THE BIOSYNTHESIS OF TREHALOSE PHOSPHATE*

By E. CABIB and LUIS F. LELOIR

(From the Instituto de Investigaciones Bioquímicas, Fundación Campomar, Obligado 2490, Buenos Aires, Argentina)

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The participation of uridine diphosphate glucose in the biosynthesis of disaccharides was first demonstrated by the discovery of an enzyme in yeast (1) which catalyzes the formation of trehalose phosphate according to the reaction

$$\text{UDPG}^1 + \text{glucose 6-phosphate} \rightarrow \text{UDP} + \text{trehalose phosphate}$$

The present paper reports a partial purification of the enzyme and a detailed study of the reaction.

Materials

UDPG was prepared as described by Pontis *et al.* (2). Part of this material was kindly furnished by Dr. H. G. Pontis. UDP was prepared by heating UDPG at 100° in 0.03 N sulfuric acid for 10 minutes, followed by neutralization with barium hydroxide and chromatography on Whatman No. 3 paper with ethanol-ammonium acetate at pH 3.8 as solvent (3). Another sample of UDP was obtained by ion exchange chromatography of an alcoholic yeast extract, followed by paper chromatography as above.

Phosphopyruvate was a gift of Dr. A. Kornberg. A crystalline sample of barium trehalose phosphate was generously provided by Dr. W. T. J. Morgan and one of crystalline albumin by Dr. D. Nachmansohn. Crystalline barium glucose 6-phosphate was obtained from the Sigma Chemical Company, St. Louis, and intestinal phosphatase from Armour and Company, Chicago. The phosphatase was dialyzed overnight against cold distilled water before use.

Glucose-6-phosphate dehydrogenase was purified according to Kornberg and Horecker (4).

UDPG dehydrogenase was obtained as described by Strominger *et al.* (5). The purification was carried on until the first ammonium sulfate

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[1] The following abbreviations are used: UDP for uridine 5′-pyrophosphate, UDPG for uridine diphosphate glucose, UMP for uridine 5′-phosphate, EDTA for ethylenediaminetetraacetic acid, and Tris for tris(hydroxymethyl)aminomethane.

precipitation step, followed by dialysis against 0.02 M acetate buffer at pH 5.9.

Pyruvate kinase was prepared from rabbit muscle (80 gm.). The extraction and precipitation with acetone were carried out as described by Kubowitz and Ott (6). The precipitate was redissolved in 4 ml. of water plus 1 ml. of 0.5 M phosphate buffer at pH 7.2. The pH was brought to 4.6 with 1 M acetic acid, and 31 ml. of ammonium sulfate solution (saturated at 4°) were added. The suspension was centrifuged in the cold, and the precipitate was taken up with 4 ml. of water and 0.5 ml. of 0.5 M phosphate buffer at pH 7.2. An insoluble portion was removed by centrifugation in the cold. This preparation was very stable when stored at −15°.

Methods

General—Phosphate was determined by the Fiske and Subbarow method (7) and fructose according to Roe and Papadopoulos (8). Reducing sugar estimations were performed either as described by Somogyi (9) and Nelson (10) or by a modification of the Schales and Schales ferricyanide procedure (11). In the analysis of the enzymatically prepared trehalose phosphate both the phosphate and reducing sugar (ferricyanide) determinations were adapted for a total volume of 1 ml., and readings were taken in the Beckman DU spectrophotometer at 660 and 420 mμ, respectively, with microcells obtained from the Pyrocell Manufacturing Company, New York.

The labile sugar of UDPG was determined as previously described for the mannose in guanosine diphosphate mannose (12).

Trehalose phosphate was estimated with anthrone according to Trevelyan and Harrison (13). In balance experiments, in which both UDPG and glucose 6-phosphate were also present, the samples were first hydrolyzed in 0.1 N HCl for 10 minutes at 100°; then enough 1 N NaOH was added to make the solutions 0.1 N in alkali; the tubes were heated again at 100° for 10 minutes, and aliquots were taken for the anthrone test. The acid hydrolysis liberated the glucose moiety of UDPG, and the alkaline treatment destroyed both this glucose and glucose 6-phosphate, while trehalose phosphate remained unaffected. Blanks and standards also contained UDPG and glucose 6-phosphate and were submitted to the same treatment as the unknowns. A similar method has been developed independently by Wyatt and Kalf (14).

Paper chromatography of nucleotides and sugar esters was performed according to Paladini and Leloir (3). Sugars were chromatographed with a solvent composed of *n*-butanol, pyridine, and water (6:4:3 parts by volume) (15). Ionophoresis of esters and free sugars was carried out with the apparatus described by Markham and Smith (16), with Whatman No. 1 paper soaked in 0.05 M sodium borate.

Ultraviolet-absorbing substances were located on paper with a Mineralight lamp and phosphate compounds by the method of Bandurski and Axelrod (17). Sugar spots were revealed according to Trevelyan *et al.* (18). The chromatograms were warmed over a boiling water bath in order to hasten the development of trehalose spots. Borate interferes with the revelation of trehalose. In order to overcome this difficulty, after ionophoresis the paper was passed twice through the silver nitrate reagent, and the alcoholic NaOH was made 1 N instead of 0.5 N. Sugar solutions were deionized before chromatography or ionophoresis with

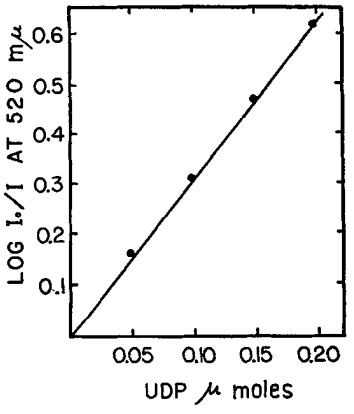

FIG. 1

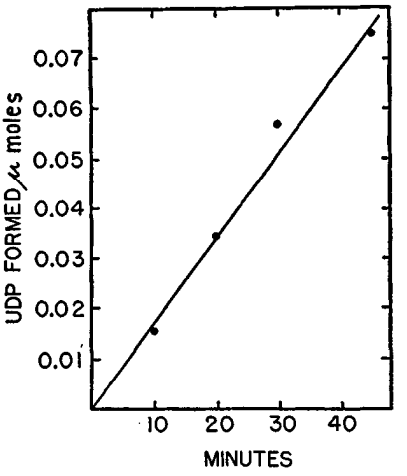

FIG. 2

FIG. 1. Determination of UDP. For the experimental details see the text.

FIG. 2. Time-course of the enzymatic reaction. The conditions are described under "Enzyme assay."

Amberlite MB-3 resin, prepared as suggested by White and Hess (19). Protein was determined according to Kunitz and McDonald (20).

Enzyme Assay—After testing several possible alternatives, the most reliable method for enzyme assay was found to be the determination of the UDP formed in the reaction. This method is based on Kornberg's observation (21) that the enzyme pyruvate kinase catalyzes the transfer of phosphate from phosphopyruvate to UDP. The pyruvate liberated can be estimated colorimetrically, after adding dinitrophenylhydrazine The presence of UMP does not interfere with the reaction.

The standard procedure was as follows: The reaction mixture contained 0.5 μmole of UDPG, 1 μmole of glucose 6-phosphate, 2.5 μmoles of magnesium sulfate, 0.1 μmole of EDTA, and 0.04 ml. of enzyme in a total volume of 0.1 ml. All solutions were previously made neutral. EDTA

and magnesium sulfate were added together from a stock solution brought to pH 7. The blanks contained the same components, except that glucose 6-phosphate was added at the end of the incubation. The tubes were incubated for 15 minutes at 37°, heated for 3 minutes at 100°, and cooled. After the addition of 0.5 μmole of phosphopyruvate in 0.05 ml., and 0.05 ml. of pyruvate kinase (diluted with water 1:20), the tubes were incubated again for 15 minutes at 37°. 1 ml. of cold 10 per cent trichloroacetic acid was added to each tube, and, after centrifuging, pyruvate was tested in 0.75 ml. of the supernatant fluid according to Friedemann and Haugen (22). The optical density at 520 mμ showed a linear dependence from the amount

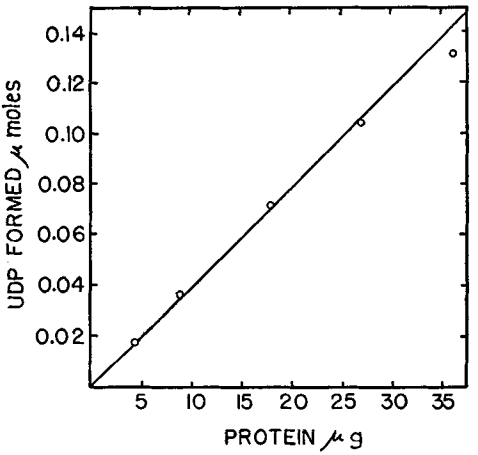

FIG. 3. Proportionality of UDP formation to the amount of enzyme. Incubation as outlined under "Enzyme assay."

of UDP, at least up to 0.2 μmole, as can be seen in Fig. 1. Figs. 2 and 3 show that plots of UDP formation *versus* time and *versus* enzyme concentration gave straight lines up to about 25 per cent of the substrate consumed.

Results

Enzyme Purification—Commercial brewers' yeast was passed through a meat chopper without a cutter, and the "noodles" thus obtained were left to dry at room temperature. The outline of a typical preparation is given below and in Table I. All operations were performed at 0–2°, unless otherwise stated. Standing for 30 minutes was allowed for ammonium sulfate precipitations. Centrifugations were carried out at approximately 15,000 × *g* for 15 to 20 minutes at 0°, except for the acetone precipitate, which was spun at 2000 × *g* for 10 minutes at −5°. Dialyses were performed in a rocking dialyzer at 2°.

360 ml. of distilled water were added to 120 gm. of dried yeast. The suspension was kept for 1 hour at room temperature with frequent shaking and then left for 20 hours in the refrigerator with occasional mixing. After centrifugation, the enzyme was precipitated from the supernatant fluid (Fraction I, 214 ml.) by adding an equal volume of 50 per cent ammonium sulfate solution (50 gm. of solid salt per 100 ml. of solution, containing 0.001 M EDTA and brought to pH 7.5 with ammonia). The precipitate was redissolved in 0.001 M EDTA (Fraction II). To this solution an equal volume of 50 per cent ammonium sulfate was added, and the precipitate was redissolved in 0.001 M EDTA. The turbid solution was again centrifuged. The supernatant liquid (Fraction III) was dialyzed for 5.5 hours against 0.001 M EDTA (three changes of 3 liters each). The di-

TABLE I

Purification of Enzyme

Fraction	Volume	Units*	Protein	Specific activity	Recovery per cent
	ml.		*mg.*	*units per mg.*	
I. Crude extract	214	25,700	29,100	0.88	100
II. 1st ammonium sulfate	62	21,400	5,150	4.15	83
III. 2nd ammonium sulfate	57	12,000	3,160	3.8	56
IV. Dialysis	69	9,770	2,660	3.7	38
V. Heating at 36°	135	8,440	2,050	4.1	33
VI. Acetone	19.5	6,520	680	9.6	25
VII. Alumina C_γ	18.3	4,460	247	18	17

* 1 unit is defined as the amount of enzyme which catalyzes the formation of 1 μmole of UDP per hour under the conditions described in the text.

alysate (Fraction IV, 69 ml.) was mixed with an equal volume of 0.04 M magnesium sulfate. The solution was kept with constant stirring in a water bath at 37° until its temperature reached 36° (about 7 minutes), and then it was rapidly cooled to 2° and centrifuged. The supernatant liquid (Fraction V) was kept in a bath at −4° until its temperature was about 0°, and 0.35 volume of cold (−4°) acetone was added slowly with stirring over a period of about 20 minutes, while the temperature was allowed to fall to −3°. After centrifugation, the precipitate was redissolved in approximately 15 ml. of 0.02 M potassium phosphate buffer of pH 7, containing 0.001 M EDTA, and dialyzed for 3.5 hours against the same buffer (three changes of 3 liters each). To the dialysate (Fraction VI) 0.2 volume of alumina C_γ (31.5 mg. dry weight per ml.) was added, and, after standing for 15 minutes with occasional stirring, the suspension was centrifuged. The supernatant fluid (Fraction VII, 18.3 ml.) was a clear, slightly yellowish solution, and the total purification achieved ranged from 15- to 20-fold in different batches.

Comments—The second ammonium sulfate precipitation usually gave a slight purification, but in the preparation described there was a small loss in purity.

The optimal amount of alumina Cγ was ascertained for every individual batch by small scale trials.

Further attempts at purification by ammonium sulfate or acetone fractionation and by isoelectric precipitation met with failure. Although the enzyme could be adsorbed on calcium phosphate or alumina gel, efforts to elute it were unsuccessful.

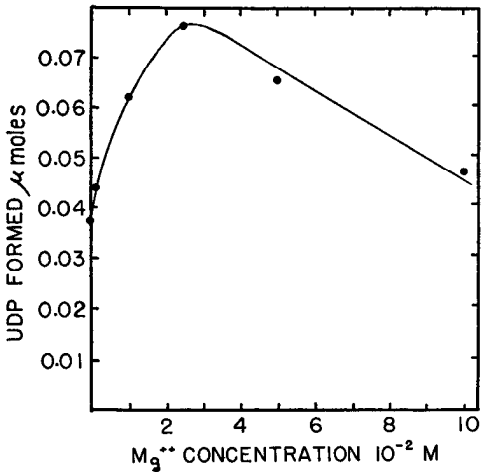

FIG. 4. Effect of Mg^{++}. The basic reaction mixture and procedure are as described in Table II.

No activity of phosphoglucomutase or UDPG pyrophosphatase could be detected in the purified enzyme. It did contain some hexosephosphate isomerase and a specific phosphatase for trehalose phosphate, which will be dealt with in more detail below.

Activators and Inhibitors—Magnesium ions at a concentration of 2.5×10^{-2} M increase the activity about 2-fold, while at higher concentration they become inhibitory, as shown in Fig. 4.

EDTA in the presence of excess Mg^{++} did not alter the activity, as illustrated in Table II. In the absence of added Mg^{++}, EDTA did not depress further the activity, and therefore it appears that the enzyme has not an absolute requirement for magnesium ions. A similar behavior has been described for acetylcholinesterase (23).

Phosphate at 3.3×10^{-2} M concentration caused a 50 per cent inhibition of the enzyme.

There seems to be a general inhibitory effect by salts; the activity was

depressed 35 per cent with 0.2 M NaCl or with 0.1 M Tris-maleate buffer (24) at pH 7.

TABLE II

Enzyme Activity in Presence of Mg^{++} and EDTA

The basic reaction mixture contained 0.5 μmole of UDPG, 1 μmole of glucose 6-phosphate, and enzyme in a total volume of 0.1 ml. Incubation time, 15 minutes at 37°. Before adding phosphopyruvate and pyruvate kinase the system was completed with magnesium sulfate when necessary.

Additions	UDP formed, μmoles × 10^2
None	3.4
2.5×10^{-2} M Mg^{++}	6.9
3.5×10^{-2} " " + 1×10^{-2} M EDTA	6.7
2.5×10^{-2} " " + 1×10^{-3} " "	6.8
1×10^{-3} M EDTA	3.4
1×10^{-2} " "	3.4

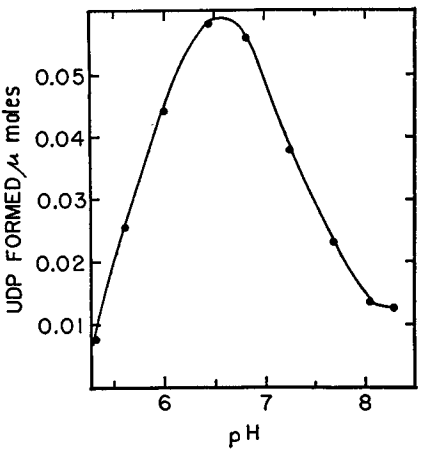

FIG. 5. pH optimum curve. System as described under "Enzyme assay" with Tris-maleate buffer (24) at 0.1 M final concentration. After 15 minutes at 37°, the reaction was stopped by heating, and the pH was brought to 7.4 by adding 0.01 ml. of either NaOH or Tris-maleic acid solution at the appropriate concentration. Then phosphopyruvate and pyruvate kinase were added, and the determination was continued as usual.

pH Optimum—As shown in Fig. 5, the highest activity with Tris-maleate buffer was obtained at about pH 6.6.

Specificity—No liberation of UDP could be detected when glucose 6-phosphate was substituted with either glucose or glucose 1-phosphate.

Fructose 6-phosphate gave rise to the formation of one-third the amount

of UDP produced by glucose 6-phosphate in the standard assay. This result can be attributed to the formation of glucose 6-phosphate catalyzed by the hexosephosphate isomerase still present in the purified enzyme. Thus, an equilibrium mixture of the two hexose phosphates gave more activity than fructose 6-phosphate alone (see Table III). Also, the activity was the same with equilibrium mixtures prepared from either glucose or fructose phosphate. This shows that the lower activity obtained with fructose 6-phosphate was not due to any inhibitor present in the sample used.

Reaction Products—When an incubated reaction mixture was deproteinized and the supernatant solution submitted to paper ionophoresis with borate buffer, in addition to the spots of nucleotides and glucose

TABLE III

Incubation of Fructose 6-Phosphate with Enzyme

All the tubes contained 0.5 μmole of UDPG, 3 μmoles of magnesium sulfate, and 0.33 unit of enzyme, plus 1.0 μmole of the hexose phosphate indicated. In Tubes 3 and 4 the corresponding ester was preincubated for 2 hours with 3.3 units of enzyme, containing enough isomerase to attain equilibrium, and, after inactivation by heating, the other components were added, making a total volume of 0.1 ml. All the tubes were then submitted to the standard assay.

Tube No.	Substrate	UDP formed, μmoles × 10^2
1	Glucose 6-phosphate	7.7
2	Fructose "	2.6
3	Equilibrium mixture from glucose 6-phosphate	5.1
4	" " " fructose "	4.8

6-phosphate a slow moving, phosphate-containing spot appeared (see Fig. 6) with the same mobility as that of authentic trehalose phosphate. In another experiment, a larger portion of the incubated mixture was run as a band, and the "trehalose phosphate" zone was eluted with water and treated with intestinal phosphatase. The product obtained moved on paper at the same rate as trehalose when submitted to chromatography or ionophoresis. The results are not given in detail, since they are similar to those described below for the isolated reaction product. One cannot distinguish clearly between UDPG and UDP by paper ionophoresis under the conditions used. Therefore, in a separate experiment, the reaction products were submitted to paper chromatography. A spot moving as UDP could be easily detected in the complete system as illustrated in Fig. 7. The results in Table IV show that the disappearance of UDPG and glucose 6-phosphate as measured by labile sugar and reducing power, respectively, was equivalent to the amount of UDP and "trehalose" formed.

Isolation of Enzymatic Disaccharide Phosphate—In order to obtain disaccharide phosphate from an incubated reaction mixture, the water-soluble,

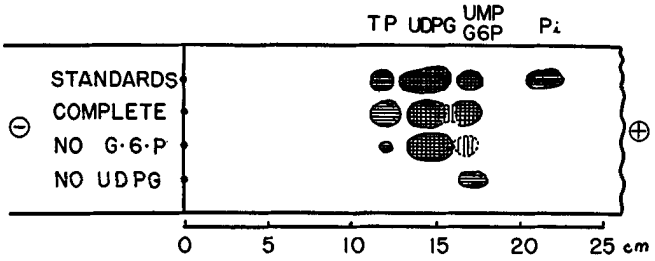

FIG. 6. Paper ionophoresis of the reaction products. The complete reaction mixture contained 6 μmoles of UDPG, 6 μmoles of glucose 6-phosphate (*G·6·P*), 30 μmoles of $MgSO_4$, 1.2 μmoles of EDTA, and 12 units of enzyme in a total volume of 1.2 ml. After 90 minutes incubation at 37°, 5 volumes of ethanol were added, and the tubes were centrifuged. Aliquots of the supernatant liquid were evaporated to dryness, redissolved in water, and spotted on paper. Ionophoresis was carried out as described in the text for 2 hours at 600 volts. Ultraviolet-absorbing spots are hatched vertically and phosphate-containing spots horizontally; *TP* trehalose phosphate, *Pi* inorganic phosphate, other abbreviations as in the text. UMP was present as impurity of UDPG. The origin of the small spot running as trehalose phosphate in the reaction mixture lacking glucose 6-phosphate remains unexplained.

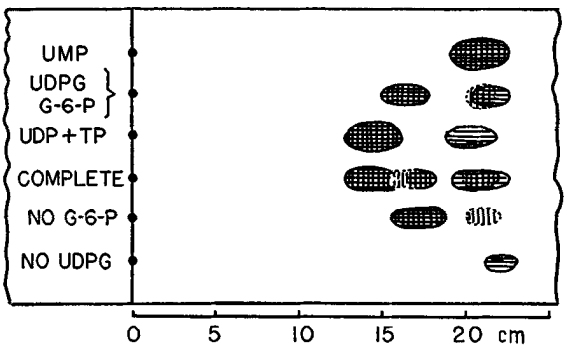

FIG. 7. Paper chromatography of reaction products. The reaction mixture was similar to that described for Fig. 6. After incubation for 90 minutes at 37°, 1 volume of 10 per cent trichloroacetic acid was added, and the tubes were centrifuged. The trichloroacetic acid was extracted thrice with ether, and the aqueous layers were evaporated to dryness, redissolved in water, and spotted on paper. Chromatography was carried out with ethanol-ammonium acetate of pH 3.8 (3). The spots are hatched as in Fig. 6.

ethanol-insoluble, barium salts were isolated and then submitted to anion exchange chromatography as described by Khym and Cohn (25).

A reaction mixture was prepared, containing 140 μmoles of glucose

6-phosphate, 70 μmoles of UDPG, 350 μmoles of magnesium sulfate, 14 μmoles of EDTA, and 137 units of enzyme in a total volume of 14 ml. After 1 hour's incubation at 37°, the mixture was heated for 2 minutes at 100°, cooled, and centrifuged for 10 minutes at 15,000 × *g*. The supernatant fluid was evaporated to dryness at 35–40°, redissolved in about 2 ml. of water, and neutralized with ammonia. A precipitate that appeared was centrifuged, washed with 0.5 ml. of water, and discarded. To the combined supernatant fluids, 0.9 ml. of 1 M barium acetate was added, and the abundant precipitate was centrifuged, washed five times with

TABLE IV

Balance of Reaction

The reaction mixture contained, per ml., 5 μmoles of UDPG, 5 μmoles of glucose 6-phosphate, 25 μmoles of magnesium sulfate, 1 μmole of EDTA, and 6.7 units of enzyme. After incubation for 45 minutes at 37°, the missing component was added when necessary, and the tubes were heated for 3 minutes in boiling water. After centrifugation, aliquots of the supernatant fluids were analyzed for the different components as described under "Methods." All the values were subtracted from those found for the tube where glucose 6-phosphate was omitted during incubation. The values refer to 1.0 ml. of reaction mixture.

Experiment No.	Component omitted during incubation	Δ reducing power	Δ labile sugar	Δ UDP	Δ "trehalose"*
		μmole	*μmole*	*μmoles*	*μmoles*
1	None	−2.2		+2.4	+2.5
	Glucose 6-phosphate	0		0	0
	UDPG	+0.1		0	0
2	None	−2.6	−2.3	+2.1	+2.4
	Glucose 6-phosphate	0	0	0	0
	UDPG	−0.4	−0.4	−0.1	0

* Measured by the anthrone test with trehalose phosphate as the standard.

0.5 ml. of water each time, and discarded. To the combined supernatant fluids, 25 ml. of 96 per cent ethanol were added, and the suspension was left overnight at −15°. After centrifugation, the precipitate was washed once with 96 per cent ethanol and once with ether and then dried in a desiccator. The solid was dissolved in 5 ml. of water, and the barium ions were precipitated with 1 M ammonium sulfate. The precipitate was washed four times with 5 ml. of water each by centrifugation. The combined supernatant liquids were brought to 30 ml., and the pH was adjusted to 8.2 with ammonia.

This solution was fed into a Dowex 1 (Cl^-) column 0.9 cm. in diameter and 10.5 cm. high. After washing the column with 75 ml. of 0.001 M ammonia, elution was started with a solution containing 0.01 M potassium tetraborate and 0.025 M ammonium chloride at a rate of 1 ml. per minute.

Fractions of 14 ml. were collected and analyzed with the anthrone reagent. A peak (A) emerged between Fractions 35 and 55, total volume 283 ml. This solution was free from inorganic phosphate and contained 36 μmoles of "organic phosphate." The elution was continued, and at Fraction 65 the solvent was substituted by 0.001 M potassium tetraborate, containing 0.025 M ammonium chloride and 0.0025 M ammonia. After another 50 fractions had emerged, a new peak (B) appeared, consisting of a reducing substance containing phosphate (49 μmoles).

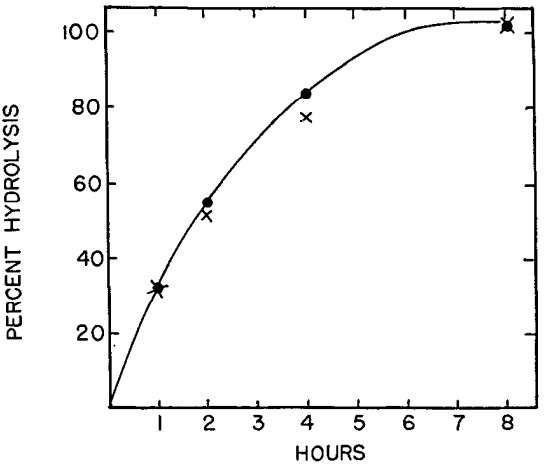

FIG. 8. Liberation of reducing power from enzymatic disaccharide phosphate and authentic trehalose phosphate in 1 N sulfuric acid at 100°. The technique was as described in Table V, except that 0.056 μmole of the substance was used in each tube and the acid was neutralized before determining sugar with ferricyanide. The data are given as per cent of the theoretical for completely hydrolyzed trehalose phosphate. ●, enzymatic product; ×, authentic trehalose phosphate.

Authentic trehalose phosphate and glucose 6-phosphate, when submitted to chromatography under the same conditions as described above, showed an identical behavior to the substances of Peaks A and B, respectively. The calcium salt of the disaccharide phosphate was isolated from Peak A by the same procedure used by Leloir and Cardini for sucrose phosphate (26). About 20 mg. of a white powder were obtained.

Properties of Disaccharide Phosphate—The product obtained did not reduce the alkaline copper reagent. The "organic phosphate" content of a sample dried over phosphorus pentoxide was 1.53 μmoles per mg. (theoretical for anhydrous Ca salt of trehalose phosphate = 2.18), corresponding to a purity of 70 per cent. The ratio "trehalose"-organic phosphate was found to be 1.0:1.0, when "trehalose" was determined with the anthrone reagent with crystalline trehalose phosphate as the standard.

The rates of liberation of reducing sugar and inorganic phosphate in 1 N acid were also similar to those of trehalose phosphate, as shown in Fig. 8 and Table V.

Ionophoresis of the intact substance gave a phosphate-containing spot with the same mobility as trehalose phosphate, while an acid hydrolysate showed a spot moving as glucose 6-phosphate (see Table VI). Inspection of Fig. 8 and Table V reveals that after hydrolysis for 8 hours in 1 N acid at 100°, all the reducing power and only about 13 per cent of the phosphate are liberated. The rest of the phosphate must therefore still be bound to glucose. A determination of glucose 6-phosphate with glucose-6-phosphate dehydrogenase in such a hydrolysate gave 98 per cent of the theoretical amount with authentic trehalose phosphate and 93.5 per cent with the reaction product.

TABLE V

Acid Hydrolysis of Reaction Product

The samples (0.21 μmole) were heated at 100° in 1 N sulfuric acid in sealed tubes.

Time of heating	Phosphate liberated	
	Reaction product	Authentic trehalose phosphate
hrs.	*per cent*	*per cent*
8	13.8	12.9
16	25.7	23.8
24	39.5	34.2

In another experiment the disaccharide phosphate was incubated with intestinal phosphatase. The free sugar thus obtained, when run on paper, gave a trehalose spot, while, after acid hydrolysis, a glucose spot was obtained (see Table VI). The solvent used does not differentiate trehalose from either cellobiose or maltose, but the three sugars can be easily separated by paper ionophoresis, as shown in Table VI. Furthermore, the sugar from the reaction product showed the same sluggishness in reacting with silver nitrate as authentic trehalose.

The rotatory power was measured on 0.3 ml. of a 0.1 N HCl solution containing 13.2 μmoles of the disaccharide phosphate, and the concentration was checked by phosphate estimation; $[\alpha]_D$ +185°, calculated for the free acid. A similar measurement on a sample of crystalline trehalose phosphate gave an identical value.

Equilibrium of Reaction—It can be seen in Table VII that the transformation of UDPG into UDP is practically complete at pH 6.1. It can also be observed that some inorganic phosphate is liberated by an enzyme to be discussed below.

No reaction in the reverse direction, that is, on starting with UDP and trehalose phosphate, could be detected, even when glucose-6-phosphate dehydrogenase and UDPG dehydrogenase were added separately or to-

TABLE VI

Paper Chromatography and Ionophoresis of Reaction Product

The butanol-pyridine-water solvent was used for chromatography. Ionophoresis was carried out with Whatman No. 1 borate-buffered paper at 600 volts for 2 hours for esters and 6 hours for free sugars. The results are given in the form $R_{standard}$ = (distance traveled by unknown)/(distance traveled by the standard substance).

	Chromatography	Ionophoresis
	$R_{glucose}$	$R_{phosphate}$
Reaction product		0.54
" " after acid hydrolysis*		0.72
Trehalose phosphate		0.54
Glucose 6-phosphate		0.72
Fructose "		0.74
		$R_{mannose}$
Reaction product after phosphatase†	0.60	0.03
" " " " and acid hydrolysis‡	0.61 (faint), 1.01	
Trehalose	0.59	0.03
Maltose	0.64	0.32
Cellobiose	0.62	0.17
Melibiose	0.44	1.07
Sucrose	0.81	
Lactose	0.48	
Galactose	0.83	
Mannose	1.12	
Fructose	1.12	

* Heated for 8 hours in 1 N HCl at 100°.

† Incubated for 16 hours at 37° with intestinal phosphatase. After deproteinizing with 5 per cent trichloroacetic acid and extracting the acid with ether, the solution was passed through Amberlite MB-3 resin.

‡ The sugar obtained by phosphatase action was hydrolyzed for 6 hours in 1 N sulfuric acid at 100° and passed through Amberlite MB-3 resin.

gether in an effort to remove the reaction products. Both crystalline and enzymatically prepared trehalose phosphate gave negative results. The experiments were rendered somewhat more difficult by the presence of slight amounts of interfering enzymes in the dehydrogenases: the glucose-6-phosphate dehydrogenase preparation slowly oxidized reduced triphosphopyridine nucleotide, while the UDPG dehydrogenase contained a system that reduced diphosphopyridine nucleotide.

Trehalosephosphate Phosphatase—The results of Table VII show that inorganic phosphate is liberated as the reaction proceeds. When UDPG,

TABLE VII

Extent of Reaction

The reaction mixtures contained per each 0.1 ml. the following: 0.444 μmole of UDPG, 0.8 μmole of glucose 6-phosphate, 2.5 μmoles of $MgSO_4$, 0.1 μmole of EDTA, and 1.0 unit of enzyme. After incubation at 37°, the tubes were heated for 3 minutes at 100° and centrifuged. Analytical determinations were carried out on aliquots of the supernatant fluids. The figures are in micromoles per each 0.1 ml.

	Incubation time		
	1 hr.	2 hrs.	3 hrs.
UDP	0.36 (80)*	0.42 (94)	0.435 (98)
Inorganic phosphate	0.04	0.1	0.16
Fructose 6-phosphate	0.049	0.064	0.069
UDPG†		0.026 (5.9)	0.01 (2.2)
pH	6.3	6.1	6.1

* The figures in parentheses refer to the per cent of UDPG added.

† Measured with UDPG dehydrogenase (5).

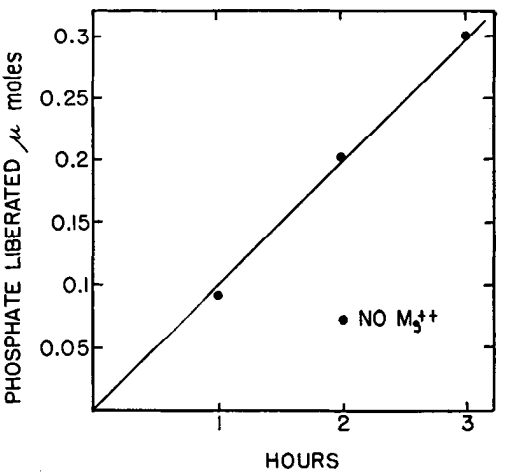

FIG. 9. Activity of trehalosephosphate phosphatase. The reaction mixture contained 1 μmole of trehalose phosphate, 2.5 μmoles of $MgSO_4$, and 54 γ of enzyme protein in a total volume of 0.1 ml. After incubation at 37°, the reaction was stopped by heating, and the tubes were centrifuged. Inorganic phosphate was determined on an aliquot of the supernatant fluid.

glucose 6-phosphate, UDP, trehalose phosphate, or glucose 1-phosphate was individually tested with the purified enzyme, only trehalose phosphate

was found to be hydrolyzed (see Fig. 9). It seems, therefore, that yeast contains a specific phosphatase for trehalose phosphate. The phosphatase is activated by magnesium ions.

DISCUSSION

The saccharide derivative formed in the reaction and its dephosphorylated product behave as trehalose phosphate and trehalose, respectively, when submitted to chromatography or ionophoresis. The rates of splitting in acid of both the glycosidic linkage and the phosphate group of the reaction product are also the same as those of trehalose phosphate. Glucose 6-phosphate and glucose were found to be present in the hydrolysates. The rotatory power of the reaction product was the same as that of an authentic sample of α,α-trehalose phosphate (27). All this evidence, together with the lack of reducing power of the intact substance, supports the conclusion that the reaction product is α,α-trehalose phosphate.

The equilibrium of the reaction is displaced far toward the synthesis of trehalose phosphate. If it is assumed in the experiment described in Table VII that equilibrium was reached after 3 hours of incubation, the value of $K_{\text{trehalose phosphate}}$ = [trehalose phosphate][UDP]/[UDPG][glucose 6-phosphate] would be 40. Since reversibility could not be demonstrated, this is probably a minimal value. It should be borne in mind, however, that the reversal experiments are not conclusive. Their failure may be partly due to the slowness of the reverse reaction or to certain experimental conditions, like the high pH (8.1), used to insure a good activity of the dehydrogenases added.

These results invite some considerations about the free energy of hydrolysis of the trehalose glycosidic bond as compared to that of sucrose. Data on the equilibrium of the analogous synthesis of sucrose phosphate are lacking, but those obtained for sucrose (28), corresponding to a $\Delta F^0_{\text{sucrose}} \simeq -1000$ calories at 37°, can be used as an approximation. A correction must be made, since the sucrose equilibrium was determined at pH 7.4, while in the trehalose phosphate reaction the pH was 6.1. The ionization of the secondary phosphate of the UDP formed drives the reaction toward the synthesis of the disaccharide phosphate (29). The corrected value for $K_{\text{trehalose phosphate}}$ at pH 7.4, assuming a pK of 6.7 for the secondary phosphate of UDP, is 192, giving a minimal

$$\Delta F^0_{\text{trehalose phosphate}} = -3200 \text{ calories}$$

The two ΔF values differ therefore by 2200 calories, and the same difference will exist between the free energy of hydrolysis of the two sugars. By taking for the ΔF^0 of hydrolysis of sucrose the value of -6600 calories (30), the *maximal* value for that of trehalose phosphate would be -4400

calories. It may be mentioned that the ΔF^0 of hydrolysis of maltose has been calculated as about -4000 calories (31).

The value of the ΔF^0 of the hydrolysis of sucrose applies, however, to the sum of two reactions, namely, the splitting of the glycosidic bond and the partial transformation of the liberated fructofuranose into fructopyranose. If it is assumed that the pyranose to furanose ratio at equilibrium is about 4:1 (32), then this would correspond to a ΔF^0 of -850 calories, to be subtracted from the value for the ΔF^0 of hydrolysis mentioned above. The ΔF^0 for the splitting of the glycosidic bond would thus be -5750 calories, a value still substantially higher than that for trehalose phosphate.

The position of privilege held by sucrose among the disaccharides from the standpoint of the free energy of hydrolysis is in harmony with its efficiency as a precursor of polysaccharides (33).

The presence in the purified enzyme preparation of a specific phosphatase for trehalose phosphate suggests the possibility that dephosphorylation is the next step in the ulterior metabolism of this substance. The hypothesis has been advanced (34) that trehalose could give rise to polysaccharide material, for instance the glucan of the yeast cell wall. This seems an interesting field for further experimentation.

SUMMARY

An enzyme which catalyzes the reaction

UDPG + glucose 6-phosphate → UDP + trehalose phosphate

has been purified 15- to 20-fold from brewers' yeast. The enzyme showed maximal activity at pH 6.6 in the presence of 2.5×10^{-2} M magnesium ions.

The disaccharide phosphate produced in the reaction was isolated and identified as trehalose phosphate by comparison of its properties with those of the authentic substance.

The equilibrium of the reaction is displaced toward the synthesis of trehalose phosphate, and reversibility could not be demonstrated. An upper limit of 4400 calories for the ΔF^0 of hydrolysis of the trehalose glycosidic bond was calculated from the experimental data.

The purified enzyme preparation contains a specific phosphatase for trehalose phosphate.

A method for the estimation of UDP is presented.

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