ISOLATION OF THE COENZYME OF THE GALACTOSE PHOSPHATE-GLUCOSE PHOSPHATE TRANSFORMATION*

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The first step in the utilization of galactose has been found to be a phosphorylation by adenosine triphosphate (ATP) catalyzed by galactokinase (1–3).

$$\text{Galactose} + \text{ATP} \rightarrow \text{galactose-1-phosphate} + \text{ADP}^1 \qquad \text{(I)}$$

Evidence has been presented (4) showing that the enzymatic transformations which follow are

$$\text{Galactose-1-phosphate} \xrightarrow[\text{(II)}]{} \text{glucose-1-phosphate} \xrightarrow[\text{(III)}]{} \text{glucose-6-phosphate}$$

In the initial studies (5) it was found that a thermostable factor was required in the over-all Reactions II and III. This fact led eventually to the discovery and isolation of glucose diphosphate (6), which acts as a coenzyme in Reaction III. These results have been confirmed by Sutherland *et al.* (7), and biological (8, 9) as well as chemical (10, 11) methods for the synthesis of glucose diphosphate have been described.

Reaction II, which consists in a Walden inversion of C-4, is catalyzed by an enzyme which is currently called "galactowaldenase" in this laboratory. The corresponding coenzyme has been named uridine-diphosphate-glucose (UDPG) for reasons which will become apparent in what follows.

Estimation and Isolation of UDPG

The method of estimation of UDPG was based on its property of accelerating Reaction II. In practice the rate of Reaction II was measured as the sum of Reactions II and III by the rate of appearance of reducing power. The reaction mixture contained galactose-1-phosphate and a maceration juice of *Saccharomyces fragilis* plus variable amounts of UDPG. Glucose diphosphate was also added; so that the velocity of the over-all reaction was limited by the rate of Reaction II. The results obtained by varying the amounts of UDPG are shown in Fig. 1.

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[1] Adenosine diphosphate.

Of the different sources of coenzyme tested as starting material for the purification, bakers' yeast was found to be the best, but it is of interest that animal tissues were also found to contain UDPG, although in slightly smaller amounts. The yeast used was not adapted to galactose and hardly fermented this sugar. This raises the question as to whether UDPG may not have some other function besides accelerating the galactose-glucose transformation.

The UDPG content of yeast was found to be increased for unknown reasons after a short incubation with toluene. The amount became at least doubled, and use of this fact was made in the large scale preparation.

Purification of UDPG was effected by extraction of the toluene-treated yeast with 50 per cent ethanol followed by precipitation with mercuric salts. The mercury precipitate was then suspended in 1 M ammonium acetate when most of the UDPG was dissolved, leaving a considerable

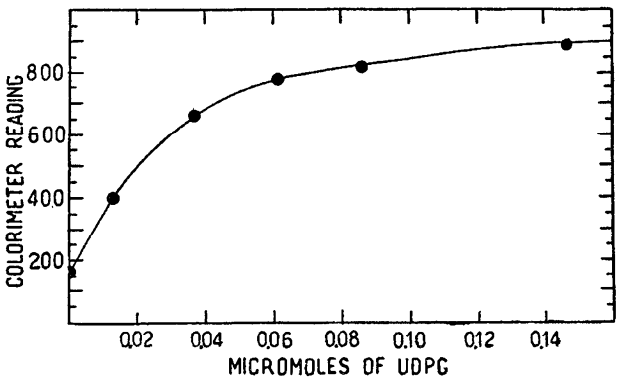


FIG. 1. The activation of galactowaldenase by UDPG

amount of impurities which were insoluble. After reprecipitation the precipitate was decomposed with H_2S in the cold. The next step was adsorption on charcoal and elution with 50 per cent ethanol. Amino acids were then removed with a cation exchange resin and the charcoal adsorption repeated.

The product obtained had maximum absorbency at 260 mμ. Many other procedures of purification were applied, but it was not possible to increase the ratio of activity-absorbency at 260 mμ. This was an indication that the absorption was due to the active substance. Confirmation of this fact was obtained by paper chromatography which showed that the activity always ran parallel with the absorbency at 260 mμ.

Presence of Uridine

The absorption spectrum of UDPG at different pH values is shown in Fig. 2. The curves agree well with those of uridine shown in the same

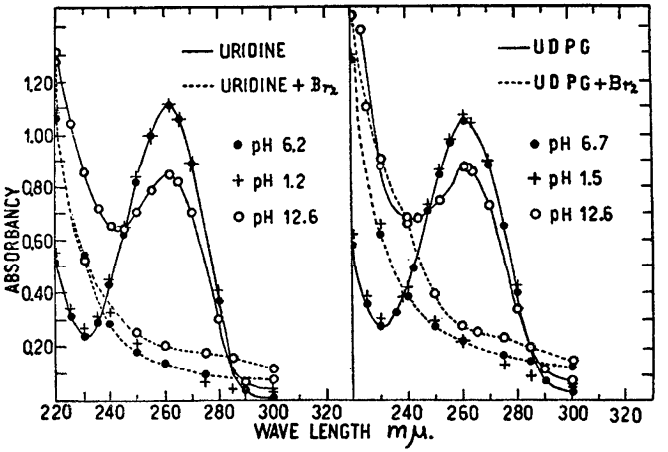


FIG. 2. Comparison of the spectra of UDPG and uridine at different pH value- and after bromine treatment. The latter was carried out in acid reaction by addis tion of a drop of bromine water followed by aeration.

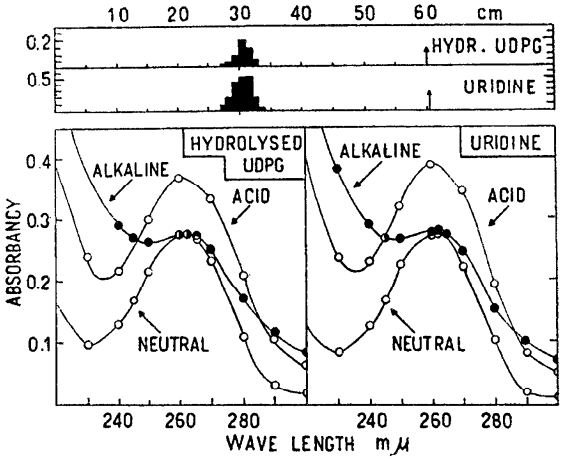


FIG. 3. Comparison of a known sample of uridine with the nucleoside obtained by hydrolysis of UDPG. The upper figure shows the position of the substances as revealed by the absorbency at 260 μM after paper chromatography with 77 per cent ethanol. Hydrolysis of UDPG was carried out for 40 hours in 0.1 N sulfuric acid, followed by removal of the acid with barium hydroxide and concentration. The chromatogram was developed according to the procedure of Hotchkiss (27). The lower figure shows the spectra of the substances extracted from the paper after the chromatography. These spectra are not exactly equal to those of non-chromatographed uridine. The difference could be produced by the action of light and probably took place during the drying of the papers.

figure and are clearly different from those of other purine or pyrimidine derivatives (12). The effect of bromine, which destroys completely the coenzymatic activity, is also shown.

As shown in Fig. 3, uridine could be identified after acid hydrolysis by comparison with a known sample with respect to behavior during paper chromatography and to the spectra at different pH values.

Uracil could also be identified by chromatography and by its spectrum after hydrolyzing UDPG 6 hours in 2.5 N sulfuric acid at 100°. Moreover, it was not precipitated by silver salts under the conditions in which purine bases are completely precipitated (13).

Estimation of the pentose in the hydrolysis products of UDPG carried out by the orcinol method after bromination as described by Massart and Hoste (14) gave the expected values. It was observed that the rate of development of the color was more rapid than with free ribose or uridine.

Presence of Phosphate

Once uridine was proved to be a constituent of UDPG, it was possible to calculate the concentration of the solutions by means of the molar absorbency index of uridine. This constant has been measured by Ploeser and Loring (12), who gave the value $A_m = 9820$. The value for uridylic acid was practically the same (10,040). Estimations of phosphate in UDPG revealed 2 molecules per molecule of uridine. Conversely, calculation of the molar absorbency on the assumption of two phosphates gave a value of 10,450. One of the phosphate groups is acid-labile and can be hydrolyzed in 15 minutes in 1 N acid at 100°. The hydrolysis curves for the labile phosphate are shown in Fig. 4. Comparison with other known compounds reveals that the rate of hydrolysis is slower than that of the labile phosphate of adenosine triphosphate and of about the same order as that of adenosine-3′-phosphate.

The rate of hydrolysis of the stable phosphate in 0.1 N acid gave values comparable with those given by Gulland and Smith (15) for uridine-2′- and 5′-phosphates, but nearer the former. The rate was definitely lower than that given for uridine-3′-phosphate.

Presence of Glucose

Besides uridine and two phosphate groups, UDPG was found to contain a reducing substance which could be split off by mild acid hydrolysis. The results of the carbazole reaction (16) on UDPG did not give clear cut results, but after acid hydrolysis and removal of contaminants with ion exchange resins, the color corresponded to that given by glucose. Moreover, the sugar was found to be fermented by *Candida monosa*, which does not act on galactose.

Paper chromatography with 77 per cent ethanol, by which galactose can be distinguished from glucose, also indicated the latter as the component of UDPG.

The rate of liberation of glucose from UDPG on acid hydrolysis is shown in Fig. 5. This rate is about 6 times higher than that of glucose-1-phos-

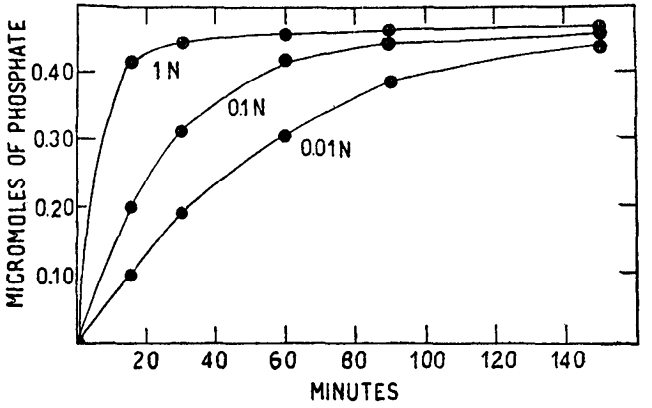


FIG. 4. Hydrolysis of the labile phosphate of UDPG in acid at 100°. Total phosphate of samples, 0.9 μM.

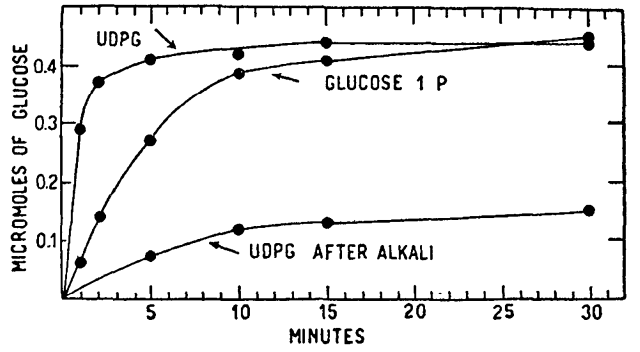


FIG. 5. Liberation of glucose in 0.01 N acid at 100°. Upper curve, untreated UDPG. Lower curve, UDPG which had been heated 30 minutes at 100° in 0.01 N sodium hydroxide. Glucose-1-phosphate is shown for comparison.

phate and about 40 times higher than that of the labile phosphate of UDPG. These differences in rates of hydrolysis allowed the isolation of a uridine diphosphate and a uridine monophosphate from UDPG.

That the bound glucose was part of the active molecule was proved by the constancy of the ratio uridine-phosphate-glucose, which always remained 1:2:1 after various fractionation procedures. Additional evidence on this point was obtained by comparing the rate of liberation of glucose

with the rate of inactivation of the coenzyme in 0.01 N acid at 100°. The results were as follows:

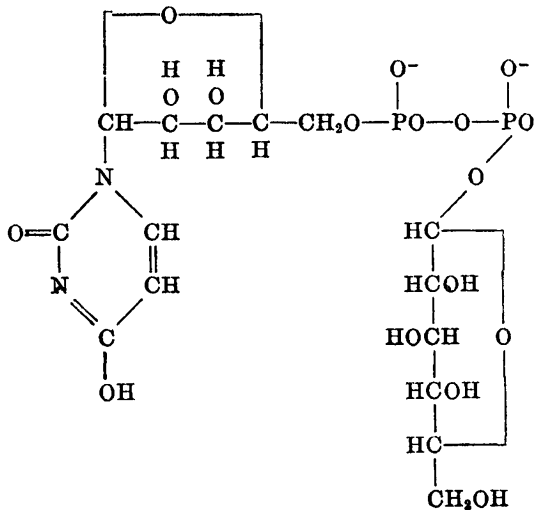
Time of heating, *min.*	1	2	5
% glucose liberated	64	82	91
" inactivation	50	75	100

Oxidation of UDPG with periodate gave 1 molecule of formic acid, as if positions 2, 3, and 4 of the glucose were free.

Linkage of Different Components

Uridine, two phosphate groups, and glucose having been identified as components of UDPG, there remained to find out how they are combined in UDPG and whether or not there is some other component. Nitrogen estimations gave 2 atoms per molecule; thus any other nitrogen-containing substance besides uridine could be excluded. Dry weight measurement gave a molecular weight of 630, calculated from the uridine content. The theoretical weight for the sum of uridine, glucose, and two phosphate groups minus 3 molecules of water is 566. The preparation would thus be 90 per cent pure.

Electrometric titration as shown in Fig. 6 indicated two acid groups in the range of the primary dissociation constant of phosphoric acid and no secondary. On treatment with acid secondary phosphoric acid groups appeared: one after hydrolysis of the glucose and another after the labile phosphate became inorganic. The accompanying formula agrees well with these findings.



The constant of hydrolysis of the labile phosphate in UDPG is about 10 times slower than that of ATP. This fact cannot be interpreted against the pyrophosphate structure of UDPG, because uridine phosphates are more stable than the corresponding adenine phosphates. The approximate values of the constants of hydrolysis are shown in Table I.

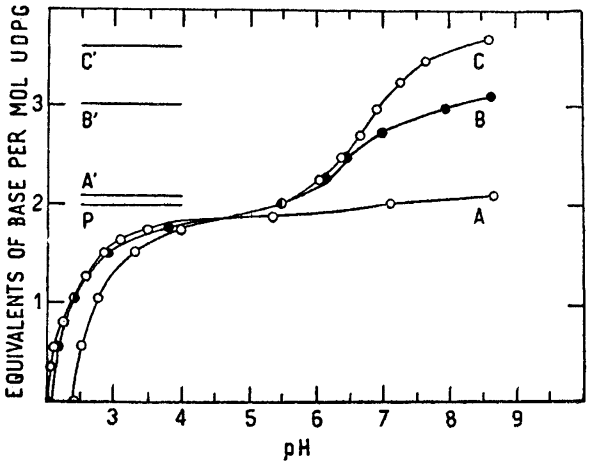


FIG. 6. Electrometric titration of UDPG before and after hydrolysis. Sample A, 14.7 μM of unhydrolyzed UDPG. Sample B, after heating the free acid 4 minutes at 100°. Sample C, after 60 minutes under the same conditions. The analytical results in micromoles were as follows:

	Sample A	Sample B	Sample C
Total phosphate (P_T)	29.52	29.52	29.5
Inorganic phosphate (P_0)	0.48	2.40	9.92
Free glucose (G)	0.84	12.48	14.04
$P_T + P_0 + G$	30.84	44.40	53.48

Levels A', B', and C' correspond to the value of ($P_T + P_0 + G$)/UDPG. Level P corresponds to P_T/UDPG. See the text.

Position of Phosphate Group in Uridine

The phosphate seems to be attached to position 5 of the pentose in uridine. One test which was applied is based on the property of substances containing neighboring hydroxyls to form a complex with copper. Klimeck and Parnas (17) observed that adenosine-5'-phosphate forms such a complex, whereas adenosine-3'-phosphate does not. Since this test requires considerable amounts of substance, it was adapted to a microscale by estimating the copper with diethyl dithiocarbamate, as described by Stiff (18) for the estimation of protein. Comparative tests with adenosine-3'-

and 5′-phosphates, uridine-3′-phosphate, and uridine monophosphate prepared from UDPG showed that the latter behaved as if the phosphate were in position 5′ (Table II).

Another test which was carried out was the measurement of the rate of color development with the orcinol reagent. This rate was found by Albaum and Umbreit (19) to depend on the position of the phosphate group in ribose derivatives. It is higher for ribose-5-phosphate than for ribose-3-phosphate or free ribose and the same is true for the corresponding adenylic acids. This test cannot be applied directly to uridylic acid due to the

TABLE I

Hydrolysis Constants (Log_{10}) for Phosphate of Some Nucleotides

The figures in parentheses refer to the bibliography.

	K
Labile phosphate of UDPG	0.0039
" " " ATP	0.038
Adenosine-3′-phosphate (33)	0.0036
Uridine-3′-phosphate (15)	0.0005
Adenosine-5′-phosphate (33)	0.00034
Uridine-5′-phosphate (15)	0.00008

TABLE II

Copper Complex Formation

	Colorimeter reading*
Adenosine-5′-phosphate, 2 μM	600
Adenosine-3′-phosphate, 2 μM	58
Uridine-3′-phosphate, 2 μM	64
Uridine monophosphate from UDPG, 2 μM	610

* Klett-Summerson photocolorimeter with Filter 44.

stability of the ribose-uracil linkage. However, the uracil nucleus can be destroyed by treatment with hydrazine (20) followed by nitrite, and then the ribose phosphate can be isolated.

As shown in Table III, the rate of color development with the orcinol reagent of the ribose phosphate isolated from UDPG follows closely that of adenosine-5′-phosphate and is different from that of free ribose or adenosine-3′-phosphate.

The only result which did not agree completely with the properties of uridine-5′-phosphate was the hydrolysis with acid. The uridine monophosphate from UDPG in 0.1 N sulfuric acid at 100° gave 18.3 and 33 per

cent liberation of phosphate after 7.42 and 21.2 hours respectively. Gulland and Smith (15) give 8.6 and 20.5 per cent for uridine-5′-phosphate, 12.5 and 29.5 for the 2′-phosphate, and 38 and 69 for the 3′-phosphate. The rate of hydrolysis was therefore more similar to that of uridine-2′-phosphate. However, there might have been some difference in the experimental procedures.

Action of Alkali

Heating with 0.01 N alkali destroys the coenzymatic activity in a few minutes. This fact does not appear to be readily explained by the above formula, since glucose-1-phosphate, the pyrophosphate group, and uridylic acid are known to be stable to alkali.

A study of the action of alkali revealed that it does not lead to the liberation of phosphate or to a permanent change in the absorption spectrum.

TABLE III

Color Development with Orcinol Reagent

Procedure as described by Albaum and Umbreit (19) but at 85°. Values in per cent of maximum.

Time, *min.*	7	15	35	70	85	95
Ribose	8.9	28.3	66	94	98.1	100
Adenosine-3′-phosphate	5.9	23.6	64.2	94.5	97.9	100
Adenosine-5′-phosphate	42.6	70.1	91.4	99.1	100	100
Ribose phosphate from UDPG	42.4	69	91.2	99.2	99.6	100

The only change which could be detected is a change in the rate of liberation of glucose by acid. This change is shown in Fig. 5. It is not due to an alteration on the glucose residue, since glucose can be recovered unchanged after hydrolysis with stronger acid. After alkaline treatment the glucose is removed in normal acid at about the same rate as the labile phosphate, whereas in untreated UDPG the glucose is removed much faster than the phosphate. Moreover, after the action of alkali the titration curve shows the presence of one secondary acid group besides the two primary groups present in untreated UDPG.

An explanation of these changes would be a migration of the glucose residue from the phosphate to the pentose.

Relationship with Substance Accumulating in Staphylococcus aureus Treated with Penicillin

Park and Johnson (21) reported the accumulation of a substance in *Staphylococcus aureus* cells when grown in the presence of penicillin. This

substance contains uracil, a reducing substance, and two phosphate groups which hydrolyze at about the same rate as in UDPG. However, other properties appear to be different, since the sugar does not appear to be glucose and the nitrogen values were higher.

Due to the similarity of the substances some experiments were carried out with the compound from *S. aureus*. By means of the procedure described by Park and Johnson (21) a partially purified substance containing uracil, labile phosphate, and a reducing substance in the relation 1.4:1:0.7 was obtained. The tests did not reveal any activating or inhibiting action on the glucose phosphate → galactose phosphate transformation. Moreover, preliminary tests agreed with the work of Park and Johnson in relation to the non-identity of the carbohydrate with glucose.

EXPERIMENTAL

Methods

The methods were as described in previous papers (1, 2, 6). Galactose phosphate was synthesized according to the procedure of Colowick (22) and Kosterlitz (23). Dry weight measurements were carried out, after drying over phosphorus pentoxide at 56° for 3 hours, on acid UDPG prepared by treating the sodium salt with Amberlite IR-100. Nitrogen estimations were carried out by the Kjeldahl method.

Enzymatic Estimation

S. fragilis was obtained as described by Caputto *et al.* (1), spread out on plates in a layer 2 to 3 mm. thick, and allowed to dry in air at room temperature. The dry yeast was extracted with 3 volumes of 2.2 per cent diammonium phosphate at 5° for 24 hours. These extracts could be stored frozen for many months. The procedure for the estimation of UDPG was as described by Cardini *et al.* (6) for glucose diphosphate but with use of a reaction mixture which contained 2 μM of galactose-1-phosphate, 2 μM of Mg^{++}, 0.01 μM of glucose diphosphate, 0.01 ml. of undiluted *S. fragilis* extract, and UDPG in amounts ranging from 0.01 to 0.1 μM, in a total volume of 0.2 ml. The reaction was allowed to proceed 20 minutes at 37° and was stopped by addition of the copper reagent.

Distribution

Estimations were carried out on the extracts obtained by the addition of 1 volume of ethanol to the fresh organs, heating to boiling, filtering, neutralizing, and evaporating off the alcohol. Due to their glucose content, the liver extracts had to be purified by precipitation with excess mercuric salts as described in "Preparation."

The results in micromoles of UDPG per gm. of fresh tissue ranged be-

tween 0.2 and 0.3 for rat kidney, brain, and muscle. For the liver the values were 0.1 to 0.2 μM. In yeast the content was somewhat variable. In bakers' yeast the content was 0.4 to 1.0 μM. After 4 to 6 days at 5° the content was the same or higher in some cases. In brewers' yeast the amount was about 0.5 μM per gm.

Action of Toluene

It was found that a treatment of yeast with toluene increased the yield of UDPG. When yeast intimately mixed with 10 per cent of its weight of toluene is incubated at 35–37°, there occurs an increase in UDPG, which attains a maximum after about 40 minutes, followed by a slow decrease. For instance, one experiment gave the following changes.

Time of incubation, *min.*	0	10	20	30	40	60
UDPG concentration, *μM per gm.*	0.6	1.7	1.9	2.0	2.1	1.8

The incubation in the absence of toluene produced no changes. The increases produced by toluene in different experiments varied from 50 to 400 per cent, and the maximum concentration attained never exceeded 2.5 μM per gm. of yeast.

Preparation

Step 1. Extraction—5 kilos of bakers' yeast were spread out in a layer about 5 cm. deep and heated in an incubator. When the temperature attained 35–36° the yeast was intimately mixed with 500 ml. of warm toluene. After 40 minutes at 35–36°, 5 liters of 95 per cent ethanol were added and the mixture heated in a water bath until it boiled. On the following day it was filtered through a 32 cm. Büchner funnel with a filter aid.

Step 2. Precipitation with Mercuric Salts—The filtrate was acidified with 5 N nitric acid until acid to Congo red paper. Then 30 ml. of mercuric acetate per liter were added. After mixing, the preparation was left overnight in the ice box. The suspension was filtered through a Büchner funnel until the precipitate was nearly dry. This precipitate was then blended in 600 ml. of 1 M ammonium acetate and left at room temperature for 2 hours. The suspension was filtered and the filtrate was acidified to Congo red paper with nitric acid, 30 ml. of mercuric acetate and 1 volume of ethanol were added, and the mixture left overnight in the cold. After filtration the precipitate was blended in 600 ml. of water and decomposed with hydrogen sulfide in the cold. The mercuric sulfide was filtered off, washed with 100 ml. of water, and the combined filtrates aerated and neutralized to pH 7. The results are shown in Table IV.

Remarks—The mercuric acetate solution was prepared by mixing 13.5

gm. of yellow mercuric oxide, 9.2 ml. of glacial acetic acid, and water to make 100 ml.

The optimum amount of mercury reagent to be added was ascertained in preliminary trials and was sufficient to precipitate nearly all the activity. Addition of more mercuric reagent gave a precipitate of inactive substances.

The extraction of the mercuric precipitate with ammonium acetate was also studied in small scale trials. A second extraction removed more activity from the precipitate, but the purity was inferior.

The decomposition with hydrogen sulfide was carried out in an ice bath and was complete in about 4 hours. The step is rather critical, since the active substance is very sensitive to acid.

TABLE IV

Results of Purification Procedure

Step No.		Volume of extract	Concentration of UDPG*	UDPG-total P ratio	Ratio, UDPG-absorbency at 260 mμ
		ml.	*μM per ml.*		
1	Yeast extract	11,000	0.65	0.072	0.016
2	After Hg^{++} and H_2S	1,500	4	0.375	0.043
3	1st charcoal elution	140	25	0.62	0.105
4	After resin	320	11	0.73	0.125
5	2nd charcoal eluates: 3rd fraction†	14.4	7.3	0.51	0.107
	2nd charcoal eluates: 4th "	12	26	0.57	0.104
	2nd charcoal eluates: 5th "	12.6	30	0.40	0.101
	2nd charcoal eluates: 6th "	10.5	10	0.36	0.081
	2nd charcoal eluates: 7th "	16.2	6.5	0.41	0.096

* Measured by enzymatic method.

† First and second fractions discarded.

Step 3. Purification with Charcoal—Two lots of extract prepared as described were mixed. 1 ml. portions were then treated with charcoal to find the amount necessary for 90 per cent adsorption of the material with absorbency at 260 mμ. This corresponded to nearly complete adsorption of the active substance. In the preparation described 103 gm. of Pfanstiehl's norit A were used for the total amount of extract. After 15 minutes at room temperature with occasional shaking the suspension was filtered. The charcoal was washed with 300 ml. of water and then suspended in 400 ml. of 50 per cent ethanol. After 15 minutes the suspension was filtered and the filtrate concentrated at about 45° under reduced pressure to one-third of its original volume.

Remarks—A second eluate of the charcoal still contained activity but the

purity was inferior. Contaminating substances such as glucose diphosphate are not adsorbed by charcoal, while others like diphosphopyridine nucleotide are adsorbed but not eluted appreciably with aqueous ethanol.

At this stage the substance is nearly pure as judged by the ratio of activity to absorbency at 260 mμ, but it still contains considerable amounts of substances giving a positive ninhydrin reaction.

Step 4. Treatment with Cation Exchange Resin—The extract from Step 3 was cooled, acidified to about pH 1.2 with hydrochloric acid, and treated five times with 15 gm. of moist Ionac C-200. The temperature was kept at 4–5° and the time of contact was 15 minutes each time. The liquid was then neutralized.

The amount of resin was that necessary to reduce the ninhydrin reaction by about 90 per cent.

Step 5. Second Adsorption with Charcoal—The liquid was treated with 26 gm. of norit. The necessary amount was ascertained as previously described. The suspension was filtered and washed with 100 ml. of water. It was then eluted by adding 50 per cent ethanol to the Büchner funnel which had been used for filtering and collecting the percolate in 10 to 15 ml. fractions.

The results of a preparation which was not one of the best are shown in Table IV.

Solubility of Different Salts

Basic lead acetate will precipitate UDPG even from very dilute solutions. With mercuric acetate in acetic acid solution it does not precipitate, but by addition of nitric acid to about pH 4 and 1 volume of ethanol complete precipitation is achieved.

The barium salt is very soluble in water. At pH 8 and after adding 2 volumes of 95 per cent ethanol the supernatant contains about 1.5 μM per ml. of UDPG. The sodium, potassium, and ammonium salts are very soluble in water. Tests with the brucine salt did not give a crystalline substance.

Correlation of Activity with Absorbency at 260 mμ

A 0.05 ml. sample containing 1.2 μM of a pure preparation was deposited on No. 1 Whatman paper and chromatographed with 77 per cent ethanol (24) by the ascending technique (25, 26). After 16 hours the paper was dried and extracted according to the procedure of Hotchkiss (27). Bands of 5 mm. were immersed in 3 ml. of water for 2 to 3 hours. The absorbency at 260 mμ was measured and then the coenzymatic activity. For the latter 1 ml. of extract was used and, owing to the dilution, the amount of enzyme and the time of incubation were doubled. The results showed a complete

coincidence between absorbency and coenzymatic activity. The R_F value was 0.44.

Impure preparations chromatographed as described and developed with ninhydrin showed several spots, but they differed in position from the active substance. Other solvents such as butanol-water with or without acetic acid hardly produced any migration of UDPG. With phenol-water the migration was satisfactory, but the solvent is difficult to eliminate and interferes with the spectral analysis.

Estimation of Pentose

Direct estimation of pentose by the orcinol method of Mejbaum (28) gave hardly any reaction, as would be expected from a pyrimidine nucleotide. However, after treatment with bromine, as described by Massart and Hoste (14), better results were obtained. With intact UDPG a green precipitate appeared. If UDPG was hydrolyzed 20 minutes in 1 N acid at 100° and then adsorbed on anion exchange resin and eluted with normal acid, then the presence of pentose was clearly detected. On comparison with a uridine solution of equal absorbency at 260 mμ after a 10 minute heating period, the ribose content was 30 per cent too high. If the heating time was prolonged to 30 or 40 minutes, then the ribose content was equal to that of uridine. The faster rate of color development is probably due to the presence of the phosphoric acid group.

Identification of Glucose

For all the tests UDPG was hydrolyzed 5 minutes in 0.01 N acid at 100°. The liquid was then treated with both an anion exchange and a cation exchange resin (Ionac).

Paper chromatography was carried out with 77 per cent ethanol, the unknown being run between known samples of glucose and galactose. In every case the sugar from UDPG moved exactly like glucose (R_F = 0.50). The position of the galactose spot was clearly different (R_F = 0.47).

The carbazole reaction as described by Gurin and Hood (16) was also carried out comparatively with different sugars. The ratios of absorbency at 520 to 420 mμ found were as follows: sugar from UDPG, 2.1; galactose, 1.5; glucose, 2.0. If the carbazole reaction was carried out directly on UDPG, the color obtained was not typical for glucose.

Fermentation tests were carried out with *C. monosa*, which does not ferment galactose, and with *S. fragilis*, which does. Known samples of glucose and galactose were run at the same time, and after fermentation and centrifugation the sugars were estimated with the Somogyi reagent (29). The sugar from UDPG behaved like glucose.

Periodate Oxidation

The procedure described by Halsall, Hirst, and Jones (30) was used. Formaldehyde was detected with chromotropic acid (31). Formic acid was detected as described by Grant (32) and measured volumetrically.

The concentration of the UDPG solutions was estimated from the glucose liberated with 0.01 N acid at 100° for 5 minutes. The action of periodate on UDPG was slow and the formic acid content of the mixture reached a limit only after 24 hours. At this point the formic acid corresponded in several experiments to 0.8 to 1.0 molecule of formic acid per molecule of UDPG. No formaldehyde was detectable.

Preparation of Uridine Diphosphate from UDPG

As can be seen in Figs. 4 and 5, boiling in 0.01 N acid for 5 minutes liberates practically all the glucose and only a very small portion of the phosphate. After such a treatment it was possible to precipitate uridine diphosphate by addition of barium acetate at pH 8 and 1 volume of alcohol. The barium salt thus obtained was free of glucose.

Preparation of Uridine Monophosphate from UDPG

Inspection of Fig. 4 shows that in order to remove the labile phosphate completely it is necessary to heat to 100° in 0.1 N acid for about 90 minutes. In order to isolate uridine monophosphate, barium hydroxide was added to pH 9 and the precipitate of barium phosphate was washed and discarded. The solution plus the wash water was then treated with sufficient alcohol to precipitate all the organic phosphate. Usually about 12 volumes were necessary. The barium salt of uridine monophosphate thus obtained was free of reducing substances and inorganic phosphate.

Isolation of Pentose Phosphate from UDPG

The destruction of the uracil nucleus was effected by an adaptation of the procedure of Levene and Bass (20), as follows: treatment of UDPG with hydrazine hydrate; destruction of the excess reagents and the urea moiety with nitrite; precipitation of the ribose-containing compound as the barium salt, followed by acid hydrolysis and isolation of barium ribose phosphate.

An experiment was carried out as follows: A mixture containing 31 μM of UDPG in 1 ml. of water plus 0.14 ml. of hydrazine hydrate was heated 90 minutes at 65°. After cooling, 0.7 ml. of 20 per cent sodium nitrite was added and adjusted to pH 1.2 with hydrochloric acid. After a few minutes saturated barium hydroxide was added to pH 8, followed by 5 volumes of 95 per cent ethanol. The suspension was left overnight in the

ice box and then centrifuged. The precipitate was dissolved in 3 ml. of water and the barium removed with sodium sulfate. The liquid was then made 1 N with hydrochloric acid, heated 10 minutes at 100°, and then neutralized. Excess barium chloride was added and after several hours in the cold the precipitate was discarded. The liquid was then evaporated to dryness. The residue was dissolved in water and precipitated with several volumes of acetone.

The product obtained gave a relation of pentose to organic phosphate of 1:1.1.

Copper Complex Formation

The procedure described by Klimeck and Parnas (17) for distinguishing adenosine-3′-phosphate from adenosine-5′-phosphate consists in the addition of a copper salt and sodium hydroxide. When two neighboring hydroxyl groups are present as in the 5′-isomer, the supernatant appears blue.

By applying approximately the procedure described by Stiff (18) for measuring the copper complex formed by proteins, it was possible to carry out a reaction similar to that of Klimeck and Parnas but with 20 times less material. The procedure was as follows: To 0.5 ml. of the sample, about 3 mg. of copper phosphate and 0.5 ml. of 20 per cent trisodium phosphate were added. The suspension was intimately mixed with a glass rod. After 1.5 hours with occasional mixing the suspension was centrifuged. The supernatant was mixed with 1 ml. of 0.5 per cent diethyl dithiocarbamate and then extracted with 6 ml. of amyl alcohol. After centrifugation the absorbency of the alcohol layer was measured with a Klett-Summerson photocolorimeter and Filter 44. The results are shown in Table II.

Electrometric Titration

The preparation of the free acid by decomposition of heavy metal salts of UDPG was not successful. The use of a cation exchange resin proved to be more convenient. The yield was about 90 per cent of UDPG, of which 94 to 98 per cent was the free acid.

In a typical experiment a 10 cm. column containing 3.5 ml. of Ionac C-200 which had been treated with 0.5 N hydrochloric acid and thoroughly washed with water was prepared. A solution of UDPG (55 μM in 2.2 ml. of water) was passed six times through the column. The latter was then washed with water, making the final volume 10 ml. The whole operation was carried out at 5° and in about 5 minutes.

The solution was then divided into three samples. One (Sample A) was titrated as soon as it reached room temperature (15°). Samples B and C were titrated after being immersed in a boiling water bath for 4 and 60 minutes respectively.

After the electrometric titration with a glass electrode to pH 8.6, each sample was analyzed for inorganic and total phosphate and glucose, and the absorbency at 260 mμ was measured. From the latter the concentration of UDPG was calculated. The results are shown in the legend to Fig. 6.

Of all the possible formulas which were tested the only one which explains the results is that shown above containing a pyrophosphate group.

According to this formula, UDPG should give two acid groups titrating to about pH 5, like the primary groups of phosphoric acid.

Curve A, Fig. 6, shows that this was the case. The small deficit in titratable groups in relation to total phosphate (about 6 per cent) may be attributed to incomplete elimination of cations. Secondary phosphoric acid groups which titrate between pH 5 and 8.6 were clearly absent.

According to the above formula, removal of the glucose should unmask one secondary phosphoric acid group. Subsequent liberation of inorganic phosphate should give one secondary and one tertiary acid group. Thus in partially hydrolyzed UDPG the primary plus secondary acid groups should be equal to the sum (in moles) of total phosphate plus inorganic phosphate plus free glucose.

The results shown in Fig. 6 agree with these predictions, as proved by the coincidence of the calculated Levels A′, B′, and C′ with the amount of base necessary to titrate Samples A, B, and C to pH 8.6.

SUMMARY

The coenzyme of the galactose-1-phosphate → glucose-1-phosphate transformation has been studied. Methods are described for its estimation and isolation from bakers' yeast. The substance contains uridine, two phosphate groups, and glucose, and has therefore been named uridine-diphosphate-glucose (UDPG). As this coenzyme has been found to be present in animal tissues and in yeast not adapted to galactose, it is suggested that it may have some other function besides being the coenzyme of galactowaldenase.

Boiling in 0.01 N acid liberates practically all the glucose from UDPG in about 5 minutes and destroys the coenzymatic activity. Half of the phosphate in UDPG can be hydrolyzed in 1 N acid in 15 minutes at 100°.

The electrometric titration curve shows two primary phosphoric acid groups and no secondary. One secondary group appears on hydrolysis of the glucose and another on hydrolysis of the labile phosphate.

Experimental evidence is given which indicates that the uridine monophosphate obtained by hydrolysis of UDPG is uridine-5′-phosphate. A uridine diphosphate and the ribose phosphate could also be isolated.

A tentative formula for UDPG is presented in which uridine-5′-phosphate and glucose phosphate are joined, forming a pyrophosphate group.

The coenzymatic activity is destroyed by bromine, which affects the uracil moiety, and by alkali, which probably brings about a rearrangement of the molecule.

A micromethod for the measurement of the copper complex formation of nucleotides is presented.

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