Studies on Ligand Binding to Bovine Liver Uridine Diphosphate Glucose Pyrophosphorylase

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A procedure for the preparation of crystalline UDP-glucose pyrophosphorylase is described. K_s values for UDP-glucose and UTP were determined as 7 and 20 μM respectively, the latter being confirmed by three methods. By assuming an octameric structure, 1 mol of enzyme subunit bound 1 mol of substrate. The metal-ion activator, Mg^{2+} , did not affect the equilibrium between nucleotide and enzyme. A substrate analogue, $\alpha\beta$ -methylene-UTP, was synthesized and had the same K_s value as UTP. In its presence, the K_s for glucose 1-phosphate decreased by two orders of magnitude, thus confirming a compulsory binding order and excluding an uridylylated enzyme intermediate. The results are discussed with respect to their implications *in vivo*.

UDP-glucose is the glycosyl donor for many reactions in polysaccharide biosynthesis in mammalian tissues. Its formation from UTP and glucose 1-phosphate is reversibly catalysed by the enzyme UDP-glucose pyrophosphorylase (UTP- α -glucose 1-phosphate uridylyltransferase, EC 2.7.7.9):

Whereas considerable data exist on the molecular weight, subunit composition and steady-state parameters of the enzyme [see review, Turnquist & Hansen (1973), and, for a comparison of the properties of the enzyme from rabbit, calf and human livers, Turnquist *et al.* (1974)] very few investigations have been made on the mechanism of reaction. The steady-state kinetics of the enzyme from human erythrocytes suggest a compulsory addition of substrate, the nucleotide binding first (Tsuboi *et al.*, 1969). This is supported by the elegant experiments of Gillett *et al.* (1971), who have also pointed to the unlikelihood of the existence of an uridyl enzyme intermediate.

The present paper discusses the equilibrium of substrates with the enzyme from calf liver, measured by a variety of means, confirming the binding order and demonstrating the value of synthetic substrate analogues for the future exploration of the reaction mechanism.

Experimental

Materials

Chemicals were obtained from the following companies: UTP, UDP-glucose, dicyclohexylcarbo-

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di-imide and calcium phosphate gel from BDH Chemicals, Poole, Dorset, U.K.; 2',3'-O-isopropylidene-uridine, salmine sulphate and Tricine [*N*-tris-(hydroxymethyl)methylglycine] from Sigma Chemical Co., London S.W.6, U.K.; glucose 1-phosphate, phosphoglucomutase and glucose 6-phosphate dehydrogenase were from Boehringer Corp. Ltd., London W.5, U.K.; [α - ^{32}P]UTP was from The Radiochemical Centre, Amersham, Bucks., U.K.; DEAE-Sephadex A-25 and Sephadex G-50 (fine grade) were from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose (DE52) from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; methylenediphosphonic acid from Miles Laboratories Ltd., Stoke Poges, Slough, Berks., U.K. All other chemicals used were AnalaR or equivalent grade.

Bovine liver UDP-glucose dehydrogenase was prepared by the method of Zalitis & Feingold (1969).

Synthesis of $\alpha\beta$ -methylene-UTP

(a) *Synthesis of uridine 5'-methylenediphosphonate.* The procedure of Myers *et al.* (1965) was followed except that 3 mmol of 2',3'-O-isopropylidene uridine were used in place of the adenine derivative, and the following procedure was adopted to separate the uridine 5'-methylenediphosphonate from the co-reactants.

After preparing the mixture as described by Myers *et al.* (1965), it was adjusted to pH 8.0 with 1 M-NH₃ and applied to a column (12 cm $\times$ 4 cm) of Dowex 1 (formate form, 200–400 mesh). The column was washed with 500 ml of 4 M-formic acid and subsequently with a linear gradient, obtained by mixing 1500 ml of 4 M-formic acid with 1500 ml of 0.6 M-ammonium formate/4 M-formic acid, at a flow rate of

80ml/h. The major u.v.-absorbing component (22500 absorption units) was contaminated with methylenediphosphonic acid in the latter half of the peak (as determined by titration). This contaminated portion was passed through a Dowex 50W (H^+) column and evaporated to dryness to remove formic acid. The residue was dissolved in 50ml of water, neutralized with 1M- NH_3 and re-run as described above.

Both fractions containing uridine 5'-methylenediphosphonate in the ammonium formate/formic acid buffer were combined and passed through a column containing an excess of Dowex 50W (H^+) resin and the eluate was evaporated to dryness. The residue was redissolved in a small volume of water and evaporated to dryness a further three times to remove all traces of formic acid. Finally, the product was dissolved in about 10ml of water, titrated with 1M-LiOH to pH7.5 and freeze-dried to obtain the lithium salt of uridine 5'-methylenediphosphonic acid (yield overall 60%).

(b) *Phosphorylation of uridine 5'-methylenediphosphonic acid.* (i) Direct condensation with P_i . The procedure used was that of Smith & Khorana (1958) for the synthesis of UDP and UTP from UMP.

A mixture containing 20 μ mol of uridine 5'-methylenediphosphonate, 200 μ mol of P_i , 400 μ mol of dicyclohexylcarbodi-imide and 420 μ mol of tri-*n*-butylamine in 500 μ l of dry pyridine was left to react for 48 h. This procedure gave an 89% yield of $\alpha\beta$ -methylene-UTP (assessed by ion-exchange chromatography). Under these conditions only 2% of higher phosphates was produced, with 9% of the starting material remaining. Although the desired product was obtained in very high yield, this was not the preferred method of synthesis, as it was contaminated with inorganic polyphosphate when purification was attempted by ion-exchange chromatography.

(ii) Phosphoromorpholidate method. The morpholine derivative of uridine 5'-methylenediphosphonate (500 μ mol) was prepared by the general method described by Moffatt & Khorana (1961). The anhydrous product, dissolved in dry pyridine was added to 1500 μ mol of P_i (mono-tri-*n*-butylamine salt) which had been dried by evaporating three times in dry pyridine. Ion-exchange chromatography of a small sample showed a 46% yield of $\alpha\beta$ -methylene-UTP, with 54% of the starting material remaining.

The reaction mixture was evaporated to dryness, dissolved in 10ml of water and then extracted with diethyl ether (4 $\times$ 20ml), the aqueous phase being retained. All traces of ether were removed by bubbling air through the solution and finally by evacuating the vessel in which it was contained. Aq. NH_3 (1M) was added to bring the solution to pH5.5 and it was then applied to a column (15 cm $\times$ 1.5 cm) of Dowex 1 resin (Cl^- form; 200–400 mesh), which was subsequently run at 5°C. The column was washed

with 100ml of water and uridine 5'-methylenediphosphonate was eluted with a linear gradient of LiCl at pH2.5 (2 litres, 0–0.2M in 3mm-HCl). $\alpha\beta$ -Methylene-UTP was eluted with 0.2M-LiCl/3mm-HCl. The eluate containing $\alpha\beta$ -methylene-UTP was titrated to pH7.5 with 1M-LiOH and the solution evaporated to dryness at 25°C. LiCl was removed by the sequential addition of dry methanol/acetone until no chloride could be detected. The insoluble product was harvested each time by centrifugation. A 34% yield of $\alpha\beta$ -methylene-UTP, based on u.v. absorption, was obtained. Ion-exchange chromatography of the product detected a u.v.-absorbing impurity of about 1–2%.

The structure of $\alpha\beta$ -methylene-UTP was confirmed as follows. (i) Potentiometric titration and u.v. absorbance gave uridine/strong acid/weak acid (1:3:1). (ii) Acid-labile phosphate (1M-HCl, 100°C for 15 min)/uridine (1:1). (iii) Elution from Dowex 1 (Cl^- form) showed it to be eluted after UDP and just before UTP (Fig. 1).

Analytical ion-exchange chromatography

A quantitative and rapid method for the separation of the 5'-phosphate derivatives of a single nucleoside was developed. A column (4 cm $\times$ 1 cm) of Dowex 1 resin (Cl^- form; 200–400 mesh) was mounted inside the cuvette housing of a Pye Unicam SP.1800. A linear gradient, created by mixing 120ml of 3mm-HCl with 120ml of 0.5M-NaCl/3mm-HCl, was passed into a reference flow-through cell (1 cm light-path, 3 mm diam.) and on to the column. The

Fig. 1. Ion-exchange separation of a mixture of uridine nucleotides and synthetic analogues

About 0.5 μ mol samples of each nucleotide were applied to a column (4 cm $\times$ 1 cm) of Dowex 1 (Cl^- form) resin and eluted with a linear gradient (240ml) of NaCl (0–0.5M) in 3mm-HCl, as described in the text. The peaks are numbered in order of emergence and are identified as follows: 1, UMP; 2, uridine 5'-methylenediphosphonic acid; 3, UDP; 4, $\alpha\beta$ -methylene-UTP; 5, UTP.

eluent from the column was passed through a second, matched flow-through cell in the sample housing. As the absorbance of the sample cell was balanced against that of the reference, a flat baseline was obtained on a chart recorder. The eluting gradient was pumped at a constant rate (20ml/h) and the eluate monitored at E_{260} . The area attributable to each peak eluted was therefore a quantitative measure of the amount of nucleotide.

Preparation of UDP-glucose pyrophosphorylase

Except where otherwise stated, the preparation was carried out at 5°C, centrifugation was at 25000g for 30min and 'buffer' refers to 10mM-Tricine/5mM-2-mercaptoethanol adjusted to pH 8.5 with 4M-NaOH. The following is a modification of the method of Gillett *et al.* (1971).

Frozen calf liver (1kg) was cut into small pieces and homogenized in 1.5 litres of 30mM-KOH/5mM-EDTA/10mM-2-mercaptoethanol in a Waring Blender. The homogenate was stirred and a suspension of 12g of salmine sulphate in 100ml of water was slowly added. The suspension was stirred for 10–15min, then centrifuged and the supernatant filtered through glass wool and immediately adjusted to pH 8.5 by the addition of conc. aq. NH_3 (sp. gravity 0.88). Finely ground solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly, with stirring, to 40% saturation, and conc. aq. NH_3 was added to maintain the pH at 8.5. The supernatant was collected by centrifugation and further $(\text{NH}_4)_2\text{SO}_4$ added to adjust it to 60% saturation. The mixture was stirred for 20min, then centrifuged, the supernatant was discarded and the pelleted precipitate redissolved in buffer (approx. 150ml). This solution was dialysed against 10 litres of buffer overnight and then centrifuged to remove any denatured protein.

Sufficient calcium phosphate gel was added to remove only about 10% of the enzyme activity (determined by trial additions to small samples) and was removed by centrifugation. The resulting clear solution (approx. pH 7.6) was titrated with DEAE-Sephadex A-25 (approx. 25g dry wt.) pre-equilibrated with Tricine buffer, pH 7.5, to the point where 10–20% of the activity was lost. Sephadex was removed by filtering through a glass-wool plug in a filter funnel. The clear solution was adjusted to pH 8.5 with 1M-NaOH and then passed through a column (22cm $\times$ 4.2cm) of DE52 cellulose. The column was washed with buffer containing 20mM-NaCl until the E_{280} of the eluate approached zero. Absorbed protein was then eluted by a linear gradient (2 litres) of NaCl (20–200mM) in buffer. Fractions with the highest specific activity (10 and over) were combined and solid $(\text{NH}_4)_2\text{SO}_4$ added to 60% saturation (maintained at pH 8.5 with conc. aq. NH_3); the precipitate was collected by centrifugation.

The protein was dissolved in the minimum amount of buffer at 25°C (about 5ml). Denatured protein was removed by a brief centrifugation at room temperature. Crystallization of the clear solution obtained occurred on dialysis against 20% $(\text{NH}_4)_2\text{SO}_4$ in buffer (adjusted to pH 8.5 with conc. aq. NH_3). The crystals obtained were harvested by centrifugation at 3000g for 10min. After recrystallization the protein was stored in 20% $(\text{NH}_4)_2\text{SO}_4$, pH 8.5.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed by the procedure of Davis (1964). Gels (5%, w/v) at pH 8.7 were developed for 2h at 4°C, by using 4mA/tube. Protein was detected with Coomassie Blue.

Ultracentrifugation

Ultracentrifugation was carried out in an M.S.E. Centriscan 75, by using the reconstructed Schlieren system. Sedimentation-velocity experiments were performed in the presence and the absence of 200 μ M-UTP, by using 2.5mg of the enzyme/ml in 100mM-NaCl/10mM-Tricine/10mM-2-mercaptoethanol, pH 8.5 at 4°C. Results were corrected to water at 20°C.

Enzyme assays

Assay 1, pyrophosphorolysis of UDP-glucose. The assay medium contained UDP-glucose (1mM), PP_i (2mM), MgCl_2 (2mM), NADP^+ (1mM) and excess of phosphoglucomutase and glucose 6-phosphate dehydrogenase in 100mM-Tricine buffer, pH 7.8 at 25°C. Formation of NADPH was followed at E_{340} . The apparent K_m values for UDP-glucose and PP_i were determined at pH 8.5 by using 2mM- PP_i and 1mM-UDP-glucose respectively, and various concentrations of the other substrate.

Assay 2, formation of UDP-glucose. The assay medium contained UTP (1mM), glucose 1-phosphate (1mM), MgCl_2 (2mM), NAD^+ (1mM) and excess of UDP-glucose dehydrogenase in 100mM-Tricine buffer, pH 8.5 at 25°C. This reaction leads to the formation of 2mol of NADH (also followed at E_{340}) from 1mol of UDP-glucose. The K_m values of UTP and glucose 1-phosphate were determined by extrapolation to infinity of the fixed substrate concentration.

Protein was measured by the methods of Lowry *et al.* (1951) and Warburg & Christian (1941), with bovine serum albumin as standard.

Determination of equilibrium binding constants

(a) *Measurement of bound ligand by gel filtration* (Hummel & Dryer, 1962). A column (15cm $\times$ 0.9cm) containing Sephadex G-50 (fine grade) was equilibrated with 50mM-Tricine/2mM- MgCl_2 buffer, pH

8.5, containing either 100 or 30 μM -UTP and sufficient [α - ^{32}P]UTP to give about 40000 c.p.m. in a 100 μl sample. A 100 μl sample of enzyme in the same buffer, containing 10% (w/v) sucrose, was layered on to the top of the column. Elution was continued with the equilibrating buffer at 11 ml/h, by using a constant-head device. Approximately 200 μl fractions were collected and 100 μl samples removed to determine radioactivity.

(b) *Spectrophotometric titration.* Titration was carried out in a Pye Unicam SP.1800 apparatus, fitted with a thermostatically controlled housing. Two 0.5 cm-path-length quartz cuvettes were placed in series in both the sample and the reference holders. In each position one cuvette contained protein in 100 mM-Tricine, pH 8.5, and the other cell an equal volume of the same buffer. Ligand was added to the buffer cuvette in the reference housing and to the protein solution in the sample housing. At each addition, an equal volume of buffer was added to the reference protein solution. Thus the same total absorbance was contained in both sets of cuvettes and any difference obtained was due to interaction between ligand and protein. E_{280} or E_{286} was recorded for each addition of ligand.

(c) *Equilibrium dialysis.* Equilibrium-dialysis cells were made by joining two Perspex half-cells, each having a reservoir of 900 μl capacity, separated by a piece of Visking tubing. A plugged hole in each side of the cell provided a means for filling the container and mixing was achieved by including a small glass bead, which stirred the contents as the cell was rotated, on each side of the membrane. The time for ligand to reach equilibrium was determined by placing buffer in one half-cell and 100 μM -UTP (containing approx. 250000 c.p.m. of [α - ^{32}P]UTP) in the other. A number of identical cells were set up and duplicate 200 μl samples removed from each side of one cell every hour. Equilibrium was complete in 4 h and subsequent experiments were therefore left for 6 h.

Various concentrations of UTP, all containing an amount of [α - ^{32}P]UTP similar to the above, were made up in 50 mM-Tricine/2 mM-MgCl₂ buffer, pH 8.5, and the radioactivity in each sample was determined. Each of the UTP solutions was added to one side of the equilibrium-dialysis cells and to the other was added a solution of pyrophosphorylase in the same buffer. Enzyme concentration was measured after termination of the experiment.

Results and Discussion

Modification of the procedure for enzyme preparation used by Gillett *et al.* (1971) was found to be necessary, since difficulty was experienced with the ion-exchange and crystallization steps. This was exacerbated by the fact that variable amounts of enzyme were found in the liver. Polyacrylamide-gel

electrophoresis and ultracentrifugation showed that the pyrophosphorylase preparation contained a single molecular species. The enzyme has been shown to be an octameric structure, with a mol.wt. of 480000 (Levine *et al.*, 1969), which is corroborated by our data. When the enzyme was subjected to ultracentrifugation in the presence of sufficient UTP to produce over 90% saturation, no other molecular species occurred. Thus the integrity of the octameric structure is preserved in the presence of bound substrate, there being no increased tendency to aggregate or dissociate.

Titration of the enzyme with ligand gave a spectrophotometric quench. At E_{280} this decrease was maximum at about 9% with uridine nucleotides and at about 7% with glucose 1-phosphate. Where high concentrations of u.v.-absorbing ligands were used it was necessary to follow the titration at E_{286} , where total absorbance was lower but only a 6% quench was obtained.

The dissociation constant, K_s , for the equilibrium between enzyme and ligand is given by:

$$K_s = [\text{E}][\text{L}]/[\text{EL}] \quad (1)$$

where E is free enzyme, L is free ligand and EL is the enzyme-ligand complex. Substitution and rearrangement of eqn. (1) leads to eqn (2):

$$[\text{L}_0] = K_s \left(\frac{\alpha}{1-\alpha} \right) + n[\text{E}_0]\alpha \quad (2)$$

where L_0 and E_0 are initial concentrations and α is the degree of saturation. Eqn. (2) was rearranged to give the expression:

$$\alpha = \frac{[\text{L}_0] + n[\text{E}_0] + K_s - \sqrt{([\text{L}_0] + n[\text{E}_0] + K_s)^2 - 4[\text{L}_0] \cdot n[\text{E}_0]}}{2n \cdot [\text{E}_0]} \quad (3)$$

such that the term containing the experimental error, α , was collected on one side. Data were fitted to eqn. (3) by using the computer program of Jones (1970). Values of K_s and $n[\text{E}_0]$ are given in Table 1 and the latter parameter is compared with the experimentally determined enzyme concentration, to give the number of mol of substrate bound/mol of enzyme subunit. It is clear that within experimental limits, 1 mol of ligand is bound per mol of enzyme subunit. The same stoichiometry has been reported for UDP-glucose binding to the rabbit liver enzyme (Turnquist *et al.*, 1974).

A direct measure of the amount of ligand bound to the enzyme was given by the gel-filtration and equilibrium-dialysis methods. By using the technique of Hummel & Dryer (1962), the amount of bound ligand was determined by measuring the peak and trough areas of the radioactivity elution profiles when the enzyme was in equilibrium with either 100 or

Table 1. Measurement of the dissociation constants and stoicheiometry of ligand binding

The data are derived from the spectrophotometric titration of the enzyme in 100mM-Tricine, pH8.5 at 25°C, as described in the text. MgCl₂ (2mM) was included, except where stated. Enzyme concentration was determined from its specific activity (240 units/mg) and from protein concentration, and by assuming a subunit mol.wt. 60000.

Ligand	$K_s \pm \text{s.d.}$ (μM)		$nE_o \pm \text{s.d.}$ (computed) (μM)	nE_o (experimentally determined) (μM)	Binding stoicheiometry (mol of ligand bound/mol of enzyme subunit)
UTP	17.9 ±	3.2	61.5 ± 9.9	70	0.88
αβ-Methylene-UTP	19.1 ±	3.5	47.0 ± 8.5	50	0.94
UDP-glucose	7.1 ±	2.0	54.9 ± 5.7	50	1.1
Glucose 1-phosphate	796 ±	189	—	30	—
Glucose 1-phosphate (plus saturating αβ-methylene-UTP)	9.5 ±	3.4	55.2 ± 10.8	50	1.1
UTP (no MgCl ₂)	13.7 ±	3.3	85.5 ± 9.3	70	1.22

Table 2. Values of the Michaelis constants, at pH8.5 and 25°C, for UDP-glucose pyrophosphorylase

Assay conditions were as described in the text. Apparent K_m values for UDP-glucose and PP_i are given, with the concentration of the second substrate as indicated. Mg²⁺ (2mM) was present throughout.

		$K_m (\mu\text{M}) \pm \text{s.d.}$			
Substrate	...	UTP	Glucose 1-phosphate	UDP-glucose (+2 mM-PP _i)	PP _i (+1 mM-UDP-glucose)
		88 ± 11	78 ± 7	26 ± 2	95 ± 13

Fig. 2. Scatchard plot of data from equilibrium dialysis of enzyme with UTP

Enzyme (12 μM) was dialysed against 200, 100, 50, 25, and 10 μM-[α-³²P]UTP, of known specific radioactivity. At equilibrium, the UTP concentration was determined on either side of the dialysis membrane.

30 μM-UTP. The values determined for K_s , 21 and 18.5 μM respectively, were only constant assuming that 1 mol of ligand was bound/mol of enzyme subunit. The Scatchard (1949) plot (Fig. 2), derived from

the equilibrium-dialysis experiments, further confirm this stoicheiometry. The experimentally determined enzyme subunit concentration was 12 μM and the computed data gave 14.1 ± 1.0 μM (± s.d., $n = 5$). K_s for UTP was 33.1 ± 3.9 μM, ± s.d., $n = 5$), which is in good agreement with determinations by the other two methods described.

A compulsory binding order, with UTP or UDP-glucose binding first, has been suggested (Gillett *et al.*, 1971). To confirm this mechanism the UTP analogue with the αβ-phosphates linked by a methylene group was synthesized. The major difference imposed by the methylene bridge is to raise the p*K* of the weak-acid dissociation of the terminal phosphate by about 1.5 pH units above that of UTP (p*K* 6.7). The similarity of the analogue to UTP is shown by the fact that it has the same K_s value (Table 1), whereas the K_s for UDP is about fourfold higher (R. A. J. Stevens, unpublished work). In the presence of glucose 1-phosphate, Mg²⁺ and UDP-glucose pyrophosphorylase, no reaction was observed with αβ-methylene-UTP and it was therefore possible to saturate the enzyme with the substrate analogue before titrating with the hexose monophosphate. Under these conditions, K_s for glucose 1-phosphate decreased by two orders of magnitude over the K_s value

for glucose 1-phosphate alone (Table 1), conclusively demonstrating the compulsory binding order. It is noteworthy that the K_s values for the three substrates (including glucose 1-phosphate in the presence of nucleotide) each bear a similar numerical relationship to their respective K_m values (cf. Table 1 and Table 2).

The mechanism of action of the pyrophosphorylase remains obscure. The possibility of a uridylylated enzyme complex is contraindicated by the work of Gillett *et al.* (1971), and is further excluded by the finding in the present paper that the hexose monophosphate could bind in the presence of $\alpha\beta$ -methylene-UTP. Uridylation would require the prior displacement of the pyrophosphate group, before glucose 1-phosphate could bind. Apart from the compulsory order of addition of substrates, which has been confirmed, limited information is also available on the role of Mg^{2+} activation, which has been speculatively reviewed (Turnquist & Hansen, 1973). It is now clear that the binding of the nucleotide is not dependent on the metal ion, the K_s for UTP being unaffected by its presence or absence (Table 1).

The amount of UDP-glucose pyrophosphorylase in bovine liver varies considerably but, assuming an upper limit of 0.3% of the extractable protein (Albrecht *et al.*, 1966), the cytoplasmic concentration of the enzyme would be approximately $10\ \mu M$ in sites. The concentration of UDP-glucose in rat liver has been determined as approx. $0.6\ mM$ (Hornbrook *et al.*, 1965; Keppler & Decker, 1969), but evidence suggests that there is rather less in bovine liver (Pieck *et al.*, 1971) where it is probably nearer $0.1\ mM$. This suggests that, as the K_s value for UDP-glucose is $7\ \mu M$, the enzyme must be approximately 90% saturated with product and, consequently, its turnover would be decreased to a low percentage of its inherent capacity (K_i $5\ \mu M$; Hopper & Dickinson, 1973).

Product inhibition appears to be of major importance in the regulation of UTP utilization, but it is far from clear what, if any, fine control exists for the maintenance of UDP-glucose concentrations which have been shown to remain constant, even during dietary stress (Phelps *et al.*, 1975). The answer may lie in the finding of Kornfeld (1965) that AMP evinced a complex form of inhibition in the rat liver enzyme. Inhibition by AMP of UDP-glucose pyrophosphorylase from bovine liver has been observed at various degrees of purification (R. A. J. Stevens,

unpublished work). Such preparations, however, were not stable to AMP sensitivity and it has never been observed with the crystalline preparation described in the present paper.

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