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Investigations of the Properties and Mechanism of the Uridine Diphosphate Glucose Pyrophosphorylase Reaction

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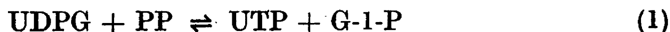
A purification procedure for the uridine diphosphate glucose pyrophosphorylase is described, and the purified enzyme preparation is used to determine equilibrium, pH dependence, Mg^{++} requirement, and Michaelis constant for the UDPG pyrophosphorylase reaction.

Isotopic exchange experiments show that the purified enzyme catalyzes an incorporation of ^{32}P-labelled pyrophosphate into uridine triphosphate in the absence of glucose-1-phosphate. Likewise ^{32}P-labelled glucose-1-phosphate is incorporated into uridine diphosphate glucose in the absence of pyrophosphate, while ^{14}C uridine monophosphate is not incorporated into either of the substrates. These findings are discussed in relation to the mechanism of the reaction.

In 1950 Leloir and coworkers [1] isolated from yeast a nucleotide, uridine diphosphate glucose (UDPG) *, consisting of a nucleoside, linked to glucose by a pyrophosphate bridge. Shortly afterwards it was shown by Kalckar and Cutolo [2] that yeast contained an enzyme, which brought about a pyrophosphorolytic cleavage of UDPG with simultaneous formation of UTP and G-1-P:



$$\text{UDPG} + \text{PP} \rightleftharpoons \text{UTP} + \text{G-1-P} \quad (1)$$

Pyrophosphorolytic cleavage of a nucleoside diphosphate compound was first demonstrated by Kornberg [3,4], who isolated from yeast and liver an enzyme which reversibly catalyzed the condensation of ATP with nicotinamide mononucleotide to split out PP and form the dinucleotide diphosphopyridine nucleotide. Later, Schrecker and Kornberg [5] demonstrated an analogous reaction with another dinucleotide, flavine adenine dinucleotide.

Reactions of this kind have subsequently been shown to be a more generally operating mechanism in group transfer reactions. Thus the biosynthesis of

* The following abbreviations are used: UTP for uridine triphosphate, G-1-P for glucose-1-phosphate, Gal-1-P for galactose-1-phosphate, G-6-P for glucose-6-phosphate, PP for inorganic pyrophosphate, ATP for adenosine triphosphate, ADP for adenosine diphosphate, UDP for uridine diphosphate, UMP for uridine monophosphate, UDPAG for uridine diphosphate N-acetyl glucosamine, TPN for triphosphopyridine nucleotide, GDPM for guanosine diphosphate mannose and Tris for tris(hydroxymethyl) aminomethane.

UDPAG and of GDPM in both cases involve a nucleoside triphosphate and the corresponding sugar phosphate ester [6,7].

Moreover, Kennedy and Weiss [8] have recently shown that in the biosynthesis of lecithin in liver a pyrophosphorylase is involved which forms cytidine diphosphate choline and pyrophosphate from cytidine triphosphate and choline phosphate. Kalckar [9] has proposed the name nucleotidyl transferases for this class of enzymes. However, for the individual enzymes of the class the term 'pyrophosphorylase' appears to be most descriptive.

The UDPG pyrophosphorylase which is abundantly present in yeast has also been shown to occur in liver [10], in muscle and brain tissue [11] and in leaves of spinach [12]. In this report a study of the purification and properties of the yeast enzyme and of the kinetics and mechanism of the reaction will be presented.

METHODS

Materials

UDPG was prepared as described by Leloir *et al.*[1] The preparation used throughout was a solution of about 60 % purity (as estimated by enzymatic analysis with the purified UDPG pyrophosphorylase and by chromatographic methods), and contained besides the UDPG some UDPAG together with small amounts of UDP and UMP.

$^{32}P^{32}P$ was obtained according to Kornberg and Pricer [13] by dehydration of ^{32}P-labelled Na_2HPO_4 and subsequent separation on a Dowex 1 Cl^- column.

^{32}P-labelled G-1-P was prepared by the method of McCready and Hassid [14], applying ^{32}P-labelled Na_2HPO_4 in the phosphorylase reaction.

ATP, ADP, UMP, and TPN were commercial products.

^{14}C-labelled UMP was prepared by incubating 2-^{14}C-labelled orotic acid with liver homogenate [15]. The sample applied in the exchange experiments was generously donated by Dr. Robert Hurlbert. The activity was 32 000 cts/min./μmole.

The *Saccharomyces fragilis* preparation was a freeze-dried product, grown on a galactose-containing medium *.

Enzymes

Glucose-6-phosphate dehydrogenase (Zwischenferment) was prepared by the method of LePage and Mueller [16]. The preparation obtained in this way was free of 6-phosphogluconic acid dehydrogenase, but contained large amounts of UDPG pyrophosphorylase. A glucose-6-phosphate dehydrogenase free of UDPG pyrophosphorylase was obtained in the following way: 100 mg of the G-6-P dehydrogenase was dissolved in 1 ml Tris buffer. The mixture was spun, and to the clear supernatant (0.9 ml) was added 1.35 ml saturated ammonium sulphate. After 20 min. at 0° C the precipitate was removed by centrifugation. To the supernatant (2.1 ml) was added 1.75 ml saturated ammonium sulphate, and after 20 min. at 0° C the precipitate was collected by centrifugation and dissolved in 0.2 ml Tris buffer. This solution was highly active with respect to glucose-6-phosphate dehydrogenase and usually devoid of UDPG pyrophosphorylase. Traces of the latter enzyme could be removed by dialysis against cold distilled water. The enzyme was stable for several weeks when kept at −20° C.

Nucleoside diphosphokinase was not prepared as such, but when the action of this enzyme was wanted, advantage was made of its abundant presence in the G-6-P dehydrogenase preparation.

Phosphoglucomutase was prepared according to Najjar [17]. In most experiments the "second heat filtrate" was used. This preparation contained sufficient glucose diphosphate for assay of G-1-P, so that extra addition of coenzyme was unnecessary.

Hexokinase was a commercial product obtained from Pabst Breweries, Inc.

* My thanks are due to Civilingeniør B. Steinhardt, Novo Terapeutisk Laboratorium, Copenhagen, for growing and harvesting a larger amount of this microorganism.

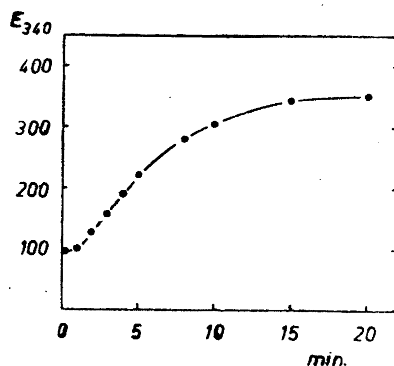


Fig. 1. Spectrophotometric demonstration of the UDPG pyrophosphorylase reaction.
Reaction mixture: 0.8 ml Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 25 µl phosphoglucomutase, 25 µl cysteine (10 mg/ml), 10 µl G-6-P dehydrogenase solution, containing also UDPG pyrophosphorylase, and 10 µl PP (0.1 M). Time zero indicates addition of PP. First reading was taken at 15 seconds.

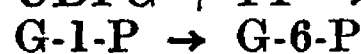
Assay of UDPG pyrophosphorylase activity

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 of protein. A rise in extinction of 0.622 per 0.1 µmole at 340 mµ was used as basis for the calculations [18].

Protein determinations in the enzyme preparations were carried out according to the method of Lowry *et al.*[19] or, in the crude extracts, according to Bücher [20].

The usually applied and most convenient assay was a spectrophotometric determination, consisting of a sequence of enzymatic processes. Care therefore had to be taken that the UDPG pyrophosphorylase was the velocity limiting factor. The following sequence of reactions took place:

UDPG + PP → UTP + G-1-P


G-1-P → G-6-P

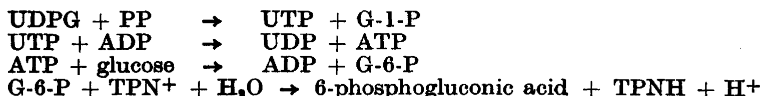
G-6-P + TPN+ + H_2O → 6-phosphogluconate + TPNH + H^+

The G-1-P formed by the pyrophosphorylase reaction was converted to G-6-P by addition of mutase, and G-6-P was oxidized by addition of TPN and glucose-6-phosphate dehydrogenase [21]. The formation of reduced TPN was finally measured by following the rise in extinction at 340 mµ. Excess of mutase and glucose-6-phosphate dehydrogenase was secured by regular checking in separate controls.

The assay was carried out in 1 ml quartz absorption cells, and the components of the assay system were as follows: 800 µl Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 25 µl mutase (3 mg/ml), 25 µl cysteine (10 mg/ml), 10 µl glucose-6-phosphate dehydrogenase (free of UDPG pyrophosphorylase), 50 µl UDPG solution (60 % purity, 7 µmole UDPG/ml), 20 µl TPN (0.01 M), and 10—20 µl of the UDPG pyrophosphorylase solution. Initial readings were taken at 340 mµ (the UDPG preparation contained minute amounts of G-1-P, which usually caused a rise in extinction of about 0.010—0.020, when the G-6-P dehydrogenase was added). When readings were constant, 10 µl 0.1 M PP was added to start the pyrophosphorylase reaction, and readings were taken every 30 seconds. Final and stable extinctions were usually obtained after 25—30 minutes.

The reaction, when demonstrated spectrophotometrically, shows a pronounced lag-period (see Fig. 1), which will be discussed later. This lag-period does not, however, influence the quantitative aspect of the assay, and the maximum rate (optical density change at 340 mµ) obtained after the lag-period was proportional to the amount of enzyme added.

Occasionally the pyrophosphorylase activity was measured by means of the nucleoside diphosphokinase-hexokinase system as described by Berg and Joklik [22]. The sequence of reactions was as follows:



UDPG + PP → UTP + G-1-P
UTP + ADP → UDP + ATP
ATP + glucose → ADP + G-6-P
G-6-P + TPN^+ + H_2O → 6-phosphogluconic acid + TPNH + H^+

It is seen that in this case it is the UTP, formed in reaction (1) which is measured. The assay system contained: 900 µl Tris (0.05 M, pH 7.2, 0.005 M $MgCl_2$), 10 µl G-6-P dehydrogenase (which also contained the nucleoside diphosphokinase), 20 µl ADP, 0.002 M, 25 µl hexokinase (3 mg protein per ml), 1.0 µl glucose, 0.01 M, 20 µl TPN (0.01 M), 10–20 µl UDPG pyrophosphorylase and 20 µl UDPG, 0.007 M. 10 µl PP, 0.1 M, started the reaction which likewise was followed at 340 mµ. The G-6-P dehydrogenase as well as the hexokinase were checked for absence of phosphoglucomutase. The assay here described may be used for demonstrating pyrophosphorolysis of UDP glycosyl compounds other than UDPG.

All enzymatic reactions were, unless otherwise stated, carried out at room temperature. In large scale experiments, when the reaction products, primarily UTP, were isolated, the reaction was stopped by adding $HClO_4$ to a final concentration of 2 %. Proteins were removed by spinning, and the nucleotides were adsorbed on a suitable amount of norite and subsequently eluted with 50 % ethanol.

Before use the norite was washed by shaking for 2 hours with 100 volumes of 2 % hexanol [23]. The suspension was filtered and washed thoroughly with distilled water, followed by drying in the air. Systematic experiments with a solution of known nucleotide content were carried out in order to fix the optimal conditions for adsorption and elution; as a result of these experiments the following standard procedure was applied throughout: To the perchloric acid solution was added 5 mg norite per 0.1 µmole nucleotide. After 2 minutes the mixture was spun; the norite was washed with 1 ml distilled H_2O per 0.1 µmole, spun again after decantation and eluted with 1 ml 50 % ethanol, 0.05 M in NH_3. After 5 min. the mixture was spun and elution was repeated with 1 ml ethanol. After evaporation of the combined eluates to a small volume, the sample was ready for chromatography. The washing of the norite with H_2O is particularly important as it removes inorganic salts, which otherwise considerably delay the drying of the spots when they are deposited on the paper.

Chromatography was carried out in ethanol-ammonium acetate solvent at pH 7.5 [24]. In this solvent UMP, UDP, UDPG and UDPAG are easily separated from one another, while G-1-P and UDPG run together. In experiments where it was necessary to separate those two compounds, the samples were chromatographed in ethanol ammonium acetate at pH 4 [24]. The nucleotide spots were localized on the paper by the "mineralight" lamp, and when desired, the chromatogram was scanned in the Beckman at 260 mµ according to the method of Leloir and Paladini [23] on strips of 1 cm width, cut out of the paper chromatogram. Impregnation of the paper with liquid paraffin was found unnecessary. In the cases, where radioactive material had been applied, the paper strips were scanned with respect to radioactivity in a Geiger-Müller counter; or the spots were cut out and eluted with H_2O, followed by estimation of ultraviolet absorption and counts/ml in the eluate.

RESULTS

Purification of enzyme

Purification procedure included the following steps: Extraction, ammonium sulphate fractionation, precipitation with protamine, ethanol fractionation and finally another fractionation with ammonium sulphate. Cuvette assays as described in Methods were the basis for activity measurements during the purification procedure. It should be mentioned that in the crude extract the presence of nucleotide pyrophosphatase [25] made activity measurements

very inaccurate. In such extracts estimations of the activity were made by comparing the amount of G-1-P formed with and without addition of pyrophosphate.

Extraction. 20 g of dried brewer's yeast (Kongens Bryghus) were autolyzed and extracted by shaking with 40 ml 0.07 M $(NH_4)_2HPO_4$ for 18 hours at 20° C. The extract was spun, whereby 23 ml of filtrate was obtained. To the supernatant was added 15.6 ml saturated ammonium sulphate to make the solution 40 % saturated. After 30 min. at 0° C a small precipitate was separated by centrifugation, and to the supernatant was added 19 ml saturated ammonium sulphate to make the solution 60 % saturated. After half an hour at 0° C a large precipitate was collected by centrifugation. Further addition of ammonium sulphate to the supernatant yielded only a slight precipitate.

The precipitate obtained at 40—60 % ammonium sulphate saturation was dissolved at zero in 25 ml 0.015 M acetate buffer, pH 6.3, and dialyzed for half an hour against tap water. To the clear solution (27 ml) was added 2 ml of a 1 % solution of protamine sulphate (salmine). After 1 hour at 2° C a small precipitate was removed by centrifugation while the main part of the uridyl transferase activity remained in the supernatant. The clear supernatant was cooled to —2° C and fractionated with ethanol. The temperature was gradually lowered to —8° C during dropwise addition of 50 % ethanol. The fractions precipitated between 20—24 %, between 24—28 % and finally between 28—31 % ethanol were collected by centrifugation at —10° C in a previously cooled centrifuge.

The ethanol precipitated fractions were kept at —20° C overnight. By this treatment, the ethanol, adhering to the precipitate and to the walls of the centrifuge tubes, evaporates completely. Traces of ethanol may otherwise cause considerable denaturing of the protein to be dissolved. Next morning the fractions were dissolved in cold distilled water. The fraction precipitated between 20—24 % ethanol usually contained a small amount of insoluble material, which was discarded by centrifugation. The other fractions yielded clear solutions.

The highest activity was usually found in the fraction precipitated at 20—24 % ethanol. To the clear solution (4 ml) was added 6 ml saturated ammonium sulphate to make the solution 60 % saturated. The precipitate was collected by centrifugation and extracted three successive times with 2.5 ml aliquots of decreasing ammonium sulphate concentrations, which were adjus-

Table 1. Purification of UDPG pyrophosphorylase.

		Vol. of fraction	Total activity	Yield %	Specific activity
Crude extract		23 ml	4 055		0.3
Amm. sulphate fraction	40—60 %	28 »	3 360	82.8	6.3
Ethanol fraction I	20—24 %	6.4 »	1 640	40.4	11.0
» » II	24—28 %	3 »	241	5.9	5.5
» » III	28—31 %	2 »	121	2.9	5.8
2nd amm. sulphate fraction					
a	40—46 %	0.5 »	196	4.8	18.3
b	46—50 %	2.5 »	693	15.3	40.7
c	50—56 %	2.5 »	618	15.0	77.2

ted to pH 7.5 with ammonia. The fraction obtained at 50—56 % saturation was the most active. It was more than 250 times as active as the crude yeast extract and represented an overall yield of 15 % (Table 1). (The concentrations of ammonium sulphate in these fractions were estimated by conductivity measurements).

The product thus obtained was free of G-6-P dehydrogenase, of 6-phosphogluconic acid dehydrogenase, of hexokinase and of phosphoglucomutase. It was stored as ammonium sulphate precipitate at —20° C.

A number of preparations were carried out from the same batch of dry yeast with approximately the same yield and enzyme activity. Other samples of dried yeast obtained from the same brewery, however, resulted in preparations with lower activities and yield.

Properties of enzyme

Stability. The purified preparation is extremely unstable in dilute solutions. Attempts to stabilize the solution by addition of crystalline bovine serum albumin (3 mg/ml) failed. It was later found that the best way of storing the enzyme was to keep the final ammonium sulphate precipitate at —20° C. Kept in this way a 3 months' old preparation still contained 60—75 % activity.

Equilibrium. The UDPG pyrophosphorylase reaction is reversible, as has earlier been shown by means of ^{32}P-labelled UTP or ^{32}P-labelled G-1-P [26, 6].

Balance studies of the reaction were done by incubating equimolar amounts of UDPG and PP with the purified enzyme and assaying the G-1-P formed in aliquots taken out at different time intervals. For this purpose a G-6-P dehydrogenase preparation, free of UDPG pyrophosphorylase, as described in Methods, was used.

The experimental incubation mixture contained 2 μmoles UDPG, 2 μmoles PP, 0.08 mg/ml of enzyme, and 0.05 M Tris, pH 7.4. Total volume was 8 ml. Aliquots of 400 μl were taken out at different times, and the reaction was stopped by heating the tube 10 sec. in boiling water, followed by rapid cooling. The protein precipitate was allowed to settle, before 300 μl of the digest were diluted with 600 μl Tris, 0.05 M, pH 7.3, in a 1 ml Beckman cuvette and analyzed for G-1-P by addition of phosphoglucomutase, cysteine, TPN and G-6-P dehydrogenase. When readings at 340 mμ were constant, indicating that all the G-1-P was used, UDPG pyrophosphorylase and more PP were added to estimate the remaining UDPG content of the digest.

In Fig. 2 is shown the equilibrium curve for the reaction. From this it may be concluded that the reaction stops at approximately 45 % conversion, yielding an equilibrium constant close to 1. Correspondingly the UDPG content of the enzyme digest decreases and reaches a final constant value of about 52 % of the initial UDPG concentration. This indicates, as could be expected, that the pyrophosphorolytic reaction involves no appreciable thermodynamic changes.

pH dependence. In the determination of the pH optimum of the UDPG pyrophosphorylase, the spectrophotometric method could not be used as this assay involves other enzyme systems, whose pH optima differ from that of the

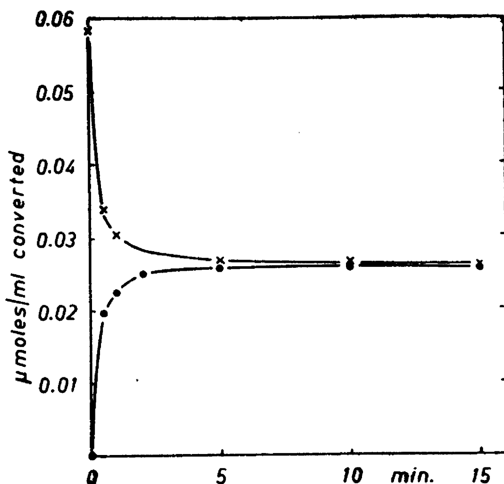


Fig. 2. Attainment of equilibrium in the reaction UDPG + PP ⇌ UTP + G-1-P.
Experimental data as described in text. ●–●–● formation of G-1-P. × – × – × disappearance of UDPG.

pyrophosphorylase. Experiments were therefore carried out in two stages by a method similar to that used in determination of the equilibrium constant.

A double series of samples with buffers of varying pH, each containing 0.1 μmole of UDPG and 0.1 μmole of pyrophosphate were incubated with the enzyme, in the first series for 30 sec. and in the second for 2 and a half min. The reaction was stopped by heating, and analysis of the G-1-P formed in the 2 min. interval was carried out according to the method described in equilibrium experiments.

In Fig. 3 the reaction velocities in the different buffers are plotted against the pH of the solution. It is seen that the reaction has a broad, but distinct optimum between pH 6.5 and pH 8.

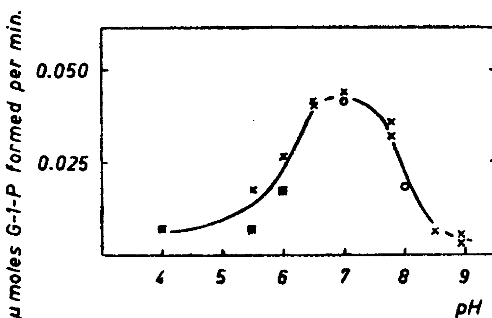


Fig. 3. Influence of pH on the UDPG pyrophosphorylase reaction.
The buffers used were 0.05 M. ○–○–○ phosphate buffer. × – × – × Tris buffer.

Mg activation. The Mg^{++} requirement was investigated by a method similar to the pH dependence measurements of the UDPG pyrophosphorylase and is shown in Fig. 4. The reaction rate was optimal at a concentration of 2×10^{-3} M Mg^{++}.

Michaelis constant. The Michaelis constant for the UDPG was found to be 0.7×10^{-4}. The constants for the other substrates have not been measured.

Lag period. The spectrophotometric method which is ordinarily used in the assay of UDPG shows a characteristic lag period (Fig. 1), which can be overcome only by addition of very large amounts of the UDPG pyrophosphorylase. Addition of excess of phosphoglucomutase and G-6-P dehydrogenase does not influence this phenomenon. For closer investigation of the lag period an experiment was set up to compare the velocity curve, obtained by the spectrophotometric assay, with a velocity curve derived from G-1-P determination on aliquots taken out with short intervals in the initial stage of the reaction. For this experiment was used a UDPG solution freed of G-1-P by paper chromatography at pH 3.9 and subsequent elution of the UDPG from the paper.

The amounts of enzyme and substrate were so adjusted that the rate of velocity was approximately the same in the two experiments and Fig. 5 shows the experimental results; it is seen that in the case where the rate of velocity is determined on aliquots of the reaction mixture there is no sign of lag period. The occurrence of a lag period in the spectrophotometric assay is most likely explained by the relatively large K_m for the G-1-P in the phosphoglucomutase reaction (see Discussion).

Influence of other factors. The rate of the pyrophosphorolytic reaction as followed by the spectrophotometric assay was found to be unaffected by the addition of sodium fluoride to a concentration of 0.05 M. Also versene (0.01 M) had no inhibitory effect. Uridine diphosphoglycosyl compounds are shown to accumulate in penicillin-treated *Staphylococcus aureus* cells [27, 28], and for this reason penicillin was tested as a possible inhibitor for the pyrophosphorylase reaction, but it was found that even in concentrations up to 10 mg/ml this substance did not materially influence the reaction.

UMP and UDP were also tested for a possible inhibitory effect, but with negative results. Cysteine does not inhibit the reaction.

Specificity of the reaction. Crude yeast and liver extracts have been shown to contain a number of pyrophosphorylases, acting on different substrates. The purified UDPG pyrophosphorylase has been tested for activity towards several nucleoside diphosphate compounds, but it seems to be quite specific with respect to the carbohydrate group as well as the nucleoside moiety. UDPAG is not attacked and neither is GDPM; also DPN is left untouched.

The reaction mechanism of UDPG pyrophosphorylase

The UDPG pyrophosphorylase belongs to a group of enzymes, which may be classified as uridyl transferases. In this particular case G-1-P and PP function as donor and acceptor, respectively, of the uridyl group.

If, in group transfer reactions, the enzyme is considered as an acceptor of

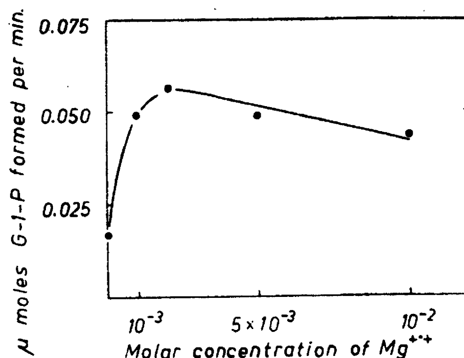
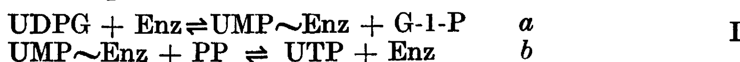


Fig. 4. Influence of Mg^{++} on the UDPG pyrophosphorylase reaction.

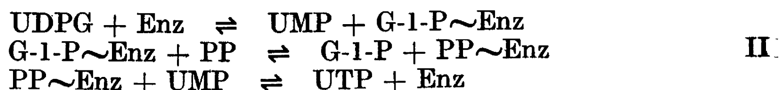
anhydride groups [29, 9, 30] the UDPG pyrophosphorylase reaction may be visualized as a two step reaction according to the following scheme:



$$\begin{array}{ll} \text{UDPG} + \text{Enz} \rightleftharpoons \text{UMP}\sim\text{Enz} + \text{G-1-P} & a \\ \text{UMP}\sim\text{Enz} + \text{PP} \rightleftharpoons \text{UTP} + \text{Enz} & b \end{array} \quad \text{I}$$

This formulation postulates the formation of a uridyl-enzyme compound.

Another possibility is that the reaction follows a three step pattern (*cf.* Kalckar [9]) with the following sequence of reactions:



$$\begin{array}{l} \text{UDPG} + \text{Enz} \rightleftharpoons \text{UMP} + \text{G-1-P}\sim\text{Enz} \\ \text{G-1-P}\sim\text{Enz} + \text{PP} \rightleftharpoons \text{G-1-P} + \text{PP}\sim\text{Enz} \\ \text{PP}\sim\text{Enz} + \text{UMP} \rightleftharpoons \text{UTP} + \text{Enz} \end{array} \quad \text{II}$$

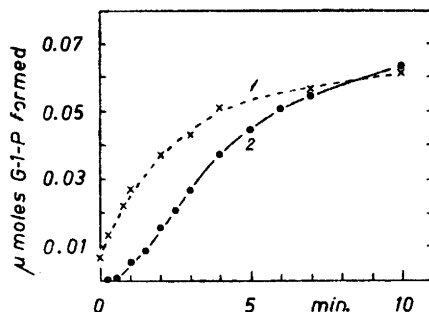


Fig. 5. Comparison between spectrophotometric and direct registration of the UDPG pyrophosphorylase reaction.

Experiment 1: 0.9 μmoles UDPG, 10 μmoles PP, 5 μl purified UDPG pyrophosphorylase (0.9 mg protein/ml), 0.05 M Tris, pH 7.3, to a volume of 1.3 ml. Aliquots of 100 μl taken out at different times and assayed for G-1-P content.

Experiment 2: 0.5 μmoles UDPG, 25 μl mutase (3 mg protein/ml), 25 μl cystein (10 mg/ml), 10 μl G-6-P dehydrogenase, 20 μl TPN (0.01 M), and 0.05 M Tris, pH 7.3, to a volume of 700 μl. The reaction was started by addition of 2.5 μl purified UDPG pyrophosphorylase and 10 μl PP, 0.1 M. Readings were at same time intervals as in experiment 1.

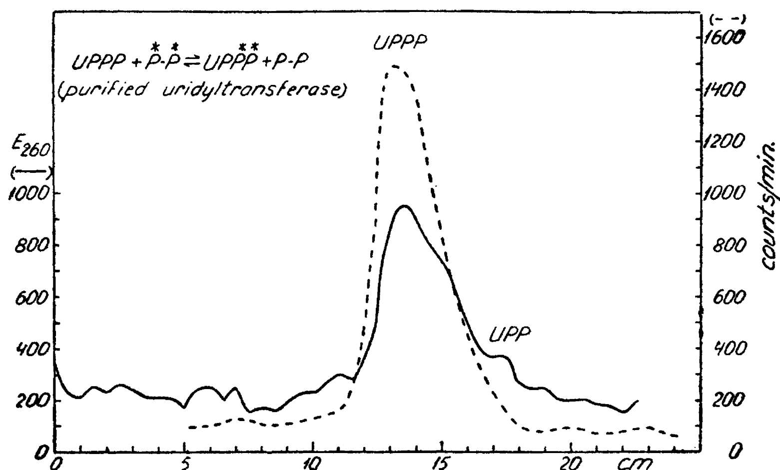


Fig. 6. Exchange experiment with UTP and $^{32}P^{32}P$.

0.25 μmoles UTP, 0.5 μmoles $^{32}P^{32}P$ (96 000 cts/min/μmole), 100 μl purified UDPG pyrophosphorylase (1.6 mg protein/ml), and Tris to a volume of 1.1 ml were incubated 1 hour. After deproteinization the nucleotides were adsorbed on norite and chromatographed. The chromatogram was scanned in the Beckman and counted in a Geiger-Müller counter.

Attempts to decide between the two possibilities were made by carrying out some isotope exchange experiments, although only indirect evidence for either of the two mechanisms can be obtained by such methods.

The following three labelled compounds were available: ^{14}C-labelled UMP [15], ^{32}P-labelled G-1-P and ^{32}P-labelled pyrophosphate. It is clear from the reaction schemes written above that if scheme I is valid, equation *a* would effect an incorporation of G-1-^{32}P in UDPG, if these two substances were incubated with the enzyme in the absence of PP; correspondingly equation *b* would cause an incorporation of $^{32}P^{32}P$ in UTP, if the two substances were incubated with the enzyme.

Table 2. Exchange experiments with ^{14}C-labelled UMP.

0.25 μmole of UTP or UDPG were incubated 60 min. with 0.1 μmole of ^{14}C-UMP (32 100 cts/min/μmole) in the presence of purified UDPG pyrophosphorylase. The reaction mixture was acidified, adsorbed on norite and chromatographed in ethanol-ammonium acetate at pH 7.5. The ultraviolet absorbing spots corresponding to UDPG, UTP and UMP were eluted and counted.

A control, containing 0.25 μmole UTP and 0.5 μmole. $^{32}P^{32}P$ (30 000 cts/min/μmole) together with the enzyme, was run simultaneously.

	cts/min/μmole		
	UTP	UDPG	UMP
UTP + ^{14}C—UMP + Enzyme	22	—	31 200
UDPG + ^{14}C—UMP + Enzyme	—	10	27 700
UTP + $^{32}P^{32}P$ + Enzyme	4 540	—	—

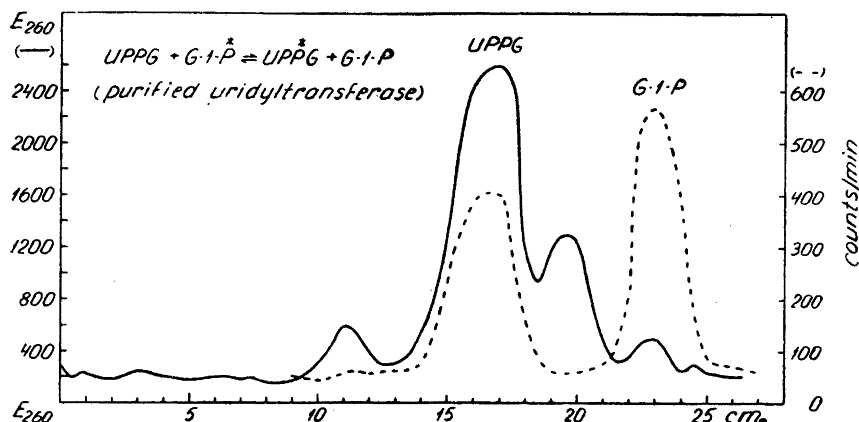


Fig. 7. Exchange experiment with UDPG and G-1-^{32}P.

0.7 μmoles UDPG, 1 μmole G-1-^{32}P (20 000 cts/min/μmole), 25 μl purified UDPG pyrophosphorylase (1.6 mg protein/ml) and 0.05 M Tris, pH 7.3, to a volume of 1.2 ml were incubated 1 hour. The reaction mixtures were treated as described in Fig. 6.

If, on the other hand, ^{14}C-labelled UMP could be incorporated in UDPG as well as in UTP in the absence of PP and G-1-P, respectively, this would indicate that the UDPG pyrophosphorylase might follow scheme II; in this case the enzyme could not be classified as a true uridyl transferase.

Enzyme preparations from brewer's yeast and of different purity were incubated with 0.2—0.5 μmoles of substrate together with the corresponding labelled uridyl acceptor. Incubation periods of 1—2 hours were chosen as it was found that further incubation destroyed the enzyme. After deproteinization of the reaction mixture the nucleotides were adsorbed on norite and chromatographed on paper. The ultraviolet absorbing spots were eluted and counted, or the chromatogram was scanned in the Beckman at 260 mμ and in a Geiger-Müller counter in the usual way. In some cases also autoradiography was applied.

In such experiments it was found that when a purified preparation of the UDPG pyrophosphorylase was incubated with UDPG and G-1-^{32}P or with UTP and ^{32}P^{32}P, a rapid exchange of G-1-^{32}P into UDPG and of ^{32}P^{32}P into UTP took place. If the same enzyme preparation was incubated with

Table 3. Incorporation of ^{32}P^{32}P into different nucleoside triphosphates.

Samples containing 0.2 μmole of GTP, ATP and UTP were incubated 60 min. with 0.5 μmole ^{32}P^{32}P (450 000 cts/min/μmole) and 1) a crude extract of *Sacch. fragilis*, 2) purified UDPG pyrophosphorylase. Reaction mixture adsorbed on norite, chromatographed and counted as described in the text.

Enzyme applied	GTP	ATP	UTP
	cts/min/μmole		
Extract of *S. fragilis*	28 100	26 700	30 000
Purified UDPG pyrophosphorylase	0	1 240	57 000

^{14}C-labelled UMP and either UDPG or UTP, no incorporation of this compound in either of the nucleotides could be detected. The results are presented in Figs. 6 and 7 and in Table 2. Control experiments where the reactants were incubated together in absence of enzyme showed that no non-enzymatic exchange occurs between these compounds.

That the incorporation is effected by the UDPG pyrophosphorylase and not by contaminating enzymes is supported by the fact that the incorporation rate of $^{32}P^{32}P$ into UTP rises with increasing purity of the enzyme (Fig. 8). Moreover, the incorporation of labelled PP into UTP is almost specific for the purified UDPG pyrophosphorylase, whereas less purified enzyme preparations also catalyze a corresponding incorporation into GTP and ATP (Table 3).

The observations made in these experiments all seem to favour scheme I in the argument above, and so far no evidence has appeared to support scheme II. All attempts, however, to isolate a uridyl enzyme compound from a digest consisting of the purified enzyme with either ^{14}C-labelled UDPG or ^{14}C-labelled UTP, have been fruitless.

DISCUSSION

The UDPG pyrophosphorylase belongs to a class of enzymes which has been named nucleotidyl transferases [9], and of which several are already known [4,6,7]. With respect to equilibrium, Michaelis constant, and the influence of factors like magnesium and fluoride ions the UDPG pyrophosphorylase shows similarities with the DPN pyrophosphorylase, described by Kornberg [4].

The characteristic lag period, which is always observed, when the reaction is followed spectrophotometrically, has been investigated more closely, and the results obtained indicate that the phenomenon does not originate from the UDPG pyrophosphorylase reaction itself, but must be ascribed to the complexity of the assay system. The phosphoglucomutase reaction is inhibited, presumably competitively, by salts [31], and as the assay mixture contains relatively large amounts of anions, it is reasonable to believe that the value of the Michaelis constant for G-1-P is considerably increased in this medium. Hence the conversion of G-1-P, formed by the UDPG pyrophosphorylase reaction, to G-6-P proceeds very slowly until the G-1-P concentration rises above the K_m of the phosphoglucomutase reaction, and the rate of velocity gradually attains the proper value.

In recent years investigators have tended to consider the enzymatic substitution reactions as analogous to the non-enzymatic substitution reactions, known from studies of organic chemical mechanisms. Such considerations have been advanced by Koshland [29], who shows that a few basic mechanisms in a number of cases can explain a variety of experimental data, obtained chiefly in exchange experiments with enzymes and labelled substrates.

In an attempt to apply considerations of this kind to the uridyl transferase reaction a number of isotopic exchange experiments have been carried out with the UDPG pyrophosphorylase. The results obtained agree readily with the assumption of an intermediate uridyl-enzyme complex in which case the transfer of the uridyl group is believed to proceed through a "double displacement mechanism" (*cf.* Koshland) as depicted in scheme I, p. 1531. It should

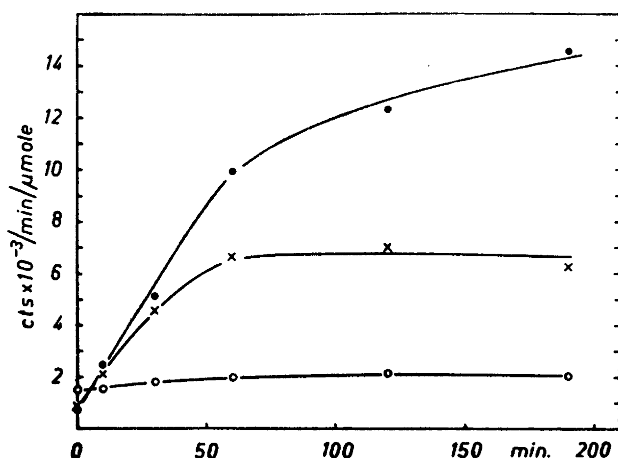


Fig. 8. Incorporation of $^{32}P^{32}P$ into UTP, catalyzed by enzyme preparations of different purity.

Three set of samples containing 1 ml Tris, 1 μmole $^{32}P^{32}P$ (90 000 cts/min/μmole), and 0.5 μmole UTP were incubated with UDPG pyrophosphorylase of different purity. The reaction was stopped at the times indicated on the curve, and the reaction mixtures were treated with norite, chromatographed and counted as described in the text.

●–●–●	0.2 mg	enzyme,	specific	activity	5
×–×–×	2.2 »	»	»	»	0.5
○–○–○	4.2 »	»	»	»	0.1

be added, though, that this interpretation of the exchange data must be taken with reservation as long as a more direct proof of the hypothesis, *e. g.* the isolation of the uridyl-enzyme complex, has not been obtained.

Trucco [32] has reported the incorporation of ^{14}C-labelled G-1-P in UDPG when these two substances are incubated with dialyzed extracts of *Saccharomyces fragilis*. This incorporation is ascribed to the combined effect of the following two enzymatic reactions, both catalyzed by extracts of *S. fragilis* [33, 34].

(1) Gal-1-P + UDPG ⇌ UDPGal + G-1-P (non pyrophosphorolytic uridyl transferase)

(2) UDPGal ⇌ UDPG (galacto-waldenase)

The fact that the purified UDPG pyrophosphorylase catalyzes a similar exchange (G-1-^{32}P into UDPG) has in the present investigation been explained by the formation of an uridyl-enzyme compound in the uridyl transferase reaction. This assumption makes the action of galacto-waldenase unnecessary to effect the incorporation of labelled G-1-P into UDPG, and such an incorporation, catalyzed by extracts of *S. fragilis*, may with equal reason be explained by the action of either the UDPG pyrophosphorylase or the non-pyrophosphorolytic uridyl transferase, both present in dialyzed extracts of *S. fragilis*.

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REFERENCES

1. Caputto, R., Leloir, L. F., Cardini, C. E. and Paladini, A. C. *J. Biol. Chem.* **184** (1950) 333.
2. Kalckar, H. M. and Cutolo, E. *IIe Congr. Intern. de Biochimie, Paris 1952*, p. 260.
3. Kornberg, A. *J. Biol. Chem.* **176** (1948) 1475.
4. Kornberg, A. *J. Biol. Chem.* **182** (1950) 779.
5. Schrecker, A. W. and Kornberg, A. *J. Biol. Chem.* **182** (1950) 795.
6. Munch-Petersen, A., Kalckar, H. M. and Smith, E. E. B. *Kgl. Danske Videnskab. Selskab, Biol. Medd.* **22** (1955) No. 7.
7. Munch-Petersen, A. *Arch. Biochem. and Biophys.* **55** (1955) 592.
8. Kennedy, E. B. and Weiss, S. B. *J. Am. Chem. Soc.* **77** (1955) 251.
9. Kalckar, H. M. and Klenow, H. *Ann. Rev. Biochem.* **23** (1954) 527.
10. Smith, E. E. B. and Mills, G. T. *Biochim. et Biophys. Acta* **13** (1954) 386.
11. Kalckar, H. M. and Maxwell, E. *Personal communication.*
12. Munch-Petersen, A. *Unpublished observations.*
13. Kornberg, A. and Pricer, W. E. *J. Biol. Chem.* **191** (1951) 535.
14. McCready, R. M. and Hassid, W. Z. *J. Am. Chem. Soc.* **66** (1944) 4.
15. Hurlbert, R. B. and Reichard, P. *Acta Chem. Scand.* **9** (1955) 251.
16. LePage, G. A. and Mueller, G. C. *J. Biol. Chem.* **180** (1949) 975.
17. Najjar, V. A. *J. Biol. Chem.* **175** (1948) 281.
18. Horecker, B. L. and Kornberg, A. *J. Biol. Chem.* **175** (1948) 385.
19. Lowry, O. H., Smith, C. A. and Cohen, D. L. *J. Biol. Chem.* **146** (1942) 519.
20. Bücher, Th. *Biochim. et Biophys. Acta* **1** (1947) 292.
21. Warburg, O. and Christian, W. *Biochem. Z.* **254** (1932) 438.
22. Berg, P. and Joklik, W. K. *J. Biol. Chem.* **210** (1954) 657.
23. Siliprandi, D. and Siliprandi, N. *Biochim. et Biophys. Acta* **14** (1954) 52.
24. Paladini, A. C. and Leloir, L. F. *Biochem. J. London* **51** (1952) 426.
25. Kornberg, A. and Pricer, W. E. *J. Biol. Chem.* **182** (1950) 763.
26. Munch,Petersen, A., Kalckar, H. M., Cutolo, E. and Smith, E. E. B. *Nature* **172** (1953) 1036.
27. Park, J. T. in McElroy and Glass: *Phosphorus Metabolism, I* (1951) 93.
28. Strominger, J. L. *Federation Proc.* **12** (1953) 277.
29. Koshland, D. E. in McElroy and Glass: *Mechanism of Enzyme Action* (1954) 608.
30. Zatman, L. J., Kaplan, N. O. and Colowick, S. P. *J. Biol. Chem.*, **200** (1953) 197.
31. Klenow, H. *Arch. Biochem. and Biophys.* (1955). *In press.*
32. Trucco, R. E. *Nature* **174** (1954) 1103.
33. Kalckar, H. M., Braganca, B. and Munch-Petersen, A. *Nature* **172** (1953) 1038.
34. Leloir, L. F. *Arch. Biochem. and Biophys.* **33** (1951) 186.

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