3): R_f 0.26. A sucrose phosphate would be expected to have a lower R_f in this solvent.

It is not possible on the available evidence to identify the position of the linkage between fructose and the pyrophosphate group in guanosine diphosphate fructose. The rate of liberation of fructose during acid hydrolysis of the nucleotide is comparable with the rate of hydrolysis of sucrose (Cardini, Leloir & Chiriboga, 1955), and this may suggest that the pyrophosphate is joined to position 2 in fructose. However, although fructose 1-phosphate is considerably more stable than is the linkage between nucleotide and fructose in guanosine diphosphate fructose it is not possible to eliminate position 1 from consideration. A fructose 1-pyrophosphate derivative may well be more labile than fructose 1-phosphate, this situation being analogous to that of glucose 1-phosphate and uridine diphosphate glucose (Caputto, Leloir, Cardini & Paladini, 1950).

The rate of liberation of glucose from guanosine diphosphate glucose has been studied by paper chromatography and it is concluded that this is consistent with the presence of the pyrophosphate at position 1 in glucose. This is also consistent with the ease of liberation of glucose from the sugar-phosphate mixture obtained by the action of the pyrophosphatase on the nucleotides.

The biochemical function of these nucleotides is not yet known. However, their general structure strongly suggests that they are coenzymes involved in the metabolism of glucose and fructose. It is known that a number of nucleoside-pyrophosphate-sugar compounds act as donors of their sugar moiety in the synthesis of polysaccharides. It is possible that one of the functions of guanosine diphosphate glucose and guanosine diphosphate fructose may be to act as intermediates in the synthesis of polysaccharides.

SUMMARY

1. Guanosine diphosphate mannose and two hitherto unidentified nucleotides have been isolated from the mould *Eremothecium ashbyii* by ion-exchange and paper chromatography.

2. Although the new nucleotides were not separated from guanosine diphosphate mannose they were shown to be guanosine diphosphate glucose and guanosine diphosphate fructose.

3. In guanosine diphosphate glucose a guanosine 5'-pyrophosphate residue is joined through its terminal phosphate, probably to position 1 of glucose.

4. The position of the linkage between fructose and the pyrophosphate in guanosine diphosphate fructose is not settled.

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