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Reversible Enzymatic Synthesis of Guanosine Diphosphate Mannose from Guanosine Triphosphate and Mannose-1-Phosphate

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From brewer's yeast an enzyme is isolated and purified, which catalyzes a pyrophosphorylation of guanosine diphosphate mannose. This reaction is demonstrated spectrophotometrically, and the reversibility of the process is proved by means of paper chromatography. Separation of guanosine diphosphate mannose from uridine diphosphate glucose on a cellulose column is described.

A number of enzyme systems, catalyzing processes which involve guanosine compounds have recently been demonstrated [1–4] and guanosine nucleotides have been isolated from liver, brain, tumor and muscle tissue [5,6]. An interesting compound, guanosine diphosphate mannose (GDPM) *, was isolated from yeast and identified by Leloir and Cabib [7]. This compound is probably identical with the mannose-containing nucleotide, noticed by Buchanan *et al.*[8] in their investigations of phosphorus compounds as intermediates in photosynthesis.

In the present paper the existence of an enzymatic pathway for the synthesis of GTP from GDPM and P—P is reported, and the reversibility of the process is proved. The reaction consists in a pyrophosphorolytic cleavage of GDPM according to the following equation:

$$\text{GDPM} + \text{P—P} \rightleftharpoons \text{GTP} + \text{M—1—P} \qquad (1)$$

and the process seems analogous to the earlier described pyrophosphorolytic cleavages of DPN [9], UDPG [10] and UDPAG [11].

* The following abbreviations are used: GDPM for guanosine diphosphate mannose; GMP, GDP, GTP for guanosine mono-, di- and triphosphate; DPN, TPN for di- and triphosphopyridine nucleotide; UMP, UDP, UTP for uridine mono-, di- and triphosphate; UDPG for uridine diphosphoglucose; UDPAG for uridine diphosphate N-acetyl glucosamine; AMP, ADP, ATP for adenosine mono-, di- and triphosphate; M-1-P for mannose-1-phosphate; G-6-P for glucose-6-phosphate and Tris for tris(hydroxymethyl)aminomethane.

The enzyme, for which the name GDPM pyrophosphorylase is proposed, has been prepared and purified from brewer's yeast, and its presence has been demonstrated in a number of microorganisms. A preliminary report of the work has appeared earlier [12].

EXPERIMENTAL

M-1-P was prepared synthetically according to Colowick [13]. Hexokinase, G-6-P dehydrogenase and nucleoside diphosphokinase were obtained as previously described [14].

GTP, isolated from muscle tissue, was a gift from Dr. Adam Deutsch, University of Lund, Sweden.

GDPM was prepared from baker's yeast using a method similar to the one described by Cabib and Leloir [7] with a few modifications in the column technique. Baker's yeast, 10 kg, was used as starting material, and the solution of nucleotides obtained by decomposition of the mercury salts (*cf.* Ref.[7]) was run through a Dowex-1 anion exchange column, which was eluted according to the principle described by Alm *et al.*[15], by gradually increasing the concentration of the eluting agent.

The column used for this purpose had a length of 25 cm and a bed volume of 64 cm^3. About 2 000 μmoles (200 ml) of the nucleotide mixture was applied in a run, and after rinsing with 25 ml H_2O the column was subjected to gradient elution. The apparatus was similar to that described by Hurlbert *et al.*[16], starting with 250 ml H_2O in the mixing flask and 800 ml 0.03 M HCl, containing 0.03 M NaCl, in the reservoir. Under these conditions nucleosides, AMP, UMP and ADP were eluted from the column. When the reservoir was empty, elution was continued by adding 500 ml 0.03 M HCl, containing 0.3 M NaCl, to the reservoir without changing the content of the mixing flask. During this step GDPM, UDPAG, UDPG, UDP and finally the nucleoside triphosphates are eluted from the column. The identity of the different peaks mentioned here was established by adsorption of each fraction on norite followed by paper chromatography together with known compounds. The eluted fractions were concentrated by adsorption on norite and subsequent elution with 50 % ethanol.

By this method GDPM is obtained, contaminated with varying amounts of UDPAG and UDPG. Complete separation of these nucleotides is difficult to accomplish on a Dowex-1 column, whereas paper chromatography in the ethanol-ammonium acetate solvent [17] at pH 7.5 yields a good separation. Separation of the compounds on a somewhat

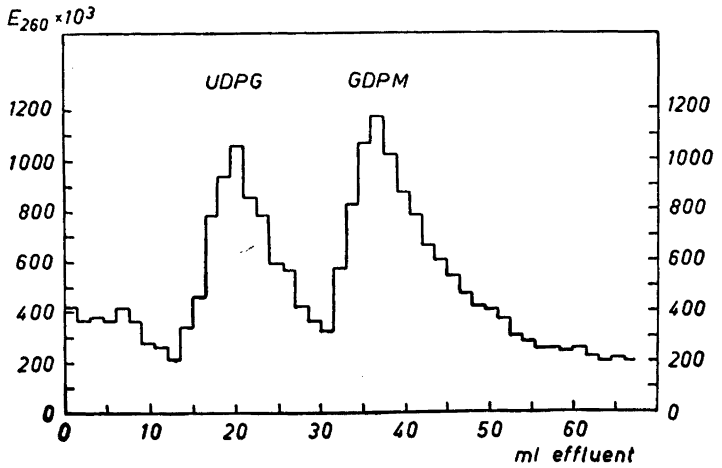

Fig. 1. Separation of GDPM from UDPG on a cellulose column.
Eluting agent: 1 M ethanol-ammonium acetate, pH 7.5 (17).

larger scale was obtained by using a cellulose column (standard grade Whatman cellulose powder), 20 cm high, with a bed volume of 22 cm^3. A solution of 10–15 μmoles of nucleotides in a volume of about 100 μl was placed on top of the column, and the column was washed with 2–3 ml ethanol. Elution was effected with 1 M ethanol-ammonium acetate, pH 7.5, the same solvent as used for paper chromatography [17]. Fig. 1 shows the separation of GDPM from UDPG by this method. The peaks were identified by paper chromatography.

The combined eluates containing the GDPM were evaporated to a small volume, and this solution was used directly in the enzymatic experiments. A salt free solution, if desired, was obtained by treating the mixture once more with norite, and subsequent elution of the norite. The technique used for adsorption of the nucleotides on norite and elution with 50 % ethanol was as earlier described [14].

Assay of enzyme activity. During the purification procedure enzyme activity was estimated by determining the GTP formed in reaction (1). The method is similar to the one used [14] for determination of UTP by pyrophosphorylation of UDPG or UDPAG. In the assay system the following reactions take place:

GDPM + P–P ⇌ GTP + M–1–P	(GDPM pyrophosphorylase)
GTP + ADP ⇌ GDP + ATP	(nucleoside diphosphokinase)
ATP + glucose ⇌ ADP + G–6–P	(hexokinase)
G–6–P + TPN + H_2O ⇌ 6-phosphogluconic acid + TPNH + H+	(G–6–P dehydrogenase)

and the reduction of TPN is followed in the Beckman spectrophotometer at 340 mμ. 1 ml quartz cells were used, and the components of the system were as follows: 100 μl GDPM, 0.002 M; 800 μl Tris, 0.05 M, pH 7.3; 5 μl $MgCl_2$, 1 M; 20 μl ADP (0.002 M); 25 μl glucose (0.01 M); 25 μl hexokinase (12 mg/ml protein); 15 μl TPN (10 mg/ml); 15 μl G–6–P dehydrogenase (1 mg/ml protein) and 5–10 μl GDPM pyrophosphorylase. 10 μl P–P (0.1 M) was added to start the reaction (*cf.* Fig. 4).

A unit of enzyme activity was defined as the amount causing the splitting of 0.1 μmole/min. Specific activity is defined as units per mg protein. A rise in extinction of 0.622 per 0.1 μmole at 340 mμ was used as basis for the calculation [18].

Protein determinations were carried out according to Bücher [19].

RESULTS

Purification of enzyme. 50 g of dried brewer's yeast (A/S Tuborgs Fabriker) was autolyzed and extracted overnight with 200 ml 0.07 M $(NH_4)_2HPO_4$ at 25° C. After centrifugation the proteins in the supernatant (150 ml) were precipitated by addition of 280 ml saturated ammonium sulphate. The mixture was left for 30 min. at 2° C before centrifugation. The supernatant was discarded, and the precipitate was dissolved in 75 ml chilled H_2O, and pH adjusted to 7.5 (volume 90 ml). To this was added 150 ml saturated ammonium sulphate. After 30 min. at 2° C the precipitate was collected by centrifugation, dissolved in 25 ml 0.02 M phosphate buffer, pH 7.5, and subjected to ethanol fractionation.

Table 1. Purification of GDPM pyrophosphorylase from brewer's yeast.

	Total units	volume ml	specific activity units / mg protein
Crude extract	145	150	0.01
Ammonium sulphate at pH 7.5	96	90	0.02
Ethanol fractionation	53	35	0.11
Calcium phosphate gel eluate	21	5	0.50

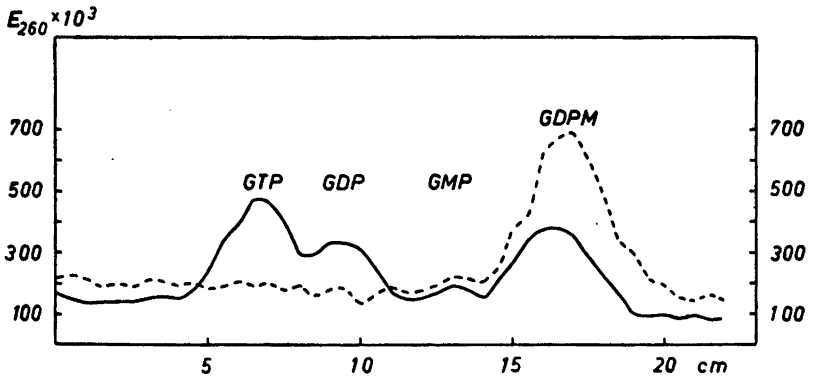

Fig. 2. Diagram of paper chromatogram from GDPM pyrophosphorylase digest.

Reaction mixture: 0.3 μmoles GDPM; 2 μmoles P—P (8 280 cts/min/μmole); 1 ml Tris; 0.05 M, pH 7.3; 0.5 μmoles $MgCl_2$.
Control: same without P—P
After 60 min. incubation the digest was acidified, adsorbed on norite and eluted with 50 % ethanol. Chromatographed 22 hours. Chromatogram scanned in the Beckman spectrophotometer at 260 mμ.

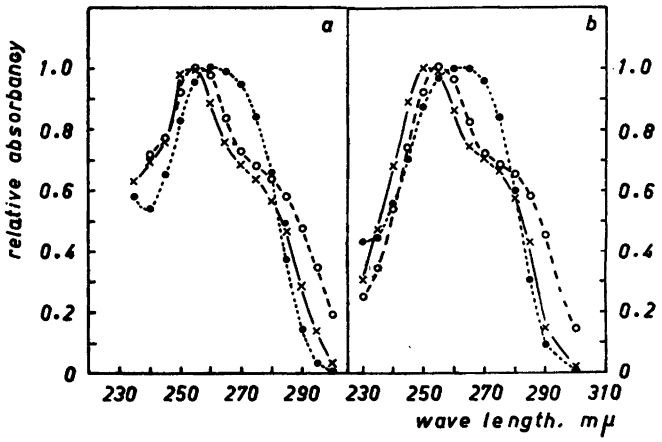

Fig. 3a. Ultraviolet absorption curves for GTP eluted from paper chromatogram.

For comparison with Hotchkiss' data [20] the relative absorbing value is used (maximum absorbancy taken as equal to 1). The values were corrected for absorption originating from the paper.

Fig. 3b. Spectra obtained for guanosine as reported by Hotchkiss [20].

Legend: O····O····O acid solution
×—×—× neutral solution
●····●····● alkaline solution

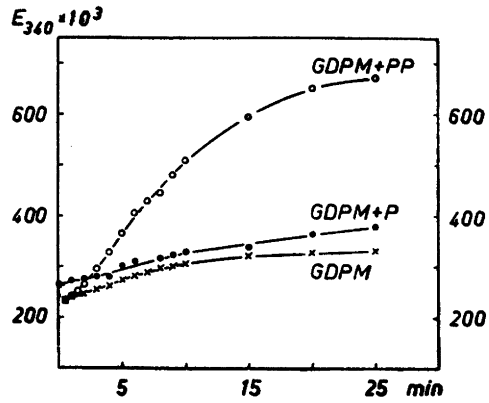

Fig. 4. Spectrophotometric demonstration of the GTP formed by pyrophosphorylation of GDPM.

Reaction mixture O—O—O: 50 µl GDPM, 0.002 M; 20 µl ADP, 0.002 M; 25 µl glucose, 0.01 M; 25 µl hexokinase (12 mg/ml); 15 µl TPN (10 mg/ml); 15 µl G—6—P dehydrogenase (0.5 mg/ml); 10 µl pyrophosphorylase; 10 µl P—P, 0.1 M and 800 µl Tris, 0.05 M, pH 7.3; 5 µl $MgCl_2$, 1 M.
Control 1 ×—×—× same without P—P.
Control 2 ●—●—● same with 2 µmoles inorganic P instead of P—P.

To 40 ml solution, cooled to —1° C were added dropwise 80 ml 50 % ethanol. During this addition the solution was stirred mechanically, and the temperature of the surrounding bath was gradually lowered to —12° C. The mixture was centrifuged 5 min. at —15° C in a previously cooled centrifuge. The supernatant was left 20 min. at —15° C and centrifuged again. The last precipitate contained the bulk of activity and was stored overnight at —20° C.

Next morning the precipitate was dissolved in 35 ml chilled Tris buffer, 0.001 M, pH 7.3, 35 ml calcium phosphate gel (16 mg/ml dry weight) was added, and the solution thoroughly mixed. After centrifugation and removal of the supernatant the precipitate was treated with 50 ml 0.05 M phosphate buffer, pH 7.2. The mixture was spun, and to the supernatant was added 80 ml ammonium sulphate. The mixture was left overnight at 2° C, and the precipitate collected by centrifugation. Stored at —20° C, this preparation retained its activity for several weeks. The preparation was soluble in 0.05 M Tris buffer. The activity was about 40 times that of the crude extract, and the overall yield was 14 % (Table 1).

The purified preparation showed no pyrophosphorolytic activity towards UDPG, but contained traces of UDPAG pyrophosphorylase.

Pyrophosphorolysis of GDPM. If GDPM, prepared from yeast, as described in Methods, was incubated with ^{32}P-labelled pyrophosphate and the enzyme, paper chromatography of the reaction mixture showed that a new ultraviolet absorbing substance had appeared (Fig. 2). The new spot was eluted with H_2O, and the ultraviolet absorption spectrum of the substance in neutral, alkaline and acid solution was determined, as presented in Fig. 3. The curves

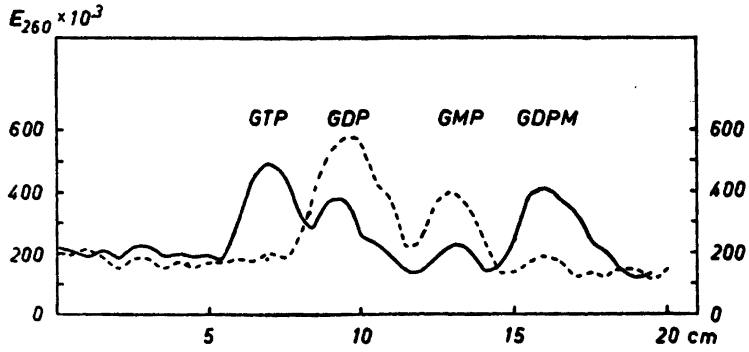

Fig. 5. Diagram of paper chromatogram from GTP—M—1—P pyrophosphorylase digest.

Reaction mixture (solid line): 0.2 μmoles GTP; 0.4 μmoles M—1—P; 1 ml Tris, 0.05 M, pH 7.3 and 25 μl GDPM pyrophosphorylase.
Control mixture (broken line): same as above, except that 0.2 μmoles GDP is substituted for GTP. After 60 min. incubation the digests were acidified and treated as described in Fig. 2.

are almost identical to those found for guanosine by Hotchkiss [20], and the changes in acid and alkaline solution are the same.

Aliquots of the solution were deposited on planchets, dried and counted in a Geiger-Müller counter. Counts per min. per μmole of the new compound (μmoles calculated from the absorption value at 257 mμ) and of the ^{32}P-labelled pyrophosphate used in the experiment were 7 800 and 8 270, respectively, and thus agree with the assumption that a pyrophosphorolysis of GDPM has occurred with simultaneous formation of GTP. The GTP formed in the reaction may be demonstrated spectrophotometrically as described in Methods (Fig. 4). As seen in Fig. 4 addition of inorganic phosphate instead of pyrophosphate causes no formation of GTP.

Reversibility of the reaction. Incubation of GTP with M-1-P and the enzyme results in formation of a compound which moves on the paper chromatogram with the same rate as known GDPM (Fig. 5). Incubation of GDP with the same M-1-P solution yields a very faint spot at the GDPM site. It is possible that a reaction exists by which GTP and GMP are formed from GDP, but this possibility has not been further investigated.

Judging from the curves in Figs. 2 and 5 the equilibrium of the reaction is at around 50 % conversion, similar to what is found for the UDPG pyrophosphorylase [14].

DISCUSSION

Nucleotidyl transferases, the group of enzymes to which the GDPM-pyrophosphorylase belongs, have been demonstrated in a number of tissue and in several microorganisms, and it is assumed that their role in the cell is the synthesis of nucleoside diphosphate glycosyl compounds from nucleoside triphosphates and glycosyl phosphates.

The function of the nucleoside diphosphate glycosyl compounds as donors of glycosyl groups has been experimentally demonstrated in the case of

UDPG[21–23] and UDPGlucuronic acid [24]. GDPM has been suggested to be active in the synthesis of mannan in the yeast cell-walls [7], but no proof of this hypothesis has so far been obtained. The abundance of mannose-containing polysaccharides in brown algae suggests these as a suitable material for further investigations on the function of GDPM.

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