Reuben, J., and Cohn, M. (1970), *J. Biol. Chem. 245,* 6539–6546.
Scopes, R. K. (1969), *Biochem. J. 113,* 551–554.
Scopes, R. K. (1971), *Biochem. J. 122,* 89–92.
Scopes, R. K. (1973), Enzymes, 3rd Ed. *8,* 335–351.
Solomon, I. (1955), *Phys. Rev. 99,* 559–565.
Tanswell, P., Westhead, E. W., and Williams, R. J. P. (1976), *Eur. J. Biochem. 63,* 249–262.
Walsh, C. T., Jr., and Spector, L. G. (1971), *J. Biol. Chem. 246,* 1255–1261.
Wendell, P. L., Bryant, R. N., and Watson, H. C. (1972), *Nature (London), New Biol. 240,* 134–136.

Galactose-1-phosphate Uridylyltransferase: Isolation and Properties of a Uridylyl-Enzyme Intermediate†

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ABSTRACT: Galactose-1-P uridylyltransferase catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P by a double displacement pathway involving a uridylyl-enzyme intermediate. The amount of radioactivity incorporated into the protein by uracil-labeled UDP-glucose is decreased by the presence of UDP-galactose, which competes with UDP-glucose for uridylylating the enzyme. The amount of glucose-1-P released upon reaction of the enzyme with UDP-glucose indicates that the dimeric enzyme contains more than one active site per molecule, 1.7 on the average for the most active preparation obtained. This suggests that there is one uridylylation site per subunit and that the subunits are similar or identical. The uridylyl-enzyme is stable to mild alkaline conditions, 0.10 M NaOH at 60 °C for 1 h, but it is very sensitive to acid, being largely hydrolyzed after 12 h at pH 3.5 and 4 °C. The principal radioactive product resulting from hydrolysis of [*uracil*-2-^{14}C]uridylyl-enzyme under the latter conditions is [^{14}C]UMP. The hydrolytic properties of the uridylyl-enzyme show that the uridylyl moiety is bonded to the protein through a phosphoramidate linkage. Complementary studies on the effects of group selective reagents on the activity of the enzyme suggest that the active site nucleophile to which the uridylyl group is bonded may be a histidine residue. The enzyme is rapidly inactivated by diethyl pyrocarbonate at pH 6 and 0 °C and reactivated by NH_2OH. UDP-glucose at 0.5 mM fully protects the enzyme against diethyl pyrocarbonate while 70 mM galactose-1-P has only a slight protective effect. Uridylyl-enzyme is inactivated by diethyl pyrocarbonate at no more than 2% of the rate for free enzyme. The enzyme is not inactivated by $NaBH_4$ or by $NaBH_4$ in the presence of UDP-glucose. It is not inhibited by 1 mM pyridoxal phosphate or by 0.5 mM 5-nitrosalicylaldehyde at pH 8.5 and it is not inactivated by $NaBH_4$ in the presence of pyridoxal phosphate. The enzyme is inactivated by 5 to 50 μM *p*-hydroxymercuribenzoate at pH 8.5, but substrates exert no detectable protective effect against this reagent. It is concluded that the enzyme contains at least one essential sulfhydryl group which is not located in the active site in such a way as to be shielded by substrates.

Galactose-1-P uridylyltransferase (EC 2.7.7.12) catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P, the second step in the Leloir pathway for converting galactose to glucose-6-P. The first step is the phosphorylation of galactose by ATP catalyzed by galactokinase and the third is the interconversion of UDP-galactose and UDP-glucose catalyzed by UDP-galactose 4-epimerase. Glucose-6-P is produced from glucose-1-P by the action of phosphoglucomutase.

Galactose-1-P uridylyltransferase activity is found in microorganisms, plants, and animals (Kalckar et al., 1953; Maxwell et al., 1955; Kurahashi, 1957; Pazur and Shadaksharaswamy, 1961). A defect in this enzyme is associated with galactosemia (Kalckar, 1960), an inherited disease of humans in which galactose cannot be metabolized to glucose at sufficient rates to prevent the accumulation of galactose and derived metabolities to toxic levels.

The close structural similarities between the uridylyl donors, UDP-glucose and UDP-galactose, and between the acceptors, glucose-1-P and galactose-1-P, as well as the chemical nature of the reaction suggested to us that it would be efficient from the standpoints of both mechanism and structural evolution for the active site to consist essentially of a UDP-hexose binding site capable of binding either uridylyl donor, such that the reaction mechanism could follow a double-displacement pathway involving a uridylyl-enzyme intermediate and ping-pong kinetics. This is a highly restrictive concept for the action of this enzyme, in that it denies the existence of productive ternary complexes and it demands that the uridylyl moiety undergoing transfer be stabilized in some way, probably by covalent bond formation to a nucleophile. This mechanism is outlined in eq 1 and 2, in which –XH symbolizes the functional group and the Michaelis complexes are omitted.

$$\text{UDP-glucose} + \text{E–XH} \rightleftharpoons \text{uridylyl–X–E} + \text{glucose-1-P} \quad (1)$$

$$\text{galactose-1-P} + \text{uridylyl–X–E} \rightleftharpoons \text{E–XH} + \text{UDP-galactose} \quad (2)$$

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We undertook to study the mechanism of this reaction and reported preliminary verification of eq 1 and 2 for the enzyme purified from *E. coli*, including the first isolation of a uridylyl-enzyme as a catalytic intermediate (Wong and Frey, 1974a). We also found that ping-pong kinetics is obeyed by this enzyme (Wong and Frey, 1974b). It was subsequently reported that the human erythrocyte enzyme also follows this pathway (Wu et al., 1974).

In this paper we describe in detail our findings to date on the isolation and properties of the uridylyl-enzyme and on some of its chemical properties.

Materials and Methods

Enzymes. Galactose-1-P uridylyltransferase was purified from a regulatory mutant of *E. coli*, ATCC-27797, by a modification of the published procedure (Saito et al., 1967). The cells were grown to the stationary phase as described (Wee and Frey, 1973) and they contained high levels of both UDP-galactose 4-epimerase and galactose-1-P uridylyltransferase. The latter was purified from freshly grown cells immediately after harvesting and essentially as described by Saito et al. (1967). We made several modifications in the procedure. We eliminated the addition of streptomycin sulfate to the crude extract because we did not observe precipitate formation in our extracts and the addition proved to be unnecessary. We also modified the calcium phosphate gel treatment, step 4 of the published procedure (Saito et al., 1967). The first addition of calcium phosphate gel was carried through as described to remove inactive protein, but the second addition, to adsorb the enzyme, was eliminated. Instead, we chromatographed the supernatant fluid obtained from the first gel addition on a column of calcium phosphate gel-cellulose. The supernatant fluid obtained at this stage from 300 g of bacterial cells was adjusted to pH 6.0 with cold 2 M acetic acid and passed into a 4.1 × 16 cm column of calcium phosphate gel-cellulose which had been equilibrated with 0.01 M potassium phosphate buffer containing 1 mM cysteine at pH 6.0. The gel-cellulose was prepared as described by Reed and Willms (1966). The column was washed with 200 mL of the above buffer followed by 600 mL of 0.02 M potassium phosphate at pH 7.0 containing 1 mM cysteine. Fractions were collected at 1.5 mL per min. Those fractions containing substantial galactose-1-P uridylyltransferase activity were pooled, made to 2 mM in EDTA[1] and 5 mM cysteine by addition of solid materials, and adjusted to pH 8.8 with 5 M ammonium hydroxide. The enzyme was precipitated by addition of solid ammonium sulfate to 68% saturation while maintaining the pH by appropriate addition of 5 M ammonium hydroxide. The precipitate was collected by centrifugation at 18 000*g* for 20 min, dissolved in a minimum volume of 0.05 M potassium phosphate buffer containing 0.5 mM cysteine and 0.2 mM EDTA, and dialyzed overnight against 3 L of the same buffer. The calcium phosphate gel adsorption and gel-cellulose chromatography effected a sixfold increase in specific activity. The balance of the purification was followed essentially as described by Saito et al. (1967) for a number of preparations. Recently we further modified the procedure by eliminating DEAE-cellulose column chromatography and proceeding directly to the hydroxylapatite column chromatography as described (Saito et al., 1967). The enzyme from the best pooled fractions was then subjected to DEAE-Sephadex A-50 chromatography with a NaCl gradient. These modifications produced higher yields of higher quality enzyme than we have been able to achieve routinely by the original procedure. The purified enzyme we obtained was not quite homogeneous, as determined by polyacrylamide disc gel electrophoresis, but passage through a column of Sephadex G-75 removed trace impurities and produced a homogeneous enzyme.

The specific activity of our best enzyme preparation was 189 units per mg of protein by the assay method given below and assuming $E_{280}^{1\%} = 16.3$, as reported by Saito et al. (1967). Our assay conditions employed lower substrate concentrations than those described by Saito et al.; however, we were able to compare the activity of our best preparation with that reported by Saito et al. by calculating the ratio of initial rates to be expected under the two conditions. When this was done using our saturation parameters and substrate inhibition constants (Wong and Frey, 1974b), we calculated that the initial rate under the assay conditions of Saito et al. (1967) would be 1.21 times larger than that we measured. Our best specific activity, when adjusted by this factor, was then 229 units per mg of protein, which appeared to be somewhat better than the 209 units per mg of protein reported by Saito et al. (1967). We concluded that our best preparations were at least the equivalent in purity of the homogeneous preparation described by Saito et al. The enzyme as isolated was frequently substantially less active than the best preparation, and even the best preparations rapidly lost activity. We have not yet found a satisfactory way to stabilize this enzyme during prolonged purification and storage.

Phosphoglucomutase, glucose-6-P dehydrogenase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase were purchased from commercial suppliers. UDP-glucose dehydrogenase was purified from beef liver through step 6 of the procedure published by Zalitis and Feingold (1969) and UDP-galactose 4-epimerase was purified from *E. coli* as described by Wilson and Hogness (1964).

Substrates and Coenzymes. [*uracil*-5,6-^{3}H]UDP-glucose and [*uracil*-2-^{14}C]UDP-glucose were prepared enzymatically from correspondingly labeled UTP. The reaction mixtures consisted of 6.75 μmol of radioactive UTP (New England Nuclear), 15 μmol of glucose-1-P, 7.5 μmol of $MgCl_2$, 5 IU of inorganic pyrophosphatase, 5 IU of UDP-glucose pyrophosphorylase, and 10 μmol of Tris-HCl buffer in a total volume of 1.0 mL and at a final pH of 7.2. The reaction mixture was placed at 27 °C for at least 75 min. The progress of the reaction was monitored by withdrawing aliquots at various reaction times and assaying for UDP-glucose enzymatically with UDP-glucose dehydrogenase and DPN^+. The conversion of UTP to UDP-glucose was quantitative and complete within 75 to 100 min. The solution was heated to destroy enzymes, clarified by centrifugation, concentrated to small volume, and subjected to descending paper chromatography on Whatman 3 MM filter paper. The developing solvent was 95% ethanol–1 M ammonium acetate at pH 3.5 (ratio 5:2). This chromatography system separated UTP, UMP, glucose-1-P, and UDP-glucose. All of the detectable radioactivity was found to be associated with UDP-glucose. This area was cut out and the radioactive UDP-glucose eluted and stored at −10 °C.

Other substrates, coenzymes, and chemicals were purchased from commercial suppliers and used without further purification. UDP-[U-^{14}C]glucose was purchased from New England Nuclear and repurified by paper chromatography in the same system used above for uracil-labeled UDP-glucose.

[1] The abbreviations are: EDTA, ethylenediaminetetraacetate; HMB, *p*-hydroxymercuribenzoate; DEPC, diethyl pyrocarbonate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN; TPNH, reduced triphosphopyridine nucleotide.

Assays. Protein was estimated routinely during enzyme purification by the Warburg and Christian method (Layne, 1957) or by the Lowry method using crystalline bovine serum albumin as the standard (Lowry et al., 1951). The protein concentration in solutions of highly purified enzyme was measured spectrophotometrically assuming $E_{280}^{1\%} = 16.3$. This value was reported by Saito et al. (1967) to be based on dry weight of homogeneous enzyme. Galactose-1-P uridylyltransferase was assayed by coupling the reaction with phosphoglucomutase and glucose-6-P dehydrogenase according to the standard activity assay method and the associated activity unit defined by Wong and Frey (1974b). The concentrations of substrates were measured enzymatically as DPNH or TPNH using phosphoglucomutase, glucose-6-P dehydrogenase, UDP-glucose dehydrogenase, UDP-galactose-4 epimerase, and galactose-1-P uridylyltransferase under appropriate conditions. Radiochemical assays were performed by liquid scintillation counting in a Packard Tri-Carb Model 3301 liquid scintillation spectrometer. Radioactive areas on paper chromatograms were detected with a Packard Model 385 radiochromatogram scanner.

Results

Isolation of a Uridylyl-Enzyme Intermediate. We have described the isolation of a [*uracil*-5,6-^{3}H]uridylyl-enzyme by gel filtration of solutions of galactose-1-P uridylyltransferase after short term reaction with [*uracil*-5,6-^{3}H]UDP-glucose. This protein was shown to be deuridylylated by glucose-1-P or galactose-1-P (Wong and Frey, 1974a). We have obtained further evidence of the involvement of this uridylyl-enzyme as an intermediate by showing that uridylylation by [*uracil*-5,6-^{3}H]UDP-glucose is inhibited by UDP-galactose. The incorporation of 0.87 uridylyl group per enzyme dimer by 0.16 mM [*uracil*-5,6-^{3}H]UDP-glucose was reduced to 0.13 uridylyl group per dimer by the additional presence of 0.08 mM UDP-galactose and to 0.03 by 0.16 mM UDP-galactose when uridylylation was carried out as described earlier (Wong and Frey, 1974a).

UDP-galactose inhibition of uridylylation by [*uracil*-5,6-^{3}H]UDP-glucose further confirms eq 1 and 2, which require that UDP-galactose and UDP-glucose both react with the free enzyme to form the common intermediate. The presence of unlabeled UDP-galactose reduces the incorporation of radioactivity from [*uracil*-5,6-^{3}H]UDP-glucose by isotope dilution in the uridylyl-enzyme.

[*uracil*-5,6-^{3}H]Uridylyl-enzyme was also subjected to 6 M urea and then reisolated by gel filtration. The urea completely and irreversibly destroyed all the catalytic activity, which is consistent with extensive structural disordering, and the reisolated protein retained 63% of its radioactivity, about the same amount as the 58% retained upon rechromatography of undenatured, active uridylyl-enzyme. We have repeatedly observed hydrolytic loss of uridylyl groups upon rechromatography of the intermediate.

The uridylyl-enzyme can be more coveniently and reliably isolated by denaturation with cold perchloric acid. In a typical experiment two solutions of enzyme were prepared, each containing 77.5 units of galactose-1-P uridylyltransferase in 0.90 mL of 0.11 M sodium bicinate buffer at pH 8.5. Thirty microliters of 8.47 mM [*uracil*-2-^{14}C]UDP-glucose (2.04×10^7 cpm μmol^{-1}) was added to one and 20 μL of 15 mM UDP-[U-^{14}C]glucose (5.49×10^6 cpm μmol^{-1}) to the other. After 15 min at room temperature, 1.3 mL of freshly prepared ice-cold 0.68 M $HClO_4$ solution containing 3.8 mM UDP-glucose and 7.7 mM UMP was added to each, and the precipitates were immediately isolated by centrifugation at 10 000*g* for 10 min at 0 °C. The precipitates were each washed four times consecutively with 1.5 mL of ice-cold, freshly prepared 0.4 M $HClO_4$ containing 0.4 mM UDP-glucose and 0.8 mM UMP and isolated by centrifugation as above. Radiochemical analysis of the precipitates dissolved in 0.1 M NaOH showed that the one labeled with [*uracil*-2-^{14}C]UDP-glucose contained 1.09×10^5 cpm while that labeled with UDP-[U-^{14}C]glucose contained only 440 cpm.

Assuming an activity of 189 units per mg of protein and a molecular weight of 8×10^4 for homogeneous enzyme, it can be calculated that 5.1 nmol of enzyme was used in this experiment. The amount of radioactivity incorporated by [*uracil*-2-^{14}C]UDP-glucose corresponds to 5.34 nmol, which is a minimum value uncorrected for protein loss and hydrolytic loss of uridylyl moieties shown in a later section to be acid labile. A rough correction for the latter can be made from the radioactivity contents of the third and fourth perchloric acid washes of the denatured [*uracil*-2-^{14}C]uridylyl-enzyme, which were 6750 and 5850 cpm, respectively. Assuming about the same loss in each of the four washes, the original precipitate probably contained about 6.5 nmol of radioactivity. This figure can be further corrected for 66% recovery of protein to 9.8 nmol, which corresponds to 1.9 nmol of radioactivity per nmol of enzyme. This estimate compares favorably with the data in Table I.

The fact that the uridylyl moiety remains associated with the protein upon perchloric acid denaturation confirms that the bonding is covalent, either to the enzyme itself or to a cofactor which is itself covalently bonded to the enzyme. In addition it is a reliable procedure for preparing the uridylyl-protein from aged preparations of the enzyme, which have low specific activities and tend to bind intact UDP-glucose molecules on gel filtration columns. As shown in a later section, the hydrolytic properties of the perchloric acid denatured uridylyl-enzyme are similar to those of the active uridylyl-enzyme isolated by gel filtration, so there is no reason to believe that perchloric acid treatment causes migration of the uridylyl moiety.

The Number of Active Sites per Enzyme Molecule. The concentration of active sites in a solution of galactose-1-P uridylyltransferase can be determined by measuring the amount of glucose-1-P produced upon adding an excess of UDP-glucose to the solution in the presence of phosphoglucomutase, glucose-6-P dehydrogenase and TPN^+. This further confirms eq 1. We can also use this method to obtain information about the number of uridylylation sites associated with each enzyme molecule, which is reported to be a dimer of subunits. Such data on two enzyme preparations are given in Table I.

Both of the enzymes in Table I appeared to be about 90% homogeneous as indicated by polyacrylamide disc gel electrophoresis, but one was substantially more active than the other. The results for both preparations were corrected by normalizing the data to enzyme of the highest specific activity, 189 units per mg of protein. In making these corrections, it was implicitly assumed that an enzyme preparation whose specific activity is less than 189 units per mg of protein consists of fully active enzyme contaminated with inactive galactose-1-phosphate uridylyltransferase or other protein.

As shown in Table I, the application of this correction to data obtained from two enzyme preparations of quite different specific activity gives consistent results, and the results consistently indicate that each dimer contains more than one active site, 1.7 on the average. The validity of the data in Table I are as dependent upon the validity of the measurement of protein

TABLE I: Titration of Galactose-1-phosphate Uridylyltransferase Active Sites.[a]

Enzyme Preparation	Sample (units)	Glucose-1-P Produced (nmol)	Active Sites / Molecule
(A) 56 units/mg of protein	12	1.40 ± 0.27	1.80 ± 0.32
	24	2.55 ± 0.28	1.64 ± 0.18
(B) 169 units/mg of protein	9.2	0.85 ± 0.05	1.5 ± 0.1
	18.4	1.98 ± 0.13	1.76 ± 0.11

[a] The reaction mixtures contained the components of the standard activity assay with the exception that galactose-1-P was omitted. The total change in A_{340} was measured upon the addition of the enzyme to the otherwise complete reaction mixtures. The enzyme preparations A and B, though quite different in specific activity, both appeared to be 90–95% homogeneous by polyacrylamide disc gel electrophoresis. The calculation of active sites per enzyme molecule in the far right-hand column assumes a dimer molecular mass of 80 000 (9) and a specific activity of 189 units per mg of protein for homogeneous enzyme.

concentration as upon the measurement of glucose-1-P produced, and the protein concentrations are calculated from A_{280} data assuming $E_{280}^{1\%} = 16.3$ and the dimer molecular mass 80 000 g per mol (Saito et al., 1967). It appears from Table I that the enzyme contains more than one active site per dimer, perhaps one per subunit.

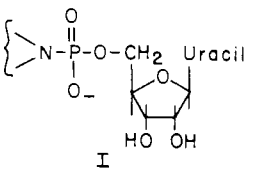
Hydrolytic Properties of the Uridylyl-Enzyme. To gain information about the chemical nature of the nucleotide–protein bond in the uridylyl-enzyme, we have studied some of its hydrolytic properties. Two identical samples of [*uracil*-5,6-^{3}H]uridylyl-enzyme were prepared by combining 56 units of galactose-1-P uridylyltransferase with 0.08 mM [*uracil*-5,6-^{3}H]UDP-glucose (2.0×10^7 cpm μmol^{-1}) in 0.3 mL of 10 mM potassium phosphate buffer containing 0.35 mM cysteine at pH 7.5 for 5 min at pH 7.5. One solution was adjusted to pH 3.5 with 1 M citric acid, and the protein was isolated by gel filtration through Sephadex G-25 equilibrated and eluted at 4 °C with 10 mM sodium citrate buffer at pH 3.5. The other solution was adjusted to pH 10.5 with 1 M Na_2CO_3 and the protein was isolated as above with the column equilibrated and eluted with 10 mM sodium carbonate buffer at pH 10.5. The protein isolated at pH 3.5 contained 2160 cpm and that isolated at pH 10.5 contained 8080 cpm. The solutions were permitted to stand at 4 °C for 15 h and then again passed through the same gel columns. The protein maintained at pH 3.5 was found to have lost all of its radioactivity whereas that maintained at pH 10.5 retained 6400 cpm. It was shown in control experiments that [*uracil*-5,6-^{3}H]UDP-glucose does not lose significant radioactivity by exchange with medium protons under these conditions, no detectable exchange at pH 3.5, and only 1.5% exchange at pH 10.5 (Wong, 1974).

The foregoing experiment showed that the undenatured uridylyl-enzyme is highly labile to weakly acidic conditions but reasonably stable at pH 10.5. Studies of hydrolytic properties at higher pH's appeared to be inadvisable with the [*uracil*-5,6-^{3}H]uridylyl-enzyme because of base-catalyzed exchange of the uracil-C-5 proton with medium protons. Therefore, more extensive studies were performed on the denatured [*uracil*-2-^{14}C]uridylyl-protein.

A sample of [*uracil*-2-^{14}C]uridylyl-enzyme was prepared as described above and dialyzed against 10 mM K_2HPO_4 at 4 °C. The following day a sample containing 2.16×10^4 cpm and 0.96 mg of protein was adjusted to pH 3.5 by addition of 0.1 M citric acid, diluted to 1.0 mL, and placed at 3.5 ± 0.5 °C for 12 h. Half of the solution was placed in a freezer at −10 °C and the other half was subjected to gel permeation chromatography through a column of Sephadex G-25 equilibrated and eluted with 10 mM K_2HPO_4. Radiochemical analysis of the fractions collected in the elution showed that 79.5% of the radioactivity was associated with small molecular weight substances in the back fractions, confirming the acid lability of the uridylyl-enzyme.

Another sample of [*uracil*-2-^{14}C]uridylyl-enzyme containing 1.08×10^4 cpm and 0.48 mg of protein in 0.25 mL was combined with 0.25 mL of 0.2 M NaOH and placed at 60 °C for 1 h. The solution was then subjected to gel permeation chromatography and analysis as described above, and 69.5% of the radioactivity was found to be associated with the protein. The balance of the radioactivity in the back fractions was attributed to hydrolytic loss of uridylyl groups, much of which no doubt occurred in the few hours subsequent to dialysis and prior to addition of base. As pointed out above we have always found some hydrolytic loss in solutions maintained in the near physiological pH range; in this experiment the loss was less than the usual because of the relatively short time between dialysis and alkaline treatment.

The fact that the chemical linkage between the protein and the uridylyl moiety is labile to acid and stable to base suggests that it may involve a phosphoramidate grouping. It is pertinent to consider whether the uridylyl-enzyme may be a uridyl phosphoramidate (structure I) or a uridyl pyrophosphoramidate (structure II). The latter would be the intermediate if the



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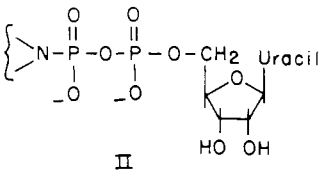

II

active site nucleophile were the phosphoryl group of a phosphorylated nitrogen atom in the enzyme. Upon mild acid hydrolysis under the foregoing conditions structure I would produce UMP while structure II would produce UDP. As shown in Figure 1 the product is chromatographically identical with UMP, which is consistent with structure I.

In a control experiment carrier UDP was included in the hydrolysis reaction mixture and shown not to be detectably converted to UMP under these conditions, although the major part of the radioactivity still cochromatographed with UMP.

Chemical Modification by Group Selective Reagents. In order to gain further information about the active site nucleophile or other catalytic groups in the active site, we have investigated the effects of several group selective reagents on the activity of galactose-1-P uridylyltransferase.

The activity was only slightly decreased by 50 mM $NaBH_4$ either in the presence or absence of 0.53 mM UDP-glucose. This reagent would probably have inactivated the enzyme in

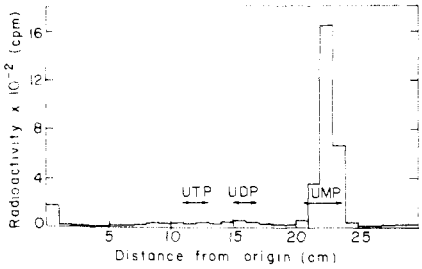

FIGURE 1: Identification of [^{14}C]UMP from hydrolysis of [*uracil*-2-^{14}C]uridylyl-enzyme. That part of the pH 3.5 hydrolysate of the [*uracil*-2-^{14}C]uridylyl-enzyme described in the text as having been stored at −10 °C was thawed, concentrated, applied as a 3-in. band on Whatman 3 MM filter paper together with carrier uridine nucleotides, and subjected to descending paper chromatography using 5:2 95% ethanol–1 M ammonium acetate at pH 3.5. A strip 1-in. in width was cut from one side of the chromatogram and cut into 1-cm sections, and each section was counted by liquid scintillation. The balance of the area corresponding to UMP was eluted and rechromatographed with 100:55.8:4.2:1.6 isobutyric acid–water–NH_4OH–0.1 M EDTA as the mobile phase. Again, all of the radioactivity coincided exactly with the position of the UMP marker.

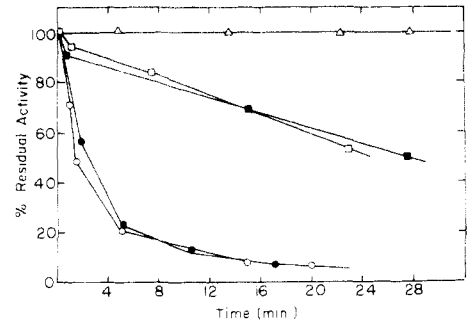

FIGURE 2: Inactivation of galactose-1-P uridylylytransferase by HMB. The reaction mixtures contained 0.66 unit of galactose-1-P uridylyltransferase in 0.23 mL of 0.01 M sodium bicinate buffer at pH 8.5 and the additions indicated below. At the indicated times, aliquots were withdrawn and assayed for enzymatic activity. Symbols: (△—△) no additions (control); (□—□) + 4.45 μM HMB; (■—■) + 4.45 μM HMB and 0.532 mM UDP-glucose; (○—○) + 43.5 μM HMB; (●—●) + 43.5 μM HMB + 0.556 mM UDP-glucose.

the presence of the substrate if the uridylyl-enzyme were a carboxylic phosphoric anhydride, by analogy with $NaBH_4$ reduction of phosphorylated sarcoplasmic reticulum (Ca^{2+}–Mg^{2+}) ATPase (Degani and Boyer, 1973) and phosphorylated acetate kinase (Todhunter and Purich, 1974).

Galactose-1-P uridylyltransferase is very sensitive to inactivation by HMB. The reagent is effective at 5 to 50 μM and its effect is reversed by cysteine. As shown in Figure 2 UDP-glucose at twice its K_m concentration has no effect on the inactivation rate, nor does 15 mM glucose-1-P (Wong, 1974). The enzyme clearly contains at least one sulfhydryl group whose integrity is essential for activity; however, the group detected in Figure 2 cannot be the active site nucleophile. This is because in the presence of twice K_m[UDP-glucose] a substantial fraction of the enzyme exists as uridylyl-enzyme or enzyme·UDP-glucose complex, yet the inactivation rate is the same as in the absence of UDP-glucose. Therefore, the uridylyl-enzyme and enzyme·UDP-glucose complex are as reactive as the free enzyme. Once inactivated by HMB, the enzyme is also inactive as a catalyst for the UDP-glucose–glucose-1-P and UDP-galactose–galactose-1-P exchange reactions (Wong, 1974). In these respects it is analogous to the mutant proteins partially purified from erythrocytes of galactosemic humans (Wu et al., 1974).

In view of the fact that the results of the hydrolytic studies strongly implied that the uridylyl group is bonded to a nitrogen atom in the protein, special efforts were made to determine whether enzyme activity involves the actions of lysine ε-amino or N-terminal amino groups in catalysis. The effects of pyridoxal phosphate and 5-nitrosalicylaldehyde at pH 8.5 were carefully evaluated. Both reagents have been found to be highly selective for amino groups in proteins, the latter as a potent inhibitor of acetoacetate decarboxylase (Frey et al., 1971). The enzyme activity was found to be unaffected by 1 mM pyridoxal phosphate and by 0.5 mM 5-nitrosalicylaldehyde as well as by the addition of $NaBH_4$ in the presence of pyridoxal phosphate. Either of the reagents should have inhibited the enzyme by forming stable, internally H-bonded aldimines with lysine ε-amino or N-terminal amino groups if they were essential for activity. In the unlikely event that dilution of enzyme–aldehyde reaction mixture aliquots into enzyme assay solutions would have reversed aldimine formation quickly enough to have been missed in activity measurements, $NaBH_4$ should have reduced such complexes to the corresponding secondary amines, resulting in irreversible inactivation. This was not detected in repeated experiments. We have found no evidence for the involvement of amino groups in catalysis.

Diethyl pyrocarbonate, DEPC, exhibits a degree of selectivity for the imidazole ring of histidine at pH 6 to produce ^1N- or ^3N-carboethoxyhistidine. It also reacts with certain primary amino groups in some proteins under these conditions, but these can be distinguished from reactions at the imidazole ring by the fact that NH_2OH can remove the carboethoxy group from ^1N- or ^3N-carboethoxyhistidine (Melchior and Fahrney, 1970). The reagent must be used with caution because of complications arising from the use of carboxylate buffers, which appear to react rapidly with the reagent to produce a reactive anhydride which acylates sulfhydryl groups at pH 6 (Garrison and Himes, 1975). Inasmuch as thioesters are deacylated by NH_2OH, the reagent can give a false indication of reaction with imidazole rings of proteins. Moreover DEPC can no doubt also react with carboxylate groups in proteins, which might also be deblocked by NH_2OH. Nevertheless the reagent can be useful as a test for histidine if these complications can be circumvented. In the case of the uridylyl-enzyme, there is no possibility that the active site nucleophile could be a carboxylate group. Cysteine present in our enzyme as a stabilizer would interfere but β-mercaptoethanol can be substituted for it.

Results of activity measurements on the effects of DEPC at pH 6 in phosphate buffer with β-mercaptoethanol as stabilizer indicate the involvement of the imidazole ring of histidine as a catalytic group at the active site in the vicinity of the uridylyl subsite. Data are given in Figures 3 and 4, which show that 1 mM DEPC inactivates the enzyme quickly at pH 6 and 0 °C while the enzyme itself is reasonably stable for 24 h in the absence of DEPC. The enzyme is reactivated to near control levels by 0.28 M NH_2OH at 0 °C, indicative of deblocking histidine. In Figure 4 the results of protection studies show that the enzyme is almost completely protected by 0.5 mM UDP-glucose. Galactose-1-P at 70 mM exerts only a slight protective effect, decreasing the initial rate of inactivation by about half, whereas the uridylyl-enzyme is very substantially protected. Its rate of inactivation is no more than 2% that of the enzyme itself. We conclude that the group modified by DEPC is at the active site and closely associated with the uridylyl subsite. This group is most likely to be the imidazole ring of a histidine residue involved in catalysis of uridylyl transfer, either as the active site nucleophile or as a general acid or base.

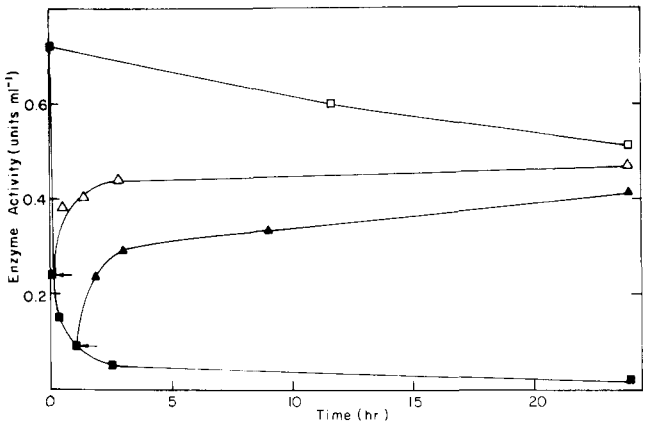

FIGURE 3: Inactivation of galactose-1-P uridylyltransferase by DEPC and reactivation by NH_2OH. The complete reaction mixture contained initially 0.73 unit mL^{-1} of galactose-1-P uridylyltransferase, 5 mM potassium phosphate buffer at pH 6.0, 0.5 mM β-mercaptoethanol, and 1 mM DEPC at 0 °C. Aliquots were removed for activity assays and quenched by dilution at the times corresponding to the data points. At the times indicated by the *arrows*, 0.2-mL aliquots of the reaction mixture were withdrawn, brought to 0.28 M NH_2OH, placed at 0 °C, and assayed for enzyme activity as described above at the times indicated by data points. Symbols: (■—■) complete reaction mixture; (□—□) minus DEPC control; (△—△) NH_2OH added after 5 min; (▲—▲) NH_2OH added after 1 h.

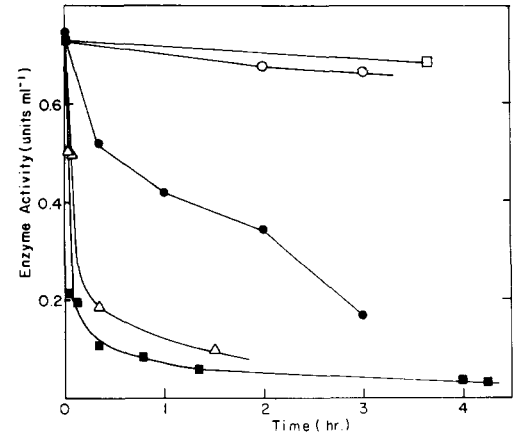

FIGURE 4: Substrate protection against DEPC inactivation. The compositions of the complete reaction mixture and the control and the reaction conditions were the same as in Figure 3. The uridylyl-enzyme was prepared by reacting 8 units of galactose-1-P uridylyltransferase with 10 mM UDP-glucose in 5 mM potassium phosphate buffer at pH 7.5 containing 0.5 mM β-mercaptoethanol at 0 °C for 20 min. The 0.5-mL reaction mixture was then passed through a 1 × 34 cm column of Sephadex G-25 equilibrated and eluted with 5 mM potassium phosphate buffer containing 0.5 mM mercaptoethanol at pH 8.5 and 4 °C. Activity and A_{280} measurements showed that the protein was well separated from UDP-glucose. An aliquot of the best protein containing fractions, containing 0.74 unit of activity, was combined with the pH 6 phosphate buffer for the 1-mL DEPC reaction mixture described in Figure 3, giving a final pH in this case of 6.72. Symbols: (■—■) complete; (□—□) minus DEPC; (○—○) plus 0.5 mM UDP-glucose; (△—△) plus 70 mM galactose-1-P; (●—●) uridylyl-enzyme.

Discussion

All lines of evidence support our contention that eq 1 and 2 reflect the reaction pathway for the galactose-1-P uridylyltransferase reaction as catalyzed by the enzyme purified from *E. coli*. A covalent uridylyl-enzyme can be isolated in both catalytically viable and denatured forms, with a uridylylation stoichiometry that is reasonable on the basis of the molecular weight and subunit composition of the enzyme, and the uridylyl group is removed by galactose-1-P. Uridylylation by one uridylyl donor is inhibited by the presence of the other. The enzyme catalyzes exchange of [^{14}C]glucose between glucose-1-P and UDP-glucose (Wong and Frey, 1974a) and of [^{14}C]galactose between galactose-1-P and UDP-galactose (Wong, 1974). It also catalyzes the exchange of uridylyl groups between UDP-glucose and UDP-galactose (Wong and Frey, 1974a). The steady-state kinetics is ping-pong as are the kinetics of the UDP-glucose–glucose-1-P and UDP-galactose–galactose-1-P exchange reactions (Wong and Frey, 1974b; Wong, 1974).

Quantitative aspects of the kinetics for the UDP-hexose–hexose-1-P exchanges constitute especially strong evidence that this pathway is the major or only pathway followed in the reaction. As pointed out previously, the fact that the maximum UDP-glucose–glucose-1-P exchange rate is larger than the maximum overall reaction rate in the direction of glucose-1-P formation means that the uridylyl-enzyme can distinguish between glucose-1-P and galactose-1-P and that formation of the uridylyl-enyzme cannot be solely rate limiting at acceptor saturation (Wong and Frey, 1974b). Therefore, at acceptor saturation the uridylyl-enzyme reacts more rapidly with glucose-1-P than with galactose-1-P, a direct consequence of which is that the maximum rate for the overall reverse reaction, in which glucose-1-P is the acceptor, must be faster than the maximum rate at which ^{14}C is exchanged from UDP-[U-^{14}C]galactose to galactose-1-P, in which galactose-1-P is the uridylyl acceptor, and this is actually the case (Wong, 1974).

A formal analysis of these relationships among exchange and overall rates, given by Janson and Cleland (1974), shows that eq 3 must be satisfied if the ping-pong pathway is followed.

$$\frac{1}{V_f} + \frac{1}{V_r} = \frac{1}{V_{ex1}} + \frac{1}{V_{ex2}} \qquad (3)$$

In this equation, V_f and V_r refer to the maximum overall rates in the forward and reverse directions and V_{ex1} and V_{ex2} refer to the maximum UDP-glucose–glucose-1-P and UDP-galactose–galactose-1-P exchange rates. The actual data at pH 8.5 and 27 °C are 4.05, 1.47, 12, and 0.88 μmol per min per μg of protein for V_f, V_r, V_{ex1}, and V_{ex2}, respectively, which are in reasonable agreement with eq 3. These quantities are calculated from published rate data (Wong and Frey, 1974b; Wong, 1974) and refer to enzyme whose specific activity is 189 units per mg of protein by the assay method referred to in the Methods section. They may all be somewhat low to the same fractional extent depending upon how closely our most highly active enzyme preparation approached maximum activity, but this would not affect the comparisons being made here.

The proposed mechanism implies that the hexose 1-phosphates bind at the same site. This is consistent with and further supported by the fact that galactose-1-P uridylyltransferase which has been inactivated by HMB is no longer active in catalyzing either of the UDP-hexose–hexose-1-P exchange reactions. If the uridylyl acceptors were to interact at separate bindings sites, any process which blocks uridylyl transfer to and from one of these sites would be sufficient to inactivate the enzyme, which might still catalyze uridylyl transfer to and from the other site. Thus an inactivated enzyme might catalyze one of the exchange reactions but not both. On the other hand if both uridylyl acceptors were to bind at the same site, blocking uridylyl transfer to this site would inactivate the enzyme as a catalyst for the overall reaction and for both exchange reactions as well. The latter is what is found, and it is consistent with the

proposal that the hexose 1-phosphates interact at the same site or at substantially overlapping sites.

The uridylylation stoichiometry shows that there are at least two uridylyl binding sites per dimer of this enzyme. The average value in Table I is 1.7 sites per dimer, and this must be considered to be a minimum value because our most highly purified preparation may not have been fully active. The correct value is probably 2. This is significant because it is a good indication that the subunits are similar or identical. They have been assumed to be identical because of their molecular weights, but more definitive proof of this has been elusive because of the intractibility of this protein in tryptic peptide mapping (Saito et al., 1967).

The hydrolytic properties of the uridylyl-enzyme and the group selective reagent studies described in this paper very strongly imply that the bond linking the uridylyl group to the protein is a phosphoramide and that there is a histidine residue in the close vicinity of this bond. Phosphoramidates are known to be labile to mild acid hydrolysis but relatively stable under alkaline conditions. Moreover, the stability of the nucleotide–protein linkage toward base is inconsistent with the nucleotide being bonded to the C-terminal carboxyl group, the β- or γ-carboxyl groups of aspartate or glutamate, the hydroxyl groups of serine or threonine, or the sulfhydryl group of cysteine. A carboxylic phosphoric anhydride would hydrolyze quickly in base (Phillips and Fife, 1969), and uridylylated serine, threonine, or cysteine could be expected to undergo β elimination in 0.1 M NaOH at 60 °C for 1 h (Perlmann, 1955; Cecil and McPhee, 1959). This leaves the phenolic hydroxyl group of tyrosine, the imidazole ring of histidine, the ϵ-amino group of lysine, the N-terminal amino group, and possibly the guanidino group of arginine as the protein functional groups most likely to be involved in binding the nucleotide. Of this listing, tyrosine can be excluded because the uridylyl-enzyme is sensitive to mildly acidic conditions. A phosphoramidate linkage to a basic amino acid, histidine, the N-terminal amino group, or an as yet undetected, covalently bonded prosthetic group remain as the most likely possibilities.

The results of the group selective reagent studies complement the findings in the hydrolytic studies. Although we find no evidence for the involvement of amino groups in catalysis, there appears to be a histidine in the active site, and it may be the active site nucleophile. It may be argued that, if the uridylyl group is bonded to such a histidine, the uridylyl-enzyme should be insensitive to DEPC, whereas Figure 3 shows that it is inactivated, albeit at a very slow rate. However, the uridylyl-enzyme is not absolutely stable to hydrolysis and may gradually have been decomposing to UMP and free enzyme under the experimental conditions. Moreover, it is not obvious that a uridylylimidazole would be completely unreactive toward DEPC. It may still react as a nucleophile at the unliganded ring nitrogen at a greatly reduced rate.

The properties of this uridylyl-enzyme complicate its detailed characterization. Enzymatic degradations produce insoluble core protein containing the uridylyl moiety. Acid hydrolysis of the protein is incompatible with the acid lability of the bond between the uridylyl moiety and the protein, and the uracil ring does not survive strong alkaline hydrolysis conditions in reasonable yield. Systematic high yield methods for chemically degrading and characterizing this intermediate, and nucleotidyl proteins in general, are currently under investigation in this laboratory.

References

Cecil, R., and McPhee, J. R. (1959), *Adv. Protein Chem. 14,* 255–389.

Degani, C., and Boyer, P. D. (1973), *J. Biol. Chem. 248,* 8222–8226.

Frey, P. A., Kokesh, F. C., and Westheimer, F. H. (1971), *J. Am. Chem. Soc. 93,* 7266–7269.

Garrison, C. K., and Himes, R. H. (1975), *Biochem. Biophys. Res. Commun. 67,* 1251–1255.

Janson, C. A., and Cleland, W. W. (1974), *J. Biol. Chem. 249,* 2567–2571.

Kalckar, H. M. (1960), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 19,* 984–990.

Kalckar, H. M., Braganca, B., and Munch-Petersen, A. (1953), *Nature (London) 172,* 1038.

Kurahashi, K. (1957), *Science 125,* 114–115.

Layne, E. (1957), *Methods Enzymol. 3,* 447–454.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Maxwell, E. S., Kalckar, H. M., and Burton, R. M. (1955), *Biochim. Biophys. Acta 18,* 444–445.

Melchior, W. B., and Fahrney, D. (1970), *Biochemistry 9,* 251–258.

Pazur, J. H., and Shadaksharaswamy, M. (1961), *Biochem. Biophys. Res. Commun. 5,* 130–134.

Perlmann, G. E. (1955), *Adv. Protein Chem. 10,* 1–30.

Phillips, D. R., and Fife, T. H. (1969), *J. Org. Chem. 34,* 2710–2714.

Reed, L. J., and Willms, C. R. (1966), *Methods Enzymol. 9,* 247–265.

Saito, S., Ozutsumi, M., and Kurahashi, K. (1967), *J. Biol. Chem. 242,* 2362–2368.

Todhunter, J. A., and Purich, D. L. (1974), *Biochem. Biophys. Res. Commun. 60,* 273–280.

Wee, T. G., and Frey, P. A. (1973), *J. Biol. Chem. 248,* 33–40.

Wilson, D. B., and Hogness, D. S. (1964), *J. Biol. Chem. 239,* 2469–2481.

Wong, L.-J. (1974), Doctoral Dissertation, The Ohio State University.

Wong, L.-J., and Frey, P. A. (1974a), *J. Biol. Chem. 249,* 2322–2323.

Wong, L.-J., and Frey, P. A. (1974b), *Biochemistry 13,* 3889–3894.

Wu, J. W., Tedesco, T. A., Kallen, R. G., and Mellman, W. J. (1974), *J. Biol. Chem. 249,* 7038–7039.

Zalitis, J., and Feingold, D. S. (1969), *Arch. Biochem. Biophys. 132,* 457–465.