# A Stable Enzyme–Phosphoenolpyruvate Intermediate in the Synthesis of Uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose 3-*O*-Enolpyruvyl Ether†

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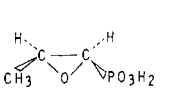
ABSTRACT: A stable enzyme–phosphoenolpyruvate complex can be formed in partially purified preparations of the phosphoenolpyruvate–uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose 3-*O*-enolpyruvyl transferase from *Micrococcus leisodeikticus*, by reaction of phosphoenolpyruvate with the enzyme, and the formation of this complex requires the initial presence of UDP-GlcNAc. A complex with similar properties may be formed by the reverse reaction, upon exposing the enzyme to the reaction products inorganic phosphate and UDP-GlcNAc-EP. When the isolated enzyme–phosphoenolpyruvate complex, prepared by either procedure, is exposed to UDP-GlcNAc, the phosphoenolpyruvate is released both as free phosphoenolpyruvate and as the reaction products, in the ratio of 1 mol of phosphoenolpyruvate per 3.0 mol of either product. The resistance of the enzyme–phosphoenolpyruvate complex to inactivation by *N*-ethylmaleimide, contrasting with the sensitivity of the free enzyme, suggests that phosphoenolpyruvate is bound to an enzymic cysteine residue. The relationship of these results to the irreversible inactivation of the enzyme by the antibiotic fosfomycin is discussed.

The chemical structure of the cell wall which imparts rigidity to bacteria and stabilizes them to their high internal osmotic pressures has been elucidated for a variety of organisms (Osborne, 1969). In each case, the wall is composed of a linear polysaccharide of alternating residues of *N*-acetyl-2-amino-2-deoxyglucose and *N*-acetylmuramic acid, cross-linked by peptide chains which are attached to the lactate portion of the muramic acid residues. The first step in the synthesis of the *N*-acetylmuramyl pentapeptide units has been shown to be the reaction of UDP-GlcNAc[1] and P-enolpyruvate to form uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose 3-enolpyruvyl ether (UDP-GlcNAc-EP) (Strominger, 1958; Gunetileke and Anwar, 1968). The enolpyruvate product of the reaction is subsequently reduced to UDP-*N*-acetylmuramic acid, and the appropriate amino acids are added to the free carboxyl of the molecule.

The enzyme which catalyzes the transfer of the enolpyruvate group to UDP-GlcNAc, P-enolpyruvate–UDP-GlcNAc 3-*O*-enolpyruvyl transferase (pyruvyl transferase), is irreversibly inhibited by the antibiotic fosfomycin[2] (I) (Hendlin *et al.*, 1969). It has also been shown (F. M. Kahan and J. E. Kahan, manuscript in preparation) that this antibiotic is bound covalently to a cysteinyl residue of the enzyme, for after proteolytic digestion of an enzyme preparation to which [$^3$H]-fosfomycin had been bound, the sole $^3$H-containing product was identified as the 2-*S*-cysteinyl-1-hydroxypropylphosphonate (II), by comparison with an authentic sample prepared chemically.



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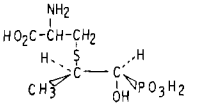

II

Because fosfomycin apparently acts as an analog of P-enolpyruvate in the enzymatic reaction, we were led to believe that P-enolpyruvate itself may bind covalently to the enzyme to form a stable intermediate as part of the natural reaction sequence. In order to help elucidate the mechanism of action of this enzyme, and thus to understand the activity of the antibiotic in more detail, we have investigated conditions for formation of a stable complex of enzyme with phosphoenolpyruvate, and have studied some of the properties of the isolated complex. Our results are presented below.

## Materials and Methods

Unlabeled P-enolpyruvate, ATP, and ADP were purchased from Calbiochem, reduced diphosphopyridine nucleotide from Pabst, and unlabeled UDP-GlcNAc from Boehringer-Mannheim Corp. Deuterium oxide of 99.7 atom % D was obtained from Merck Sharp and Dohme of Canada, and $\gamma$-labeled [$^{32}$P]ATP, [$^{32}$P]orthophosphate, 2-[$^{14}$C]pyruvate, [$^{14}$C]-UDP-GlcNAc, and tritiated water were from New England Nuclear Corp. Pyruvate kinase and lactate dehydrogenase were purchased from Boehringer, bovine serum albumin from Sigma, and Triton X-100 and other liquid scintillation supplies from Packard. Protamine sulfate was from Eli Lilly & Co.

Phosphocellulose P-1 floc was obtained from Reeve-Angel and washed by settling sequentially from 100 vol each of 0.1 M HCl, water, 0.5 M NaOH, and water several more times.

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[1] Abbreviations used are: UDP-GlcNAc, uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose; P-enolpyruvate, phosphoenolpyruvate; UDP-GlcNAc-EP, uridine-5′-diphospho-*N*-acetyl-2-amino-2-deoxyglucose 3-enolpyruvyl ether; ATP, adenosine 5′-triphosphate; NEM, *N*-ethylmaleimide; TME, 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

[2] Previously called phosphonomycin (Hendlin *et al.*, 1969; Christensen *et al.*, 1969).

Potassium phosphate buffer was added to 0.05 M and adjusted to pH 7.2 with KOH and HCl. The final slurry was used to pack the column.

DEAE-cellulose (Bio-Rad Cellex D) was washed with NaOH and HCl as described by Peterson and Sober (1962), and fines removed by several cycles of settling and resuspension in $H_2O$. The appropriate buffer was added before packing into the column.

Sephadex resins were obtained from Pharmacia, and Bio-Gel P-2 from Bio-Rad. These resins were treated and packed as recommended by the manufacturers.

The charcoal used for adsorption experiments was Norit A, obtained from Fisher Scientific Co., and was washed by suspending in 10 vol of distilled water and centrifuging for 30–60 sec at 1000 rpm in an International SBV centrifuge. The centrifugations were repeated 12 times. One volume of water was added to the final pellet to give the standard 50% slurry. This slurry was thoroughly suspended before each use. The adsorptive capacity of the charcoal slurry was tested by adding varying volumes of slurry to 1-ml aliquots of a 1 mM solution of UDP-GlcNAc in 0.05 M Tris, pH 7.5, stirring the solution several times over a period of 5 min at room temperature, and centrifuging. Measurement of the absorbance of the supernatants at 260 nm permitted calculation of the fraction of UDP-GlcNAc adsorbed. With 10 $\mu$l of slurry added, 25% of the UDP-GlcNAc was removed, with 50 $\mu$l added, 83% was removed, and with 100 $\mu$l added, 98.8% was removed. This slurry was used for all charcoal adsorption steps described below.

The preparation of [$^{32}$P]P-enolpyruvate was accomplished by phosphate exchange between $\gamma$-$^{32}$P-labeled ATP and unlabeled P-enolpyruvate, in the presence of pyruvate kinase, as follows. The mixture contained 120 $\mu$mol of Tris-HCl buffer pH 8.2, 10 $\mu$mol of P-enolpyruvate·$Na_3$, 0.10 $\mu$mol of unlabeled ATP, 30 $\mu$mol of $MgCl_2$, 910 $\mu$mol of KCl, and 117 $\mu$mol of sodium pyruvate in a total volume of 5.80 ml. To this solution were added 0.22 $\mu$mol of $\gamma$-$^{32}$P-labeled ATP (3 mCi/$\mu$mol specific activity) and 0.1 ml of a solution of pyruvate kinase containing 7 mg/ml of enzyme of specific activity 110 units/mg (previously dialyzed into a buffer containing 0.02 M Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 0.15 M KCl). The reaction mixture was incubated at 37°, and progress of the reaction was monitored by observing the decrease in charcoal-adsorbable radioactivity. Equilibrium was achieved by 90 min, and the solution was chilled. The pH was reduced to 7.6 by addition of HCl, and 0.45 ml of charcoal slurry was added to remove ATP. After allowing the ATP to adsorb for 15 min at 0° with occasional stirring, the charcoal was removed by centrifugation and the supernatant again treated with charcoal in an identical fashion. The P-enolpyruvate was precipitated by first adding 30 $\mu$mol of KOH to raise the pH, and then adding 0.13 ml of 1 M barium acetate, followed by 13 ml of anhydrous ethanol. The solution was stored at −20° overnight. After centrifugation and removal of the supernatant, the P-enolpyruvate precipitate was metathesized with Dowex 50 ($H^+$), the resin filtered off and rinsed, and the combined filtrate and rinse were neutralized to pH 7.5 with KOH. This solution was concentrated by rotary evaporation to a final volume of 0.5 ml. Final concentration was 15 mM, measured by coupled enzyme assay (Czok and Eckert, 1963). The specific activity was 128,000 cpm/nmol when counted in Triton scintillation fluid.

[$^{14}$C]P-enolpyruvate was prepared by pyruvate kinase catalyzed exchange of phosphate between P-enolypyruvate and 2-[$^{14}$C]pyruvate, as described by Harrison *et al.* (1955). Final specific activity was 2380 cpm/nmol in Triton scintillation fluid.

Radioactivity was measured in a Packard Tri-Carb scintillation counter, Model 3320. Aqueous solutions of no more than 2.5 ml of $^{14}$C, $^{3}$H, and $^{32}$P were counted in 20 ml of Triton scintillation fluid. When less than 2.5 ml of aqueous solution was to be counted, sufficient water was added to the vial to bring the total quantity of water added to 2.5 ml.

Proton magnetic resonance spectra were obtained in $D_2O$ using a Varian HA-100D spectrometer.

For the series of experiments in which the enzyme was labeled from [$^{32}$P]orthophosphate, the $^{32}$P was counted directly in aqueous solution by Cerenkov radiation, without the use of scintillation fluid. The $^{32}$P counts appeared in the lowest ($^{3}$H) channel at approximately 30% efficiency.

Fosfomycin labeled with $^{3}$H in $\alpha$ and $\beta$ positions was prepared by Dr. H. Mertel of the Merck Institute for Therapeutic Research.

For measurements of enzyme specific activities, protein was assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Column eluates were monitored by absorbance at 280 nm.

Pyruvyl transferase activity was assayed by two procedures. For enzyme fractions up to the phosphocellulose eluate, the assay mixture (total volume = 50 $\mu$l) contained 1 mM UDP-GlcNAc, 0.5 mM [$^{14}$C]P-enolpyruvate, and enzyme in TME, and was incubated for 10 min at 37° in 15-ml Corex centrifuge tubes. The reactions were terminated by the addition of 1 ml of cold 5% trichloroacetic acid, and 0.1 ml of standard charcoal slurry was added. After standing at 0° for 15 min with occasional stirring, the solution was centrifuged at 2000 rpm, the charcoal was washed with 0.1 M ammonium acetate, and the radioactivity eluted with ethanol–ammonia as described by Ito and Strominger (1962) for their enzyme assays. Only 60–70% of the radioactive product is recovered from the charcoal, and the quoted activities are corrected for this incomplete recovery.

For the fractions subsequent to the phosphocellulose step, the assay measured release of inorganic phosphate from P-enolpyruvate under conditions similar to those of Gunetileke and Anwar (1968), but using a modification of the phosphate assay of Chen *et al.* (1956). Enzyme reactions were carried out in 0.25-ml aliquots of a solution containing 0.5 mM P-enolpyruvate, 1 mM UDP-GlcNAc, 0.05 M Tris-HCl, pH 7.5, and 10 mM 2-mercaptoethanol. Less than 60 $\mu$g of protein was added to each 0.25-ml assay, which was contained in a 10 × 75 mm plastic tube, and incubation was for 30 min at 37°. Reactions were terminated by the addition of 0.75 ml of cold molybdate–ascorbate reagent, prepared by mixing a solution of ascorbic acid (40 g/l) with 2 vol of ammonium molybdate (5 g/l.) in 1.9 N sulfuric acid. The terminated reactions (total volume = 1 ml) were incubated for exactly 10 min at 50°, and the absorbance at 820 nm was read immediately. Using this procedure, the background from the acid hydrolysis of P-enolpyruvate and UDP-GlcNAc is equal to the release of 4 nmol of phosphate. The apparent specific activity decreases slightly with increasing enzyme concentration in the range from 3 to 40 nmol of released phosphate. The specific activities quoted are based on assays in which 40 nmol of $P_i$ is released. When more than 60 $\mu$g of enzyme is added, the results are erratic due to a high background and adsorption of the color onto precipitated protein. Therefore, the convenient phosphate release assay cannot be used at stages cruder than the phosphocellulose fraction. Values for enzyme activities obtained by this and by the radioactive

assays were essentially identical. One unit of enzyme activity is defined as the quantity of enzyme releasing 0.5 μmol of phosphate in 30 min at 37°.

*Purification of the Enzyme.* Several batches of enzyme were prepared for use in binding experiments and the purification procedure was slightly different for each batch. The procedures employed for purifying the enzyme used in most binding experiments are described below.

All operations were carried out at 0–5° and all centrifugations were at 15,000*g* for 15 min unless otherwise noted.

*Micrococcus lysodeikticus* strain MB-1784 was obtained from Mr. Karl Prescott. The cells had been grown in a medium containing 2% glucose and 1% yeast extract, supplemented with iron, magnesium, and manganese, and buffered at pH 9 with 8.5 g/l. of $NaHCO_3$. Cells were harvested in mid-log phase by passage through a Sharples centrifuge, quick-frozen in trays, and dried in a Hull vacuum chamber. The freeze-dried cells were stored at −20° for 18–36 months prior to use.

The cells (65 g) were suspended in 1.5 l. of a solution containing 0.1 M NaCl–20 mM potassium phosphate buffer at pH 7.4, and 2 mM 2-mercaptoethanol, at 37°. After addition of 300 mg of lysozyme the suspension was incubated at 37° for 20 min, and then 1.4 ml of 1 M $MgCl_2$ and 10 mg of pancreatic DNase were added, and incubation was continued for an additional 5 min. The suspension was then chilled.

Aliquots of this suspension (15 ml each) were sonicated for 30 sec, in 30-ml Corex centrifuge tubes immersed in ice, using a Branson Model LS-75 Sonifier at full power. After sonification was complete, the debris was removed by centrifugation. The crude extracts had a specific activity of *ca.* 0.15 μmol per hr per mg by the [$^{14}$C]P-enolpyruvate assay.

*Protamine Fractionation.* The extract (1400 ml) was diluted with 1 l. of distilled water and 200 ml of a 2% solution of protamine sulfate was added with stirring. After addition was complete, stirring was continued for 5 min, the solution was centrifuged, and the supernatant was discarded. The precipitate was resuspended thoroughly in 300 ml of 1 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, by stirring overnight. The suspension was diluted with an equal volume of distilled water and centrifuged. The supernatant was collected and the pellet was resuspended in 100 ml of 1 M potassium phosphate buffer containing 10 mM 2-mercaptoethanol. This resuspension was diluted with 100 ml of distilled water and centrifuged. This second supernatant was combined with the first to give a final volume of 760 ml.

*Ammonium Sulfate Precipitation.* To the 760 ml of protamine eluate was added 326 ml of saturated ammonium sulfate solution, and the precipitate was centrifuged off and discarded. An additional 1447 ml of saturated ammonium sulfate solution was added to the supernatant, and this solution was centrifuged and the pellet was redissolved in 100 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol to give a final volume of 112 ml.

*Phosphocellulose Column.* The 112 ml of redissolved ammonium sulfate precipitate was diluted to 400 ml by addition of 10 mM 2-mercaptoethanol and passed through a column (5.2 × 15 cm) of phosphocellulose which had been previously equilibrated with 0.05 M potassium phosphate, pH 7.2, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The fractions containing the main peak of absorbance at 280 nm were pooled, giving a final volume of 440 ml. The protein was precipitated by addition of 230 g of solid ammonium sulfate, and after centrifugation the precipitate was redissolved in 40 ml of 0.05 M potassium phosphate, pH 7.5, containing 10 mM 2-mercaptoethanol. The redissolved protein was dialyzed against 2 l. of the same solvent. The volume after dialysis was 75 ml and the absorbance at 280 nm was 15.1, and at 260 nm was 10.6.

A portion of this (25 ml) was reprecipitated with ammonium sulfate and resuspended in 4 ml of the phosphate–mercaptoethanol buffer and dialyzed twice against 2 l. of the same buffer. The final volume after this concentration step was 8 ml, the final absorbance at 280 nm was 40, and the 280/260 absorbance ratio was 1.43. The protein concentration by the procedure of Lowry *et al.* (1951) was found to be 37 mg/ml, and the specific activity was 1.5 units/mg. The low 280/260 absorption ratios could be increased to about 1.6 by treatment with one-fifth volume of charcoal slurry without adsorption of any enzyme activity. Hence, this fraction, which is designated as the *phosphocellulose enzyme*, still contained substantial quantities of 260-nm absorbing material.

*DEAE-Cellulose Column.* The remainder of the phosphocellulose eluate (50 ml of $OD_{280}$ = 15.1) was adsorbed directly onto a column (3.8 cm × 20 cm) of DEAE-cellulose that had been previously equilibrated with TME. The column was washed with 100 ml of TME and was eluted with a linear gradient of 0–0.4 M NaCl in TME, the total gradient volume being 2 l. Protein and enzyme both eluted as broad peaks, with the peak of enzyme activity slightly behind the protein peak. Fractions with specific activity greater than 0.5 unit/mg were pooled, giving a final specific activity of 4.5 units/mg. The 280/260 absorbance ratio was increased to 1.65 by this procedure. The pooled enzyme fractions were concentrated to a final volume of 10 ml by precipitation with solid ammonium sulfate (0.50 g/ml of enzyme) and by redissolving in TME.

*Sephadex G-100 Column.* The concentrated DEAE-cellulose eluate was applied to a column (2.7 cm × 90 cm) of Sephadex G-100 which had been equilibrated with TME, and was eluted with the same buffer. Both protein and enzyme activity eluted in a narrow peak between 170 and 209 ml of effluent, with no purification achieved. All tubes containing enzyme activity were pooled and the enzyme was concentrated by precipitation with solid ammonium sulfate, redissolved in TME, and dialyzed twice against 2 l. of the same buffer. The final volume was 26 ml. The final concentration was 18 mg/ml by the Lowry procedure and the absorbance at 280 nm was 23 and at 260 nm was 14. This fraction is designated the G-100 enzyme and had a specific activity of 4.6 units/mg.

*G-200 Enzyme.* In a separate preparation starting with the same quantity of cells and using the same procedure, the total protein recovered at the phosphocellulose stage was 3800 mg with a specific activity of 0.8 unit/mg. For this preparation, the dialyzed phosphocellulose fraction was adsorbed to a DEAE-cellulose column (4.3 × 35 cm) and eluted with a gradient of 0.10–0.40 M NaCl in TME. The total gradient volume was 14 l. Fractions with specific activity higher than 0.5 unit/mg were pooled. Because of the low protein concentration, the pooled fractions (2 l. of 0.3 mg/ml) were concentrated to 200 ml in an Amicon Model 400 Dia Flo apparatus with a PM30 membrane before concentration by precipitation with ammonium sulfate, followed by dialysis against TME. The specific activity of this DEAE-cellulose fraction was 4.6 units/mg. The 10 ml of concentrated enzyme was chromatographed on a column (2.7 × 80 cm) of Sephadex G-200 as described for the G-100 column. The peak of enzyme activity eluted between the two major peaks of protein on this column. Fractions with specific activity greater than 1.5 units/mg were pooled, concentrated by precipitation with ammonium sulfate, and dialyzed twice against TME. The

final specific activity was 13 units/mg, the final volume was 7.5 ml, and the concentration of protein was 13 mg/ml. The G-200 enzyme preparation lost 65% of its initial activity after storage for 5 months at 0°.

*Preparation of UDP-GlcNAc-EP.* The synthesis of unlabeled as well as deuterium- or tritium-labeled UDP-GlcNAc-EP was carried out in solutions containing 20 mM Tris-HCl buffer, pH 7.5, 5 mM P-enolpyruvate, 5 mM UDP-GlcNAc, 10 mM 2-mercaptoethanol, and pyruvyl transferase at a concentration of 1 unit/ml. EDTA at 2 mM was also added to some reactions. Reactions were performed at 37° and were monitored by measuring the phosphate released. Reaction rates slowed considerably after 2–3 hr and the reactions were terminated by chilling.

After termination, the reaction mixtures were diluted threefold with water and the pH was adjusted to 3 by the addition of HCl. The solutions were then applied to a column (3.8 × 20 cm) of DEAE-cellulose which had been previously equilibrated with 0.001 M HCl. The column was washed with 100 ml of water and eluted with a linear gradient of NaCl from 0 to 0.12 M in 0.001 M HCl, total gradient volume being 4 l. The column was monitored by absorbance at 260 nm. The UDP-GlcNAc-EP chromatographed as the second major peak, containing 50–70% of the starting ultraviolet (uv) absorbance at 260 nm, following an initial peak containing the UDP-GlcNAc. The UDP-GlcNAc-EP peak fractions were pooled and neutralized with NaOH to pH 7.5, diluted fourfold with water, and applied to an identical column of DEAE-cellulose which had been equilibrated with 0.005 M Tris-HCl buffer, pH 7.5. The second column was eluted with a linear gradient of 0–0.15 M NaCl in 0.001 M Tris-HCl buffer, pH 7.5. The total gradient volume was 4 l. The main peak containing UDP-GlcNAc-EP followed a minor unidentified peak. Peak fractions were pooled and concentrated to 5 ml by rotary evaporation under reduced pressure, and applied to a column (3 × 32 cm) of Bio-Gel P-2, 200–400 mesh, and eluted with distilled water. Because of a slight overlap between the salt and product peaks, as measured by conductivity and uv absorbance, only about 80–90% of the product applied to the column was usually pooled as the final product, which contained less than 1 mol of salt/10 mol of product.

*Conditions for Phosphoenolpyruvate Binding.* To 0.5-ml aliquots of G-100 enzyme (18 mg/ml) or G-200 enzyme (13 mg/ml), 10 μl of 0.24 M dithiothreitol was added, and the enzyme was incubated for 20 min at 25°. The enzyme was chilled to 0° and 15 μl of 0.2 M EDTA was added, and, to the appropriate samples, 5 μl of 0.1 M UDP-GlcNAc was added. No additional buffer beyond that in the dialyzed enzyme preparation was included. To each sample in turn was added 30 μl of 15 mM P-enolpyruvate (either $^{14}C$ or $^{32}P$), and, after 60 sec at 0°, 0.25 ml of charcoal suspension, with stirring. Samples were agitated occasionally for 10 min at 0° and were then centrifuged to remove the charcoal, and the supernatants were pipetted into 15-ml Corex centrifuge tubes. The volume of the supernatant was brought to 1 ml by the addition of TME, and the enzyme in the supernatants was precipitated by the addition of 5 ml of saturated ammonium sulfate (pH 8.0, containing 10 mM 2-mercaptoethanol). Tubes were centrifuged at 12,000 rpm for 7 min, the supernatants were pipetted off, and the protein pellet was redissolved in 1 ml of TME. The precipitation and redissolution cycle was repeated an additional six times and the final solutions were counted in Triton scintillation fluid.

*Fosfomycin Binding.* To a 1-ml aliquot of G-100 enzyme, pretreated for 20 min at 25° with 5 mM dithiothreitol as above, was added 50 μl of a 7 mM solution of [$^{3}H$]fosfomycin (specific activity $6 \times 10^{6}$ cpm/μmol). A control aliquot of 0.25 ml was removed, and to the remaining 0.75 ml was added 15 μl of 0.1 M UDP-GlcNAc. The two samples were incubated at 37°. From the samples containing UDP-GlcNAc, 0.25-ml portions were removed and chilled after 10, 20, and 35 min of incubation, and the enzyme in each portion was precipitated by the addition of 0.75 ml of TME, and then 5 ml of saturated ammonium sulfate solution containing mercaptoethanol as described for the P-enolpyruvate binding. The control without UDP-GlcNAc was chilled and precipitated at 35 min of incubation. The precipitated samples were centrifuged and redissolved in Tris buffer and reprecipitated seven times, as above, and the final 1 ml of solution in each case was precipitated with 5 ml of 5% trichloroacetic acid solution and redissolved in 1 ml of 0.05 M ammonia. The 20- and 35-min samples had identical activities, indicating that the enzyme had been saturated with fosfomycin by 20 min.

*Reverse Reaction.* The basic mixture for observing reaction of [$^{32}P$]orthophosphate with UDP-GlcNAc-EP contained 5 mM UDP-GlcNAc-EP, 0.1 M [$^{32}P$]phosphate, 10 mM 2-mercaptoethanol, 0.4 mM EDTA, and 0.05 M Tris-HCl, pH 7.5. In addition, UDP-GlcNAc at 0.5 mM and P-enolpyruvate at 0.1 mM were included in some reactions. To 0.2-ml aliquots of this reaction mixture, 190 μg of phosphocellulose enzyme was added and the reactions were incubated at 37° for 30 min. At the end of this time, the radioactive phosphorus of the [$^{32}P$]P-enolpyruvate formed was transferred to ADP, forming [$^{32}P$]ATP, by adding 0.10 ml of a pyruvate kinase solution containing 0.3 mM ATP, 3 mM ADP, 20 mM $MgCl_2$, 100 mM KCl, 50 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 8 units (70 μg) of pyruvate kinase. After additional incubation for 5 min, the reactions were terminated by the addition of 5 ml of cold 0.1 M perchloric acid, followed by 0.3 ml of charcoal slurry. The terminated reactions were agitated with the charcoal for 5 min at 0°, centrifuged, the supernatant removed, and the charcoal resuspended in 5 ml of 0.2 M NaCl containing 0.02 M Tris-HCl, pH 7.5. The centrifugation and resuspension were repeated five times and the charcoal was finally resuspended in 2.5 ml of 50% ethanol containing 0.05 M ammonia. This final resuspension was agitated several times over 5 min and centrifuged. The supernatants were counted in a Triton scintillator. Controls in which the pyruvate kinase solution was added at the start rather than at the end of the 30-min incubation period gave similar results, indicating that there was little destruction of P-enolpyruvate or ATP by possible contaminating enzymes.

*Binding of [$^{32}P$]Phosphate to the Enzyme.* The reaction mixture contained 1 ml of either phosphocellulose enzyme (37 mg/ml) or G-200 enzyme (13 mg/ml), which had been dialyzed into TME, and which was incubated with 5 mM dithiothreitol at 20° for 20 min immediately prior to addition of the reactants. To 1 ml of either enzyme was added 5 μmol of potassium [$^{32}P$]orthophosphate in 0.2 ml, pH 7.5. The solution was divided into two portions of 0.6 ml each and to one of the portions 10 μl of 0.1 M UDP-GlcNAc-EP was added. Both portions were kept at 0° for 30 min, after which both samples were subjected to eight cycles of precipitation with 5 ml of saturated ammonium sulfate–mercaptoethanol solution, followed by redissolution in 1 ml of TME, as described for the P-enolpyruvate binding experiments. Both reaction mixtures were then dialyzed for 30 hr against 1 l. of 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, and then for an additional 3 days against 1 l. of TME.

*Release of $^{32}P$ and $^{14}C$ Label.* In a typical experiment, 1.0 ml of labeled enzyme previously dialyzed into TME and containing 1.0 nmol of bound $^{14}C$ or $^{32}P$ was brought to 10 mM in P-enolpyruvate and 5 mM in nonradioactive $P_i$, in an Amicon Centri-Flo filter cone at 0°. To this UDP-GlcNAc was added to a final concentration of 2 mM, and, after 1 min at 0°, the filter cone was centrifuged for sufficient time to pass more than two-thirds of the initial volume (10–25 min). To the enzyme remaining in the cone, 1 ml of TME was added and centrifugation repeated. Another 1 ml of TME was added, and after a final centrifugation, the passed solution was collected. The filtered samples were free of pyruvyl transferase and the transferase retained in the filter cone had its full initial enzymatic activity.

*Identification of Released Products.* Procedures for identification of the released label differed for the $^{14}C$- and $^{32}P$-labeled enzyme preparations. For release of label from enzyme labeled with [$^{14}C$]P-enolpyruvate, adsorption of label from the solution onto charcoal was employed to discriminate between P-enolpyruvate and product UDP-GlcNAc-EP, as in the enzyme assay system. In this procedure, the label remaining in the supernatant after treatment with charcoal slurry was assumed to be P-enolpyruvate, and the adsorbable label was assumed to be UDP-GlcNAc-EP. The adsorbed label could be released from the charcoal under the conditions used in the enzyme assay.

The identification of products released from the enzyme labeled with [$^{32}P$]orthophosphate was more detailed. The solution passing through the Amicon Filter Cone was diluted to 10 ml with water and applied to a column (0.5 × 5 cm) of Dowex 1-X2 ($Cl^-$), 200–400 mesh, and the column was washed with several milliliters of water. All of the radioactivity adsorbed to the column. The total radioactivity was eluted with 0.2 M NaCl in 0.01 M Tris buffer, pH 7.5. The eluted radioactivity appeared in two peaks corresponding to the positions of inorganic phosphate and P-enolpyruvate. The radioactivity in the two peaks was verified as being exclusively phosphate and P-enolpyruvate, as follows: to 1 ml of pooled phosphate peak fractions were added 6 ml of water, 2.5 ml of 5% ammonium molybdate, and 0.5 ml of 10 N $H_2SO_4$, to form the isobutyl alcohol soluble phosphomolybdate complex (Berenblum and Chain, 1938). Upon subsequent extraction with isobutyl alcohol, less than 0.2% of the radioactivity remained in the aqueous phase, verifying that essentially all of the radioactivity in that peak was present as inorganic phosphate. Under the same conditions, 7% of the radioactivity in the P-enolpyruvate peak is apparently extracted into the isobutyl alcohol. The radioactivity in the P-enolpyruvate peak was verified as P-enolpyruvate by bringing the pooled P-enolpyruvate fractions to 0.05 M in Tris, pH 7.5, 5 mM in $MgCl_2$, 1 mM in ADP, and 0.04 M in KCl. The resulting solution (11.4 ml) was divided into two equal portions. To one portion 70 μg of pyruvate kinase was added, and the second portion was left untreated as a control. Both portions were allowed to stand at 23° for 40 min and then 0.5 ml of charcoal suspension was added to each sample and the samples were centrifuged. More than 99% of the radioactivity in the pyruvate kinase treated sample was removed by charcoal, whereas less than 5% of the radioactivity of the untreated sample was removed. Hence more than 99% of the $^{32}P$-containing soluble products produced by treatment of the [$^{32}P$]phosphate-labeled enzyme with UDP-GlcNAc was identified as inorganic phosphate and P-enolpyruvate.

*Acid Lability of Enzyme–Phosphoenolpyruvate Complex.* The enzyme–P-enolpyruvate complex generated by reaction of enzyme with UDP-GlcNAc-EP and [$^{32}P$]$P_i$ was diluted with 5 vol of 5 mg/ml of bovine serum albumin in TME. To 1-ml aliquots of the diluted complex were added 4 ml of 0.2 M perchloric acid at 0°. Each tube was held at 0° for 1 min less than the nominal exposure time desired, and was then centrifuged for 3 min at 10,000 rpm, and the supernatant pipetted off. The precipitates were redissolved in 5 ml each of 0.15 M ammonia, and the radioactivity in both supernatant and precipitate was measured.

Within 30 min after each supernatant was obtained, 1.5 ml of water, 2.5 ml of 5% ammonium molybdate, and 0.5 ml of 10 N sulfuric acid were added, and these reactions were left for 5 min at room temperature. The solutions were then shaken with an equal quantity of isobutyl alcohol, and the radioactivity in each phase was measured.

*NEM Inactivation of Enzyme and Enzyme–Phosphoenolpyruvate Complex.* The enzyme and the enzyme–P-enolpyruvate complexes produced by both forward and reverse reactions were tested for susceptibility to inactivation by NEM as follows: enzyme or enzyme–P-enolpyruvate complex in TME was treated with 5 mM dithiothreitol for 20 min at 25° and diluted 100-fold into 0.25-ml aliquots of 0.05 M Tris-HCl buffer, pH 7.5, with or without 1.0 mM UDP-GlcNAc, at 0°. After 1 min at 0°, NEM was added to a final concentration of 1 mM and the solution was kept at 0°. At various times, the reactions were quenched by addition of 2-mercaptoethanol to 10 mM, which rapidly destroys the NEM. Then P-enolpyruvate was added to 1 mM, and UDP-GlcNAc to 1.0 mM in cases where it was not already present. The reactions were then incubated at 37° for 30 min and assayed for phosphate release as usual.

## Results

*Binding of Phosphoenolpyruvate to Enzyme.* In the course of their work on the inactivation of the pyruvyl transferase by fosfomycin, it was observed by Kahan and Kahan (manuscript in preparation) that approximately 20% of the activity of the enzyme was resistant to inactivation by NEM, and that this resistant fraction could be rendered sensitive to NEM by the addition of UDP-GlcNAc to the enzyme preparation. This finding was believed to indicate that 20% of the enzyme, as isolated, contained bound P-enolpyruvate which protected the enzyme from inactivation by NEM, and which was removed (by conversion to product) upon exposure of the enzyme to UDP-GlcNAc. Therefore, we expected that if a stable enzyme–P-enolpyruvate complex would form, it would probably be destroyed in the presence of UDP-GlcNAc. However, no formation of an enzyme–P-enolpyruvate complex could be observed after incubation of enzyme with P-enolpyruvate in the absence of UDP-GlcNAc. This was not unexpected, since binding of fosfomycin likewise does not proceed in the absence of UDP-GlcNAc.

From the foregoing we believed that the preparation of a stable complex of P-enolpyruvate and enzyme would require the incubation of enzyme simultaneously with P-enolpyruvate and UDP-GlcNAc, followed by a rapid selective removal of the UDP-GlcNAc. We therefore sought procedures for selective removal of UDP-GlcNAc from a reaction mixture.

An unambiguous binding was observed when the UDP-GlcNAc was removed from an incubation mixture by the addition of charcoal. Conditions were first found, using [$^{14}C$]UDP-GlcNAc, under which 99.8% of a 1 mM solution of the UDP-GlcNAc could be removed from a solution of concentrated enzyme, while less than 15% of the enzyme was

TABLE I: Binding of Phosphoenolpyruvate and Fosfomycin to Enzyme.

| Compd Bound | nmol Bound per 7 mg of G-100 Enzyme | | |
|---|---|---|---|
| | −UDP-GlcNAc | +UDP-GlcNAc | Difference |
| [$^{14}$C]P-enolpyruvate | 0.049 | 1.25 | 1.20 |
| [$^{32}$P]P-enolpyruvate | 0.088 | 1.09 | 1.00 |
| [$^{3}$H]Fosfomycin | 0.047 | 1.55 | 1.50 |

TABLE II: Reverse Reaction of Phosphate with UDP-GlcNAc EP.

| Reaction Components | nmol of $^{32}$P Recovered[a] |
|---|---|
| Basic mixture[b] | 0.54 |
| Basic + 0.5 mM UDP-GlcNAc | 2.48 |
| Basic + 0.5 mM UDP-GlcNAc + 0.1 mM P-enolpyruvate | 2.44 |
| Basic + 0.5 mM UDP-GlcNAc, without addition of pyruvate kinase solution | 0.56 |
| 0 time blank (no incubation) | 0.36 |

[a] Tests with [$^{14}$C]ATP indicate that recovery of ATP from charcoal is only 50%. Hence apparent recoveries should be multiplied by 2 to give the actual quantity of [$^{32}$P]ATP formed. [b] The basic mixture contained 5 mM UDP-GlcNAc-EP, 100 mM [$^{32}$P]phosphate, 0.4 mM EDTA, and enzyme in TME. For details see Materials and Methods.

removed. We then employed these conditions to remove unlabeled UDP-GlcNAc from concentrated enzyme solutions containing 1 mM UDP-GlcNAc, and P-enolpyruvate labeled with $^{14}$C or $^{32}$P (Materials and Methods). Even after 1 min of incubation at 0°, it was found that removal of UDP-GlcNAc with charcoal left a substantial quantity of $^{14}$C or $^{32}$P bound to enzyme, and this label was not removed from the enzyme either by dialysis or by precipitation with ammonium sulfate. In contrast, similar treatment of a solution containing P-enolpyruvate and enzyme, but no UDP-GlcNAc, resulted in very little binding of label to enzyme (Table I). Thus, binding of P-enolpyruvate to enzyme under these conditions did indeed require the initial presence of UDP-GlcNAc. The quantities of $^{32}$P and $^{14}$C bound were identical within experimental error, and were also close to the quantity of [$^{3}$H]fosfomycin which could be bound to the same quantity of enzyme. The apparent differences in the capacity of the enzyme to bind fosfomycin or P-enolpyruvate could be due to inaccuracies in the experimental procedures, or to nonstoichiometric binding of P-enolpyruvate under these conditions.

*Lack of Binding of UDP-GlcNAc.* In a control to test whether UDP-GlcNAc is bound together with P-enolpyruvate, a set of parallel experiments was performed under the conditions described above using either [$^{14}$C]P-enolpyruvate or [$^{14}$C]UDP-GlcNAc as the labeled compound, with 0.5 ml of G-200 enzyme (13 mg/ml) in each reaction. In this experiment, 1.85 nmol of [$^{14}$C]P-enolpyruvate was bound in the presence of UDP-GlcNAc, less than 0.003 nmol was bound in the absence of UDP-GlcNAc, and less than 0.005 nmol of [$^{14}$C]-UDP-GlcNAc was bound in the presence of nonradioactive P-enolpyruvate. Thus, the isolated complex is free of detectable UDP-GlcNAc.

*Binding by Inactivated Enzyme.* When enzyme is first inactivated by incubation in the presence of fosfomycin, the binding capacity for P-enolpyruvate is essentially eliminated. Thus, when concentrated G-200 enzyme was incubated with 1 mg/ml of disodium fosfomycin and 1 mM UDP-GlcNAc at 37° for 10 min in TME, and then dialyzed against TME to remove the fosfomycin, the residual enzyme activity was found to be about 0.6–1% of the original value, and the specific UDP-GlcNAc-dependent binding of [$^{14}$C]P-enolpyruvate was 0.6% of the initial value when measured under the usual conditions.

*Reverse Reaction.* If the enzyme–P-enolpyruvate complex which could be isolated as described above is a true reaction intermediate, it should be possible to prepare the same intermediate by the reverse enzymatic reaction, in this case between inorganic orthophosphate and the UDP-GlcNAc-EP. Evidence for reverse reaction was obtained by Gunetileke and Anwar (1968), who demonstrated incorporation of label from [$^{32}$P]orthophosphate into P-enolpyruvate in the presence of UDP-GlcNAc. However, their data do not permit calculation of a specific reaction rate. In order to estimate the optimal conditions for labeling by reverse reaction, we first wished to estimate the rate of reverse reaction at convenient substrate concentrations. Results are listed in Table II.

The data in Table II reveal that apparent formation of [$^{32}$P]P-enolpyruvate in the absence of UDP-GlcNAc is no higher than the background noise of the reaction, demonstrating that the presence of UDP-GlcNAc is required for the reverse reaction to proceed at the optimal rate. This is consistent with our observation (below) that bound P-enolpyruvate is released from enzyme only in the presence of UDP-GlcNAc. The rate of reverse reaction in the presence of UDP-GlcNAc under these particular conditions is approximately 3% of the rate of the forward reaction for the same quantity of enzyme. The apparent rate of reverse reaction under these conditions was therefore considered sufficient to produce a noticeable labeling of enzyme with [$^{32}$P]phosphate under attainable conditions. Furthermore, the apparent lack of reaction in the absence of UDP-GlcNAc suggested that removal of UDP-GlcNAc-EP would not be necessary to stabilize the resulting enzyme–P-enolpyruvate complex, since P-enolpyruvate will not be released from the enzyme at an appreciable rate. This proved to be the case.

*Binding of [$^{32}$P]Phosphate to the Enzyme.* The binding of [$^{32}$P]phosphate to the enzyme could be demonstrated after simply exposing the enzyme to [$^{32}$P]$P_i$ in the presence of UDP-GlcNAc-EP, and removing the reactants by repeated precipitation with ammonium sulfate. However, in contrast to the binding of [$^{14}$C]P-enolpyruvate, there was a significant background binding of [$^{32}$P]$P_i$ in the absence of UDP-GlcNAc-EP. After the ammonium sulfate precipitation step in these binding experiments (Materials and Methods), 0.5 ml of phosphocellulose enzyme was found to bind 0.75 nmol of $^{32}$P in the absence of UDP-GlcNAc-EP, and 1.68 nmol in its presence. After several days of dialysis the binding without UDP-GlcNAc-EP had been reduced to 0.28 nmol and with UDP-GlcNAc-EP to 1.28 nmol. The difference in

TABLE III: Release of Products from the Enzyme–Phosphoenolpyruvate Complex.

| Enzyme Labeled with | % of Total Label Released | Obsd Distribution of Products (% of Total Released Label) | | Calcd Distribution of Products[a] | |
|---|---|---|---|---|---|
| | | P-enolpyruvate | $P_i$ or UDP-GlcNAc-EP | P-enolpyruvate | $P_i$ or UDP-GlcNAc-EP |
| [$^{14}$C]P-enolpyruvate | 81 | 23 | 77 | 25 | 75 |
| [$^{32}$P]Phosphate[b] | 80 | 21 | 79 | 31 | 69 |

[a] Corrected for conversion of P-enolpyruvate to products by enzyme. [b] Average of two different preparations.

incorporation attributable to the UDP-GlcNAc-EP remained constant at about 1.0 nmol. This would correspond to 1.16 nmol bound to the quantity of enzyme activity used for the P-enolpyruvate binding experiments (Table I). Thus, the presence of a UDP sugar causes the binding of an equal quantity of either P-enolpyruvate or $P_i$ to a given quantity of enzyme activity.

The background binding of [$^{32}$P]$P_i$ in the absence of UDP-GlcNAc-EP is probably unrelated to the excess binding observed in its presence because the background label is not released in the presence of UDP-GlcNAc, whereas the additional 1.0 nmol bound in the presence of UDP-GlcNAc-EP is released. Furthermore, when [$^{32}$P]$P_i$ is bound to the more purified G-200 enzyme under the same conditions as to the phosphocellulose enzyme, the background binding to G-200 enzyme after dialysis is 13% of the total rather than the 22% observed for phosphocellulose enzyme. It thus appears likely that enzyme(s) other than the pyruvyl transferase are responsible for the [$^{32}$P]$P_i$ binding in the absence of UDP-GlcNAc-EP.

*Release of Bound Label by Addition of UDP-GlcNAc.* One of the criteria for identification of a true enzyme–substrate intermediate is that it must proceed to form product upon addition of a second reactant (if any). We found that the radioactivity in the enzyme–P-enolpyruvate complexes, whether formed by forward or reverse reactions, can be recovered both in free P-enolpyruvate and in the products, UDP-GlcNAc-EP and inorganic phosphate. An additional criterion requires that the rates of these partial reactions be at least as rapid as the overall reaction; however, we have not examined the kinetics of these reactions.

The release of label from the enzyme could be effected by simply adding UDP-GlcNAc to the labeled enzyme. However, in order to discriminate between released P-enolpyruvate and released products, it was necessary to add unlabeled P-enolpyruvate to dilute any P-enolpyruvate released. Otherwise, in the presence of high concentrations of UDP-GlcNAc and of enzyme, any traces of P-enolpyruvate which might be released from the enzyme upon first exposure to UDP-GlcNAc would subsequently be rapidly converted to products. Because a portion of the excess P-enolpyruvate was nevertheless converted to products, the observed values of P-enolpyruvate release had to be corrected to obtain the actual values, after finding the concentration of unreacted P-enolpyruvate by enzymatic assay (Czok and Eckert, 1963). The ratio of products released to P-enolpyruvate released was about 3.0 (Table III).

*Resistance of Enzyme–Phosphoenolpyruvate Complex to NEM Inactivation.* Exposure of free enzyme or enzyme–P-enolpyruvate complex to 1 mM NEM at 0°, with or without added UDP-GlcNAc, causes the enzyme activity to drop to a lower value within 2 min, and this lower value then remains constant for at least an additional 30 min. Increasing the NEM concentration does not change the value of the residual activity.

When freshly isolated phosphocellulose enzyme is treated with NEM in the absence of UDP-GlcNAc, the residual activity is 20%. If UDP-GlcNAc is included in the reaction, or if the enzyme is first exposed to UDP-GlcNAc and then dialyzed to remove the UDP-GlcNAc, in either case subsequent treatment with NEM now leaves only 0.5–5% of the activity intact.

In a similar fashion, when enzyme–P-enolpyruvate complex is exposed to NEM in the absence of UDP-GlcNAc, 65–95% of the activity remains after 30 min, whereas in the presence of UDP-GlcNAc, only 4–11% remains. The degree of protection of the activity by bound P-enolpyruvate varies somewhat with the preparation; there is 75–95% residual activity when the complex is prepared from $P_i$ and UDP-GlcNAc-EP, or 65% when prepared from P-enolpyruvate and UDP-GlcNAc. Since the residual activity does not decrease perceptibly with time, the incomplete protection is probably due to incomplete saturation of enzyme binding sites by P-enolpyruvate. Incomplete binding is also suggested by the data of Table I.

From these results it appears that the bound P-enolpyruvate confers essentially complete resistance to NEM inactivation, and most of the residual activity of freshly isolated enzyme treated in the absence of UDP-GlcNAc is probably due to bound P-enolpyruvate present on a portion (10–20%) of the enzyme when isolated.

The small variable quantities (0.5–11%) of enzyme activity which resist NEM inactivation in the presence of UDP-GlcNAc may be caused by the formation of a protective disulfide form of the active SH group of the enzyme, at the low thiol concentrations present during NEM treatment. Without dithiothreitol preincubation, this residual activity can be larger than 50% for enzyme stored for several months.

*Acid Lability of Bound Phosphoenolpyruvate.* When the enzyme–P-enolpyruvate complex generated by reaction of enzyme with UDP-GlcNAc-EP and [$^{32}$P]$P_i$ is exposed to 0.16 N acid at 0°, the $^{32}$P is released over a period of time and at each time point essentially all of the released radioactivity is present as $P_i$ (Table IV). In this particular preparation, the background $P_i$ binding which is not dependent on UDP-GlcNAc-EP was 13% of the total, and 43% of this background was released after 80 min at 0° in 0.16 N acid.

In another experiment, samples of G-200 enzyme labeled with [$^{14}$C]P-enolpyruvate or [$^{32}$P]$P_i$ were maintained separately without added bovine serum albumin in 0.24 M perchloric acid, for 100 min at 0°. After centrifugation, 19% of the $^{14}$C

TABLE IV: Acid Lability of Bound [$^{32}$P]Phosphate.

| Time at 0° (min) | % Label in Supernatant | % of Released Label Extracted into Isobutyl Alcohol[a, b] |
|---|---|---|
| 5 | 20 | 97.6 |
| 10 | 33 | 100.0 |
| 20 | 47 | 99.8 |
| 40 | 61 | 99.3 |
| 80 | 80 | 100.0 |

[a] After treatment with molybdate–$H_2SO_4$ (Materials and Methods). [b] Calculated as 100 − (% of total label remaining in aqueous phase).

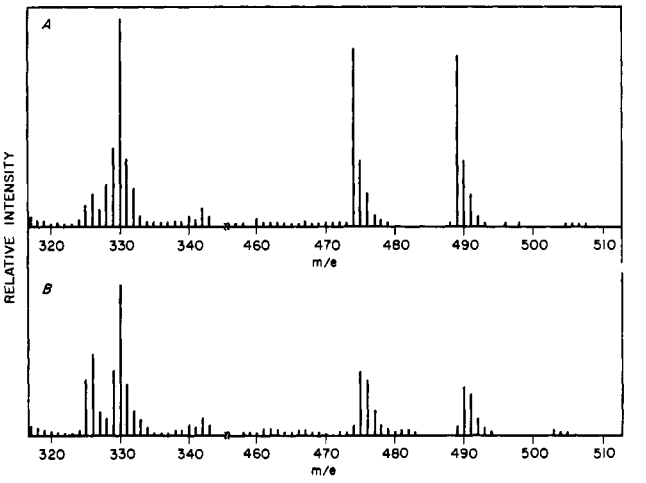


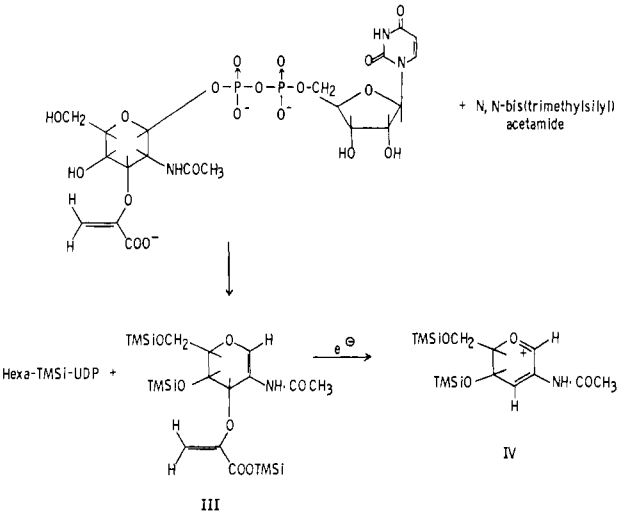

FIGURE 1: Mass spectra of deuterated and undeuterated UDP-GlcNAc-EP. For interpretation, see text.

and 5% of the $^{32}$P were retained in the precipitates. Thus the carbon portion of the P-enolpyruvate is released significantly more slowly than is the phosphorus. However, an accurate ratio of labilities cannot be calculated from this experiment because of the uncertain contribution of the background $^{32}$P binding to the residual $^{32}$P found in the precipitate.

*Position of Attachment of Phosphoenolpyruvate; Deuterium and Tritium Incorporation into Product.* We interpreted the foregoing data as evidence for covalent attachment of P-enolpyruvate to an enzymic cysteine. By analogy with the formation of the cysteine–fosfomycin adduct II, we presume that the P-enolpyruvate binds by covalent addition of cysteinyl sulfhydryl across the P-enolpyruvate double bond. However, sulfhydryl can add across the double bond of P-enolpyruvate in two orientations, with the sulfur atom attached to either C-2 or C-3 of P-enolpyruvate, and we wished to discriminate between these two possibilities. If the sulfur atom adds to position 2, the added proton should randomize with the protons already on C-3, and a portion of the protons added to C-3 would be expected to remain when the product enolpyruvate leaves the active site. Thus, if covalent addition of the sulfhydryl group does occur, with the sulfur added at C-2 of P-enolpyruvate, one would expect incorporation of deuterium or tritium from water into the product UDP-GlcNAc-EP. On the other hand, addition of sulfhydryl with sulfur attached to C-3 would leave no hydrogen from water in the final product.

When the UDP-GlcNAc-EP was prepared in deuterated or tritiated water, deuterium and tritium incorporation could indeed be observed. Measurements of the $^{3}$H content of the purified product showed that each molecule of product had incorporated 0.21 atom of tritium. This less than stoichiometric amount of tritium incorporation was due to a kinetic isotope effect, as could be demonstrated by preparing UDP-GlcNAc-EP in water of greater than 95 atom % deuterium, under similar conditions, and measuring the D content of the purified product by mass spectroscopy.

Mass spectrographic analysis on products prepared in deuterated and undeuterated water (Materials and Methods) was performed by Dr. George Albers-Schonberg of the Merck Institute for Therapeutic Research, with the following results. Trimethylsilylation of the ammonium salt of the UDP-GlcNAc-EP results in fragmentation of the molecule to hexatrimethylsilyluridine diphosphate and compound III, which gives a molecular ion cluster at *m/e* 489–493 (Figure 1A). The relative intensities of the peaks in this ion cluster are in the ratios expected from the natural abundances of the isotopes of carbon and silicon. The corresponding cluster for the deuterated product (Figure 1B) has a maximum peak at *m/e* 490, and an analysis of the ratios of the isotopic peaks demonstrates a content of 11% undeuterated, 60% monodeuterated, and 29% doubly deuterated product. The absence of a deuterium shift in the peak at *m/e* 330, corresponding to compound IV (lacking the enolpyruvate residue), localizes the incorporated deuterium on the vinyl atom of the enolpyruvate portion of the molecule.




Proton magnetic resonance spectra of deuterated and undeuterated products in $D_2O$, obtained by Dr. Byron Arison of the Merck Institute, demonstrate that the deuterated product has only 40 ± 10% of the protons initially present on the 3 position of the enolpyruvate group, with deuterium substitution occurring equally on both vinyl positions.

Mass spectrographic analysis of the residual P-enolpyruvate isolated from the reaction in $D_2O$ shows 1.6 atom of deuterium incorporated per molecule of P-enolpyruvate.

## Discussion

*Identity of the Binding Enzyme.* The enzyme preparations used in this study were relatively crude. We have roughly estimated the molecular weight of the enzyme as 100,000 from

its position of elution on Sephadex G-200. If there is only one P-enolpyruvate binding site per enzyme molecule, then only 1.6% of the protein in the G-100 enzyme fraction is the pyruvyl transferase. It is therefore useful at this point to summarize our reasons for believing that essentially all of the bound P-enolpyruvate is attached to the pyruvyl transferase.

The primary control is the lack of P-enolpyruvate binding in the absence of UDP-GlcNAc, and the release of bound P-enolpyruvate upon exposure of the enzyme–P-enolpyruvate complex to UDP-GlcNAc. The binding enzyme must have recognition sites for both P-enolpyruvate and UDP-GlcNAc. Only one such enzyme is currently known.

In addition, P-enolpyruvate binding can also be achieved by exposing the enzyme preparation to UDP-GlcNAc-EP and $P_i$. A small amount of $P_i$ which binds in the absence of UDP-GlcNAc-EP (13–22% of the total) is probably unrelated to the P-enolpyruvate binding because this background binding component cannot be released by exposure to UDP-GlcNAc. The excess [$^{32}$P]$P_i$ bound in the presence of UDP-GlcNAc-EP appears to be bound in the same chemical form as P-enolpyruvate is bound, because upon exposure to UDP-GlcNAc, 31% of the $^{32}$P is released as P-enolpyruvate, essentially the same as with P-enolpyruvate bound in the presence of UDP-GlcNAc. Most significantly, the binding enzyme is producing P-enolpyruvate from UDP-GlcNAc-EP and $P_i$, and this net reaction is by definition one of the activities of the P-enolpyruvate–UDP-GlcNAc enolpyruvyl transferase.

Furthermore, when P-enolpyruvate is bound to the enzyme preparation, the transferase is protected from inactivation by NEM, and removal of the P-enolpyruvate by exposure to UDP-GlcNAc restores the NEM sensitivity. Thus, most of the pyruvyl transferase is in some way modified by the bound P-enolpyruvate.

Finally, enzyme inactivated by fosfomycin loses its capacity to bind P-enolpyruvate even after removal of free fosfomycin by dialysis. Fosfomycin irreversibly inactivates the P-enolpyruvate–UDP-GlcNAc pyruvyl transferase, but has either no detectable activity or a weak competitive inhibition on other P-enolpyruvate-utilizing enzymes examined, namely enolase, pyruvate kinase, P-enolpyruvate carboxykinase, and P-enolpyruvate–shikimate-5-phosphate enolpyruvyl transferase (Kahan, F., Kahan, J., and Cassidy, P., unpublished observations). This irreversible loss of P-enolpyruvate-binding capacity after fosfomycin treatment implicates the P-enolpyruvate–UDP-GlcNAc pyruvyl transferase as the binding species.

For these reasons we considered it unnecessary to completely purify the enzyme in order to establish the identity of the main binding species. We cannot rule out that a small fraction of the UDP-GlcNAc releasable P-enolpyruvate may be bound to some other enzyme.

*Nature of the Enzyme–Phosphoenolpyruvate Bond.* The bond between enzyme and P-enolpyruvate is sufficiently tight to survive multiple precipitations with ammonium sulfate and extensive dialysis. Nevertheless, this does not by itself prove that the bond is covalent, since we may be observing a very tightly bound or a "trapped" molecule. The P-enolpyruvate might, for example, enter or leave a cavity in the enzyme which is open only in the presence of UDP-GlcNAc.

The most direct evidence which we have obtained for a covalent bond is the acid lability of the bound phosphate. The [$^{32}$P]phosphate bound in the presence of UDP-GlcNAc-EP may be released upon exposure to UDP-GlcNAc, and 31% of the released label is found in P-enolpyruvate. However, in dilute acid, all of the $^{32}$P released is found to be inorganic phosphate. Since less than 3% of free P-enolpyruvate is hydrolyzed to phosphate under these conditions, the $^{32}$P cannot be bound as intact P-enolpyruvate.[3] The $^{14}$C of bound P-enolpyruvate is also released by acid treatment, but somewhat more slowly. The explanation most consistent with our other results is that the P-enolpyruvate is covalently bound and the complex is first decomposed in acid to free phosphate and a bound carbon fragment, and the carbon fragment is also subsequently released. The elements of P-enolpyruvate might also be bound separately as phosphate and a carbon fragment.

The resistance of the enzyme–P-enolpyruvate complex to inactivation by NEM, and the incorporation of deuterium and tritium into the enol position of the product, are consistent with our postulate of a covalent bond to an enzymic cysteine. Although there are alternative possible explanations for these observations, they tend to support the covalent binding interpretation.

*Implications of the Enzyme–Phosphoenolpyruvate Complex for the Mechanism of the Pyruvyl Transferase.* The type of reaction catalyzed by the P-enolpyruvate–UDP-GlcNAc enolpyruvyl transferase is unusual among enzymatic reactions involving P-enolpyruvate, which may be classified into three general types according to the formal chemical reaction which the enzyme catalyzes: (1) the hydration of the double bond (enolase); (2) a larger class in which addition of a positively charged atom (proton or carbonyl carbon) to C-3 of P-enolpyruvate is coupled to the transfer of phosphate to a nucleophile (*e.g.*, $H_2O$, ADP, hexose); and (3) the displacement of phosphate by a nucleophile at C-2 of P-enolpyruvate with retention of the double bond.

The reaction catalyzed by P-enolpyruvate–UDP-GlcNAc pyruvyl transferase falls into the third category, which also includes one other known example, the transfer of the enolpyruvate group to the 3 position of shikimate-5-phosphate (Levin and Sprinson, 1964). A related reaction might also be involved in the generation of a postulated enzyme–P-enolpyruvate intermediate in the DAHP synthetase reaction (DeLeo and Sprinson, 1968), although the interpretation of these data is still uncertain (Floss *et al.*, 1972). The formal reactions involved in these cases can be written as a nucleophilic displacement at C-2 of P-enolpyruvate, but the actual mechanism is more likely on thermodynamic grounds to involve an addition–elimination sequence. One example of such a sequence is that proposed for the P-enolpyruvate–shikimate-5-phosphate enolpyruvyl transferase by Levin and Sprinson (1964).

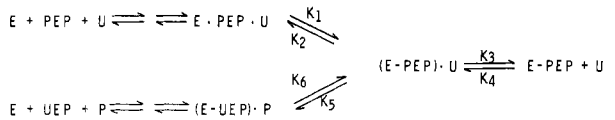
In view of our evidence for a stable enzyme–P-enolpyruvate intermediate in the synthesis of UDP-GlcNAc-EP, we believe that the most likely mechanism for the enzyme would involve the covalent attachment of P-enolpyruvate to enzyme by addition of enzymic sulfhydryl across the P-enolpyruvate double bond, with the sulfur attached to position 2 of P-enolpyruvate. Subsequent stages in the reaction sequence would presumably involve either a direct displacement of phosphate by the 3-hydroxyl of UDP-GlcNAc, followed by elimination of enzymic sulfhydryl, or alternatively, an elimination of phosphate to re-form the double bond followed by addition of UDP-GlcNAc hydroxyl and then elimination of sulfhydryl. Our data do not permit a clear discrimination between these two possibilities, but if phosphate is released before addition

[3] A reviewer has pointed out that a ketal phosphate of the kind envisaged would be expected to be hydrolyzed much more readily than free P-enolpyruvate by dilute acid.

of UDP-GlcNAc to the enolpyruvate residue, it must be released only in the presence of UDP-GlcNAc, since dialysis for 24 hr against phosphate buffer causes no change in the UDP-GlcNAc releasable [$^{32}$P]$P_i$ bound to the enzyme.

The data also do not strictly rule out certain other possible mechanisms, such as the formation of an adduct of P-enolpyruvate and UDP-GlcNAc followed by elimination of phosphate, by analogy with the mechanism suggested by Levin and Sprinson (1964) for the P-enolpyruvate shikimate-5-phosphate 3-enolpyruvyl transferase; however, such a mechanism would probably not give rise to an enzyme–P-enolpyruvate intermediate and the deuterium incorporation results of Bondinell *et al.* (1971) are adequately explained by a mechanism similar to that suggested here.

If our interpretation of the P-enolpyruvate binding observations is correct, the reaction steps involving the intermediate would be written




where U, UEP, and PEP are UDP-GlcNAc, UDP-GlcNAc-EP, and P-enolpyruvate, respectively; a dash indicates covalent attachment and a dot, noncovalent attachment to enzyme.

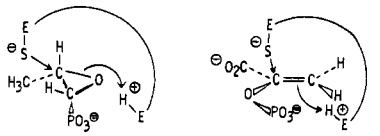
In this scheme, the fact that little reverse reaction is observed in the absence of added UDP-GlcNAc would indicate that $K_3$ is substantially larger than $K_2$. In this situation, one would expect the reverse reaction of UDP-GlcNAc-EP and $P_i$, in the absence of UDP-GlcNAc, to start out very slowly and accelerate in an autocatalytic fashion until the concentration of UDP-GlcNAc generated by the reaction approaches its $K_m$ for binding to the enzyme–P-enolpyruvate complex. Under the conditions of our reverse reaction experiments, this apparently takes more than 30 min.

This interpretation is also consistent with our observation that most of the enzyme may be isolated as an enzyme–P-enolpyruvate complex upon charcoal treatment of the reaction mixture. If the predominant enzyme species in the forward reaction, in the presence of 1 mM UDP-GlcNAc, is the (E-PEP)·U, then rapid removal of UDP-GlcNAc should leave E-PEP as the predominant species if $K_3$ is significantly larger than $K_2$ and $K_5$.

*Related Enzyme–Substrate Complexes.* In a recent survey of the literature on covalent enzyme–substrate complexes, Bell and Koshland (1971) found no direct evidence reported either for the binding of a substrate containing a double bond by addition of an enzyme residue to the double bond, nor for the covalent binding of P-enolpyruvate in any fashion. Covalent intermediates of one or the other type have been proposed (DeLeo and Sprinson, 1968; Nagano and Zalkin, 1970; Kalman, 1971), but none have as yet been isolated.

Our deuterium and tritium incorporation results are similar to those reported recently by Bondinell *et al.* (1971) for hydrogen isotope incorporation into shikimate-5-phosphate 3-enolpyruvyl ether and P-enolpyruvate in deuterated or tritiated water. The enzyme catalyzing the reaction, the P-enolpyruvate–shikimate-5-phosphate 3-enolpyruvyl transferase, is related to the UDP-GlcNAc pyruvyl transferase, in that these two enzymes are the only known examples of a class of enzymes catalyzing the transfer of the enol pyruvate portion of P-enolpyruvate to an acceptor alcohol. It is possible that the reaction mechanisms of the two enzymes are identical.

*Steric Relationship of Phosphoenolpyruvate to Fosfomycin.* Our evidence implicating a sulfhydryl addition–elimination sequence as part of the normal reaction mechanism of the pyruvyl transferase suggests a specific relationship between P-enolpyruvate and fosfomycin, as they appear on the active site of the enzyme. The attack of an enzymic cysteinyl sulfur at C-2 of fosfomycin can be viewed as an addition of sulfhydryl group across the $C_2$–O bond, in analogy with our proposed addition of sulfhydryl across the C=C bond of P-enolpyruvate in the physiological reaction. This would require that the C-2 atoms of each compound be equivalent and that the C=C bond of P-enolpyruvate be analogous to the C–O bond of fosfomycin. Thus, the relationship between the two compounds at the active site would be visualized schematically as depicted below.



The lack of exact isostery of the two compounds as written is also apparent in three-dimensional models, and may indicate that the structure of P-enolpyruvate is substantially distorted in the transition state to approach a structure closer to that of fosfomycin.

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References

Bell, R. M., and Koshland, D. E., Jr. (1971), *Science 172*, 1253.
Berenblum, I., and Chain, E. (1938), *Biochem. J. 32*, 295.
Bondinell, W. E., Vnek, J., Knowles, P. F., Sprecher, M., and Sprinson, D. B. (1971), *J. Biol. Chem. 246*, 6191.
Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1754.
Christensen, B. G., Leanza, W. J., Beattie, T. R., Patchett, A. A., Arison, B. H., Ormond, R. E., Kuehl, F. A., Jr., Albers-Schonberg, G., and Jardetzky, O. (1969), *Science 166*, 123.
Czok, R., and Eckert, L. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. V., Ed., New York, N. Y., Academic Press, p 229.
DeLeo, A. B., and Sprinson, D. B. (1968), *Biochem. Biophys. Res. Commun. 32*, 873.
Floss, H. G., Onderka, D. K., and Carrol, M. (1972), *J. Biol. Chem. 247*, 736.
Gunetileke, K. G., and Anwar, R. A. (1968), *J. Biol. Chem. 243*, 5770.
Harrison, W. H., Boyer, P. D., and Falcone, A. B. (1955), *J. Biol. Chem. 215*, 303.
Hendlin, D., Stapley, E. O., Jackson, M., Wallick, H., Miller, A. K., Wolf, F. J., Miller, T. W., Chaiet, L., Kahan, F. M., Foltz, E. L., Woodruff, H. B., Mata, J. M., Hernandez, S., and Mochales, S. (1969), *Science 166*, 122.
Ito, E., and Strominger, J. L. (1962), *J. Biol. Chem. 237*, 2689.
Kalman, T. I. (1971), *Biochemistry 10*, 2567.
Levin, J. G., and Sprinson, D. B. (1964), *J. Biol. Chem. 239*, 1142.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Nagano, H., and Zalkin, H. (1970), *Arch. Biochem. Biophys. 138*, 58.
Osborne, M. J. (1969), *Annu. Rev. Biochem. 38*, 501.
Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol 5*, 3.
Strominger, J. L. (1958), *Biochim. Biophys. Acta 30*, 645.

# Biochemical Variability of Human Erythrocyte Membrane Preparations, as Demonstrated by Sodium–Potassium–Magnesium and Calcium Adenosine Triphosphatase Activities†

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ABSTRACT: Membranes have been prepared from human erythrocytes, by hypotonic and isotonic hemolysis. These preparations show considerable variability in (enzymatic) behavior as compared to each other and to the parent cell. The $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPases have proven satisfactory as monitors for these changes and in illustrating that hemoglobin-free membranes have biochemical properties quite different from those of the parent cell membrane. Hemoglobin-free membranes prepared at pH 7.6 in 20 mosm Tris buffer allow easy access of ATP to the $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ stimulated ATPases. This is contrary to the behavior of intact erythrocytes which show little such activity unless the cells are ruptured, for example, by freezing and thawing. On the other hand, membranes preprared by hypotonic hemolysis at pH 5.8 showed minimal ATPase activities toward extracellular ATP and a significant retention of hemoglobin, Freeze-thaw treatment of the pH 5.8 membranes, however allowed expression of these enzymatic activities. The degree of availability of ATPases was found to be influenced by the composition of the hemolysis buffer, its osmolarity, and pH. No alterations in the availability of acetylcholine esterase in the pH 7.6 or 5.8 membranes were noted. Divalent cations, such as $Ca^{2+}$ and $Mg^{2+}$, had a definitive effect on membranes prepared in hypotonic buffer in that retention of hemoglobin was promoted and the availability of ATPase activities was reduced. A particularly provocative observation was that incubation of human erythrocytes at 44° in isotonic Tris buffer, pH 7.6, led to a time-dependent hemolysis with release of >95% of the hemoglobin in 2 hr. These hemoglobin-depleted membranes developed ATPase sites accessible to extracellular ATP. However, at shorter periods of incubation, significant quantities of hemoglobin could be removed without development or expression of the ATPase activities. It was observed that a 60–75-min incubation at 37° would allow release of nearly 90% of the hemoglobin, with only a slight increase in accessible $Ca^{2+}$-stimulated ATPase. Other buffers such as Hepes, Mes, histidine–imidazole, or $NaHCO_3$, at isotonic levels at pH 7.6, did not cause hemolysis or allow expression of ATPase activities on incubation with human erythrocytes at 37 or 44°. On the other hand, hemolysis of intact erythrocytes in 60 and 100 mosm Tris buffer, pH 7.6 at 4°, yielded membranes with low levels of hemoglobin, *i.e.*, 4 and 20% mean corpuscular hemoglobin, respectively, with low $Na^+$–$K^+$–$Mg^{2+}$ and $Ca^{2+}$ ATPase activites. These experimental findings provide possible routes to further study of some of the molecular changes occurring in the erythrocyte membrane during preparation by hypotonic and isotonic hemolysis. A possible role of hemoglobin in providing structural and functional integrity to the erythrocyte membrane is provocative, yet under these experimental conditions it may serve merely as an indicator of the state of the membrane at any one time.

The biological and biochemical characterization of the plasma membrane of mammalian cells has become the subject of intense interest in many laboratories today. Among the choices of cells available to investigators probably the one most widely used for membrane preparation has been the mammalian erythrocyte. This has not occurred by sheer experimental accident, but has developed due to the fact that this cell has no detectable subcellular organelles and provides, by using a very simple osmotic lysis procedure (Dodge *et al.*, 1963; Weed *et al.*, 1963), a structure considered by many investigators as representative or at least reminiscent of the plasma membrane of the intact cell.

In an earlier study in this laboratory on possible changes in the level of certain enzymes and proteins in membranes obtained by hypotonic hemolysis of human erythrocytes (Mitchell *et al.*, 1965) it was evident that depending on the conditions, *e.g.*, osmolarity and pH, varying quantities of these components could be found in the isolated membranes. Further, as had been suggested by these studies, and more recently emphasized by further experimental evidence (Bramley *et al.*, 1971; Hanahan and Ekholm, 1972), there are very definitive and important biochemical changes occurring during preparation of these membranes. As proposed in the latter study (Hanahan and Ekholm, 1972), the membranes obtained by osmotic lysis must be considered at best as a derivative of the intact erythrocyte membrane. Zwaal *et al.* (1971) and Laster *et al.* (1972) using phospholipase C and Woodward

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