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Stereochemical Courses of Nucleotidyltransferase and Phosphotransferase Action. Uridine Diphosphate Glucose Pyrophosphorylase, Galactose-1-phosphate Uridylyltransferase, Adenylate Kinase, and Nucleoside Diphosphate Kinase†

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ABSTRACT: Reactions catalyzed by UDP-glucose pyrophosphorylase, galactose-1-P uridylyltransferase, and nucleoside diphosphate kinase can be analyzed for their stereochemical courses, by using nuclear magnetic resonance and mass spectroscopic techniques with either sulfur or sulfur and ^{18}O-substituted nucleotides as substrates. The UDP-glucose pyrophosphorylase reaction proceeds with inversion of configuration at P_α of uridine-5′-(1-thiodiphospho)glucose, and the galactose-1-P uridylyltransferase reaction proceeds with retention. Therefore, the bond cleavage/formation mechanisms for these chemically matched reactions cannot be identical. The simplest explanation for the stereochemical results is that the former reaction involves a single displacement at P_α and the latter involves a double-displacement mechanism. Thiophosphoryl group transfer by nucleoside diphosphate kinase acting on the [^{18}O]phosphorothioate substrate analogue of ATP chirally substituted at P_γ proceeds with retention of configuration, in contrast to the inversion of configuration observed earlier in the adenylate kinase reaction [Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757]. As in the uridylyltransferases, these chemically matched reactions occur by different mechanisms, the former by a double and the latter by a single displacement at P_γ of ATP. UDP-glucose pyrophosphorylase accepts uridine 5′-(1-thiophosphate) having the *R* configuration at P_α and uridine 5′-(1-thiodiphospho)-glucose with the *S* configuration at P_α as substrates, while galactose-1-P uridylyltransferase accepts only the *R* configuration at P_α of uridine 5′-(1-thiodiphospho)glucose or -galactose. By use of these stereochemical preferences P_α epimers of uridine 5′-(1-thiotriphosphate) and uridine 5′-(1-thiodiphosphate)glucose can be prepared in pure form from synthetic mixtures.

Phosphotransferase and nucleotidyltransferases are large and interesting classes of enzymes whose mechanisms have been extensively studied. In enzymatic group transfer reactions many questions arise concerning the precise functional role of the enzyme. One question is whether the enzyme catalyzes the direct transfer of the group between acceptors bound at the active site or whether the enzyme mediates the transfer by providing a functional group to which the group being transferred is covalently bonded as a transient intermediate.

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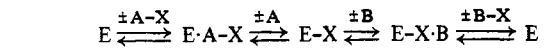
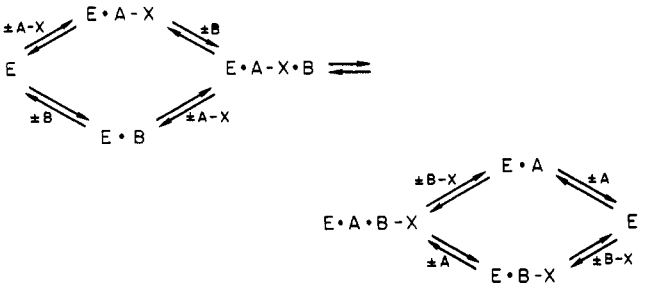
Scheme I



Scheme II



The determination of which prevails for a given enzyme or class of enzymes remains a challenging task for mechanistic enzymologists.

Kinetic and radiochemical techniques can provide important information on this question. They are capable of distinguishing double-displacement pathways involving *free* covalent intermediates from sequential pathways involving the compulsory formation of ternary or higher order complexes (Alberty, 1956; Cleland, 1970). The general patterns are illustrated by Schemes I and II for bisubstrate reactions, where X refers to the group undergoing transfer, A and B refer to the group acceptors, and A–X and B–X refer to the group donor substrates. Scheme II is intended to be general for pathways involving ternary complexes; the binding may be random as illustrated or one or more of the binding steps may not be detectable experimentally, in which case binding of substrates, release of products, or both occur in a definite order. The essential distinguishing features between the schemes are the compulsory occurrence of the free, covalently bonded intermediate E–X in Scheme I and of the ternary complexes in Scheme II.

A pathway such as Scheme I can be verified by the isolation and structural characterization of the intermediate E–X, by the fact that the steady-state kinetics is ping pong bi bi, by the observation of certain exchange reactions at rates compatible with the overall forward and reverse rates, and by the kinetic competency of the rate of E–X formation. Scheme II is characterized by the observation of sequential steady-state kinetics, the absence of exchange reactions such as A* + A–X $\rightleftarrows$ A*–X + A at rates comparable to overall rates, where A* represents radiochemically labeled A, and by the absence of a detectable intermediate E–X (Cleland, 1970).

The assignment of the double-displacement pathway is unambiguous in cases in which the experimental criteria for Scheme I are met. When the criteria for Scheme II are met the assignment of a single or double displacement is ambiguous, because the occurrence of an intermediate E–X·A·B complex linking the ternary complexes, as shown in eq 1, would

$$\text{E·A–X·B} \rightleftarrows \text{E–X·A·B} \rightleftarrows \text{E·A·B–X} \qquad (1)$$

not be detected in the kinetics, and it would be missed in radiochemical experiments if it existed at a small steady-state concentration.

We have undertaken a program of research designed to provide an additional experimental criterion for distinguishing between the single- and double-displacement pathways for phospho- and nucleotidyltransferases which is independent of their kinetic pathways. It produces information about the stereochemical courses of these reactions. In our work the P_α phosphorus atoms of substrates for nucleotidyltransferases are made chiral by the substitution of sulfur for one of the two prochiral oxygens bonded to P_α. These are prepared by the methods introduced by Eckstein & Goody (1976), and the configurations at P_α of substrates and products are related by the ^{31}P NMR chemical shifts of these atoms (Sheu & Frey, 1977, 1978). In our phosphotransferase work the terminal phosphoryl groups of nucleotides are made chiral by substitution of one of the three equivalent oxygen atoms by sulfur and the enrichment of one of the remaining two with ^{18}O to give chiral [^{18}O]phosphorothioates. The configurations are related by stereospecific enzymatic phosphorylation of terminal [^{18}O]phosphorothioate groups and mass spectral analysis for bridging or nonbridging ^{18}O in the products (Richard et al., 1978; Richard & Frey, 1978).

In undertaking this work we were motivated in part by the fact that knowledge of the stereochemical courses of such reactions would be essential to understanding the mechanisms by which they occur. We were also hopeful that such studies would yield clear-cut evidence on the issue of whether they proceed by single- or double-displacement mechanisms, i.e., that the occurrence of inversion of configuration at phosphorus would signal a single displacement whereas net retention would indicate a double-displacement mechanism.

In this paper we present the results of our studies on two enzymes known to catalyze group transfer according to Scheme I, galactose-1-P uridylyltransferase, whose catalytic pathway has been elucidated in our laboratory (Wong & Frey, 1974a, 1974b; Wong et al., 1977; Yang & Frey, 1979), and nucleoside diphosphate kinase which has been studied in other laboratories (Mourad & Parks, 1966; Garces & Cleland, 1969). We also confirm our earlier work on UDP-glucose pyrophosphorylase (Sheu & Frey, 1978) by showing that the stereochemistry in the reverse direction is the same as that in the forward direction. The action of this enzyme is known to involve compulsory formation of ternary complexes (Tsuboi et al., 1969).

Experimental Procedures

Enzymes, Coenzymes, and Chemicals. Rabbit muscle adenylate kinase, pyruvate kinase, phosphoglucomutase, and lactate dehydrogenase were purchased from Sigma Chemical Co. Yeast hexokinase, glucose-6-P dehydrogenase, UDP-glucose pyrophosphorylase, and inorganic pyrophosphatase as well as *Escherichia coli* acetate kinase and beef liver nucleoside diphosphate kinase were also purchased from Sigma Chemical Co. Galactose-1-P uridylyltransferase was purified from *E. coli* cells by Dr. Sue-Lein Lee Yang of this laboratory by a modification of the procedure of Saito et al. (1967).

Commercial nucleoside diphosphate kinase contained sufficient myokinase activity to interfere with the stereochemical study, so this was removed. The enzyme in 50% glycerol solution was applied directly to a 2 × 100 cm column of Sephadex G-100 and eluted with 10 mM Tris-HCl buffer at pH 7.0. The lower molecular weight myokinase was eluted well behind nucleoside diphosphate kinase, which was used without further purification.

All nucleotides and nucleosides were purchased from Sigma as were acetyl phosphate, triethyl phosphate, diphenyl phosphorochloridate, D_2O, tris(hydroxymethyl)aminomethane, *N*,*N*-bis(2-hydroxymethyl)glycine, phosphoenolpyruvate, α-D-glucose-1-P, α-D-galactose-1-P, and α-D-glucose 1,6-bisphosphate. DEAE-Sephadex A-25 was purchased from Pharmacia or Sigma and thiophosphoryl chloride was purchased from Ventron. 2,5-Diphenyloxazole and *p*-bis[2-(5-phenyloxazolyl)]benzene were purchased from New England Nuclear. Cation-exchange resin AG50W was obtained from

Bio-Rad Laboratories. [*adenine*-8-^{14}C]ADP was a generous gift from Professor David H. Ives. All other chemicals were obtained in reagent grade from commercial suppliers.

Analytical Procedures. UDP-glucose pyrophosphorylase activity was measured by a modification of the method of Tsuboi et al. (1969) in which glucose-1-P formed is detected as NADPH in the presence of phosphoglucomutase, glucose-6-P dehydrogenase, NADP,[1] and glucose 1,6-bisphosphate. The assay solution contained in 1.0 mL and at 27 °C 2 mM sodium pyrophosphate, 2 mM $MgCl_2$, 0.4 mM UDP-glucose, 1.4 mM cysteine, 1 μM glucose 1,6-bisphosphate, 0.2 mM NADP, 0.2 unit/mL of phosphoglucomutase, 0.2 unit/mL of glucose-6-P dehydrogenase, 50 mM Tris-HCl buffer at pH 7.6, and limiting UDP-glucose pyrophosphorylase. Galactose-1-P uridylyltransferase was assayed by a similar procedure in the direction of glucose-1-P formation as described by Wong & Frey (1974b). Nucleoside diphosphate kinase was assayed as the rate of NADH disappearance in reaction mixtures consisting of 50 mM Tris-HCl buffer at pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM KCl, 0.1 mM NADH, 1 mM ATP, 0.4 mM dTDP, 1 mM phosphoenolpyruvate, excess pyruvate kinase and lactate dehydrogenase, and limiting nucleoside diphosphate kinase at 27 °C.

Radiochemical assays were made by liquid scintillation counting in a Beckman Model LS-100C spectrometer. Aqueous samples (1 mL) were combined with 15 mL of a scintillation medium consisting of 7 g of 2,5-diphenyloxazole, 300 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene, and 100 g of naphthalene in 1 L of dioxane solution.

^{31}P nuclear magnetic resonance spectra were obtained on a Bruker HX-90 spectrometer operating at 36.43 MHz and equipped with a Fourier transform accessory, Bruker BSV-3B decoupler, and Nicolet DNC-12 computer. The spectrometer was field-frequency locked at deuterium resonance with D_2O used as the solvent. Proton noise decoupled spectra were recorded after 5000–10 000 scans on 5 mM solutions of nucleotides in 10 mm Wilmad tubes at 32 °C. Chemical shifts were related to an external 85% H_3PO_4 standard.

Syntheses of Nucleotides. UMPαS was synthesized by a modification of the procedure described by Murray & Atkinson (1968). Uridine (20 mmol, 4.88 g) was dissolved in 20 mL of trimethyl phosphate by heating to 100 °C. After the solution was cooled, thiophosphoryl chloride (93 mmol, 16.6 g) was added gradually at 0 °C, and the solution was then permitted to stand at room temperature for 2 days. The barium salt was formed by adding 200 mL of 10% barium acetate and adjusting the pH to 9 with triethylamine. The barium salt was precipitated by adding 600 mL of 95% ethanol, and the precipitate was collected by centrifugation and washed with 75% ethanol. The product was dissolved from the precipitate by repeated extraction with water. The 500-mL extract was chromatographed by passage into a 3 × 30 cm column of DEAE-Sephadex A-25, which was eluted with a 1.4-L linear gradient of $(C_2H_5)_3NH^+HCO_3^-$ from 0.05 to 0.55 M at pH 7.8 and 4 °C. The product, obtained in 44% yield, was eluted between 0.2 and 0.3 M $(C_2H_5)_3NH^+HCO_3^-$. Pooled fractions were evaporated to dryness by rotary evaporation in vacuo, and the residue was twice taken up in ethanol and evaporated to dryness to remove the last traces of $(C_2H_5)_3NH^+HCO_3^-$.

(α-*R*+*S*)UDPαS was synthesized from UMPαS as follows. UMPαS, 0.8 mmol in aqueous solution, was converted to its mono(trioctylammonium) salt by passage through a 2 × 20 cm column of AG-50 cation-exchange resin in the pyridinium form. The effluent was concentrated to a gum by in vacuo rotary evaporation, and 0.8 mmol of trioctylamine was added together with 10 mL of methanol. The mixture was shaken until it clarified, and the solvent was removed by in vacuo rotary evaporation. To remove traces of H_2O we twice dissolved the residue in dry dimethylformamide and rotary evaporated to dryness. A solution of diphenyl phosphorochloridate (1.38 mmol, 390 mg) in 0.2 mL of tributylamine and 2 mL of triethyl phosphate was added to the residue, and this was kept at room temperature for 3 h. Solvent was removed by rotary evaporation, and 30 mL of cold, dry diethyl ether was added with swirling and allowed to stand at 0 °C for 20 min. The ether was decanted, and the residue was taken up in dioxane and again evaporated to dryness. Phosphoric acid (2.1 mmol, 0.206 g) was converted to its mono(tributylammonium) monopyridinium salt by adding tributylamine (2.1 mmol, 0.389 g) to the solution of acid in pyridine. Traces of H_2O were removed by 3 times evaporating this solution to dryness in vacuo and redissolving in dry pyridine. The material was finally dissolved in 5 mL of dry pyridine, which was then used to dissolve the activated UMPαS and permitted to react at room temperature for 10 h. The pyridine was removed by in vacuo rotary evaporation, and the residue was extracted with 30 mL of cold ethyl ether. The product was dissolved in H_2O and loaded onto a 3 × 28 cm column of DEAE-Sephadex A-25 which was eluted with a 3.8-L linear gradient of $(C_2H_5)_3NH^+HCO_3^-$ from 0.07 to 0.55 M at 4 °C and pH 7.8. UDPαS was eluted as a band at about 0.45 M $(C_2H_5)_3NH^+HCO_3^-$. It was eluted well behind UDP which was formed as a result of S activation in UMPS and eluted from the column as a side product at about 0.36 M gradient. Some UMPαS also was eluted even earlier as well as several other very minor bands. The appropriate fractions were pooled and rotary evaporated to dryness in vacuo. The residue was twice dissolved in ethanol and again evaporated to dryness. The yield was 30%, and upon dissolution in D_2O and adjustment to pD 8.4 the proton noise decoupled ^{31}P NMR spectrum consisted of P_α doublets at −40.81 and −40.33 ppm and a P_β doublet at 7.02 ppm, $J_{\alpha,\beta}$ = 32.96 Hz.

(α-*R*+*S*)UTPαS was prepared by substituting pyrophosphate for phosphate in the above procedure and extending the reaction time to 12 h at room temperature. $Na_4P_2O_7 \cdot 10H_2O$ (3 mmol, 1.34 g) was converted to its di(tributylammonium) salt by first passing a 50-mL aqueous solution through a 2 × 30 cm AG-50 cation-exchange column in the pyridinium form. The effluent was evaporated to dryness, and 6 mmol of tributylamine was added together with pyridine, prepared for reaction as described above for tributylammonium phosphate, and finally reacted with activated UMPαS as

[1] Abbreviations used: NADH, 1,4-dihydronicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; dTDP, 2′-deoxythymidine diphosphate; ADP, adenosine 5′-diphosphate; NADP, nicotinamide adenine dinucleotide phosphate; ATPγS,γ^{18}O, adenosine 5′-(3-thio[3-^{18}O]triphosphate); ADPβS,β^{18}O, adenosine 5′-(2-thio[2-^{18}O]diphosphate); ATPβS,β^{18}O, adenosine 5′-(2-thio[2-^{18}O]triphosphate); UMPαS, uridine 5′-phosphorothioate; UDPαS, uridine 5′-(1-thiodiphosphate); UTPαS, uridine 5′-(1-thiotriphosphate); UDPαS-glucose, uridine-5′-(1-thiodiphospho)glucose; UDPαS-galactose, uridine-5′-(1-thiodiphospho)galactose. The appearance of α-, β-, or γR or α-, β-, or γS before the abbreviated name of a thionucleotide designates the absolute configuration at the chiral phosphorus in the nucleotide. These configurations are related to that of ATPαS diastereomer A which has been reported by Burgers & Eckstein (1978) to be *S* on the basis of a kinetic correlation. This designation is tentative pending a direct determination of the absolute structure of ATPαS, and it is used here for convenience in discussing our results. Our conclusions are independent of absolute configurations.

described above, but for 12 h. The product was chromatographed as described for UDPαS, except that the elution gradient consisted of 2.8-L total volume from 0.15 to 0.8 M $(C_2H_5)_3NH^+HCO_3^-$ and the product was eluted as a clean band at 0.57–0.61 M gradient. The product was the last band to be eluted from the column, well separated from UTP, and it was isolated from the gradient salt as above. The proton noise decoupled ^{31}P NMR spectrum consisted of P_α doublets at −42.72 and −42.45 ppm, a P_β doublet of doublets at 23.32 ppm, and a P_γ doublet at 6.99 ppm, $J_{\alpha,\beta}$ = 28.38 Hz and $J_{\beta,\gamma}$ = 21.06 Hz.

(α-*R*+*S*)UDPα*S*-glucose was synthesized in low yield by a procedure analogous to those used for UDPαS and UTPαS by substituting α-D-glucose-1-P for phosphate or pyrophosphate. UMPαS (0.84 mmol) was activated with diphenyl phosphorochloridate (1.2 mmol) as described above for the synthesis of UDPαS and finally dissolved in 3 mL of dry dimethylformamide. The dipotassium salt of α-D-glucose-1-P (3 mmol) was converted to its di(tributylammonium) salt by the same procedure described above for pyrophosphate in the UTPαS synthesis. This was finally dissolved in 3 mL of dry pyridine and combined with the 3-mL dimethylformamide solution of activated UMPαS. The reaction proceeded for 3 days at room temperature. The solvent was removed by rotary evaporation in vacuo, and the residue was extracted with cold diethyl ether as described above in the synthesis of UDPαS. The residue was finally dissolved in water and applied to a 2 × 24 cm column of DEAE-Sephadex A-25. The column was eluted with a 1.4-L gradient of 0.15–3.0 M triethylammonium acetate at pH 4.2 and 4 °C, and effluent fractions were collected. The fractions corresponding to the major band of A_{260}-positive material eluting at 1.75–1.95 M gradient contained the product and were pooled. These were rotary evaporated in vacuo to a small volume and then lyophilized. This material was not pure and so was further purified by descending paper chromatography on Whatman 3 MM filter paper using as the mobile phase 70:30:20 ethanol–methyl ethyl ketone–0.5 M morpholinium tetraborate at pH 8.6 and containing 10 mM ethylenediaminetetraacetate. The product was eluted from the paper with H_2O and further chromatographed on a 1.5 × 14 cm column of DEAE-Sephadex A-25, which was eluted with a 300-mL linear gradient of 0.1–0.8 M triethylammonium bicarbonate, to remove borate and other residual impurities introduced by paper chromatography. (α-*R*+*S*)UDPαS-glucose was obtained in 12% overall yield after volatilizing the eluting buffer. This was a much lower yield than we found in synthesizing other nucleotides. The reaction of glucose-1-P appears to be slower than phosphate or pyrophosphate, and so the reaction required a long time to complete, during which significant deterioration of product probably also became a significant problem. UDP-glucose formed quickly from sulfur-activated UMPαS, so after shorter reaction times UDP-glucose was the only detectable nucleotide sugar. The uridine/glucose ratio in the (α-*R*+*S*)UDPαS-glucose was 1.00:1.04, and the proton noise decoupled ^{31}P NMR spectrum consisted of two P_α doublets at −43.54 and −43.41 ppm and a P_β doublet at 13.68 ppm, $J_{\alpha,\beta}$ = 28.08 Hz.

(α*R*)UDPαS and (α*S*)UTPαS were prepared from synthetic (α-*R*+*S*)UDPαS by stereospecific phosphorylation of the *S* isomer with phosphoenolpyruvate catalyzed by pyruvate kinase. The procedure is similar to that described by Eckstein & Goody (1976) in the adenine thionucleotide series. The reaction mixture contained, in 30 mL, the following: 1.63 mM (α-*R*+*S*)UDPαS, 1.85 mM phosphoenolpyruvate, 1.9 mM NADH, 5 mM $MgCl_2$, 67 mM KCl, 45 mM mercaptoethanol, 27 units/mL of pyruvate kinase, 7 units/mL of lactate dehydrogenase, and 50 mM Tris-HCl buffer at pH 8.0. The progress of the reaction was monitored as the decrease in A_{340}, which reflected the production of pyruvate from phosphoenolpyruvate. The pyruvate/UDPαS ratio reached 0.44 in 12 h at 25 °C. The solution was applied to a 1.5 × 30 cm column of DEAE-Sephadex A-25, and the nucleotides were eluted with a 1.0-L linear gradient of 0.05–0.75 M $(C_2H_5)_3NH^+HCO_3^-$ at pH 7.8, and 17-mL fractions were collected at 4 °C. (α*R*)UDPαS emerged in fractions 29–31 and (α*S*)UTPαS emerged in fractions 37–39, in yields of 16.3 and 13.5 μmol, respectively. The appropriate fractions were pooled and evaporated to dryness by rotary evaporation in vacuo. The residues were twice taken up in ethanol and again evaporated to remove the last traces of gradient buffer. The proton decoupled ^{31}P NMR spectrum of (α*R*)UDPαS consisted of a P_α doublet at −40.48 ppm and a P_β doublet at 6.97 ppm, $J_{\alpha,\beta}$ = 31.73 Hz. The spectrum of (α*S*)UTPαS consisted of a P_α doublet at −42.81 ppm, a P_β doublet of doublets at 23.32 ppm, and a P_γ doublet at 6.99 ppm, $J_{\alpha,\beta}$ = 28.08 Hz and $J_{\beta,\gamma}$ = 20.75 Hz.

(α*S*)UTPαS and (α*S*)UDPαS-glucose were prepared by the action of yeast UDP-glucose pyrophosphorylase on (α-*R*+*S*)UTPαS. The reaction mixture consisted of the following in 140 mL of solution: 0.6 mM (α-*R*+*S*)UTPαS, 1.53 mM α-D-glucose-1-P, 2 mM $MgCl_2$, 1.83 mM cysteine, 0.28 unit/mL of UDP-glucose pyrophosphorylase, 0.28 unit/mL of inorganic pyrophosphatase, and 30 mM Tris-HCl buffer at pH 7.6. After 4 h at 25 °C the reaction reached completion with the conversion of 46% of the UTPαS to UDPαS-glucose. The reaction mixture was chromatographed on a 2.3 × 28 cm column of DEAE-Sephadex A-25 which was eluted with a 1.3-L linear gradient from 0.085 to 0.85 M $(C_2H_5)_3NH^+HCO_3^-$ at pH 7.8 and 4 °C. Fractions of 11-mL volume were collected, and (α*S*)UDPαS-glucose was eluted in fractions 29–33 (0.3 M gradient) and (α*S*)UTPαS was eluted in fractions 48–58 (0.5 M gradient). The yields were 53 and 65 μmol, respectively, and they were desalted as described above. The proton-decoupled ^{31}P NMR spectrum of (α*S*)UDPαS-glucose consisted of a P_α doublet at −43.62 ppm and a P_β doublet at 13.68 ppm, $J_{\alpha,\beta}$ = 28.08 Hz. The spectrum of (α*S*)UTPαS consisted of a P_α doublet at −42.64 ppm, a P_β doublet of doublets at 23.41 ppm, and a P_γ doublet at 6.66 ppm, $J_{\alpha,\beta}$ = 28.08 Hz and $J_{\beta,\gamma}$ = 20.60 Hz.

(α*R*)UTPαS and (α*R*)UDPαS-glucose were prepared by the action of UDP-glucose pyrophosphorylase on (α-*R*+*S*)-UDPαS-glucose. The 60-mL reaction mixture consisted of 0.4 mM (α-*R*+*S*)UDPαS-glucose, 1.25 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 1 mM cysteine, 0.33 μM glucose 1,6-bisphosphate, 0.09 unit/mL of UDP-glucose pyrophosphorylase, 0.2 unit/mL of phosphoglucomutase, and 25 mM Tris-HCl buffer at pH 7.6. The progress of the reaction was monitored by measuring the production of glucose-6-P by using NADP and glucose-6-P dehydrogenase. The reaction reached completion after 4 h at 25 °C when about 45% of the UDPαS-glucose had been converted to UTPαS. The reaction mixture was chromatographed on a 1.5 × 24 cm column of DEAE-Sephadex A-25 which was eluted with a 0.9-L linear gradient of 0.1–0.8 M $(C_2H_5)_3NH^+HCO_3^-$ at pH 7.8 and 4 °C. Fractions of 13-mL volume were collected, and (α*R*)-UDPαS-glucose emerged in fractions 16–18 (0.29 M gradient) and (α*R*)UTPαS appeared in fractions 39–42 (0.53 M gradient). The yields were 10.5 and 11.5 μmol, respectively, and the samples were desalted as described above. The proton-decoupled ^{31}P NMR spectrum of (α*R*)UDPαS-glucose con-

sisted of a P_α doublet at −43.43 ppm and a P_β doublet at 13.74 ppm, $J_{\alpha,\beta}$ = 29.30 Hz. The spectrum of (αR)UTPαS consisted of a P_γ doublet at −42.62 ppm, a P_β doublet of doublets at 23.20 ppm, and a P_γ doublet at 6.62 ppm, $J_{\alpha,\beta}$ = 28.33 Hz and $J_{\beta,\gamma}$ = 20.75 Hz.

(αR)UDPαS-galactose was prepared from (αR)UDPαS-glucose by the action of galactose-1-P uridylyltransferase. The reaction mixture contained the following in 30 mL: 0.67 mM (αR)UDPαS-glucose, 1.27 mM galactose-1-P, 1.0 mM $MgCl_2$, 3.3 mM cysteine, 0.33 μM glucose 1,6-bisphosphate, 0.4 unit/mL of phosphoglucomutase, 0.68 unit/mL of galactose-1-P uridylyltransferase, and 66 mM sodium Bicinate buffer at pH 8.5 and 25 °C. The progress of the reaction was monitored by assaying for glucose-6-P formation by using NADP and glucose-6-P dehydrogenase. The reaction was complete after 30 h. The solution was chromatographed on a 1.5 × 28 cm column of DEAE-Sephadex A-25 which was eluted with an 0.8-L gradient of 0.1–0.65 M $(C_2H_5)_3NH^+$-HCO_3^-. (αR)UDPαS-galactose was eluted as a single band in a yield of 20 μmol at a gradient concentration of 0.31 M. After desalting as described above, we found that it gave a proton-decoupled ^{31}P NMR spectrum consisting of a P_α doublet at −43.48 ppm and a P_β doublet at 13.59 ppm, $J_{\alpha,\beta}$ = 28.08 Hz.

P^1,P^5-Diadenosine pentaphosphate used as an inhibitor of adenylate kinase was synthesized by the following new procedure. AMP (4 mmol) was activated with diphenyl phosphorochloridate according to Michelson (1958, 1964) as described above for UMPαS. This was coupled to 1 mmol of di(tri-*n*-butylammonium) tripolyphosphate in 5 mL of dry pyridine for 12 h at 25 °C. The nucleotides were separated on a 2.2 × 45 cm column of DEAE-Sephadex A-25 by elution with a 4-L linear gradient of triethylammonium bicarbonate from 0.3 to 0.9 M. Fractions eluting in a band at 0.65 M gradient exhibited potent inhibitory action against rabbit muscle adenylate kinase and were pooled. The yield was about 0.11 mmol, and after desalting by rotary evaporation of the gradient in vacuo the proton-decoupled ^{31}P NMR spectrum consisted of multiplets at 11.5 and 22.5 ppm upfield from H_3PO_4, as reported earlier (Nageswara Rao & Cohn, 1977). The compound contained about 10% of a polyphosphate impurity as determined by integration of the NMR spectrum; however, since it did not interfere with our use of this compound as an inhibitor of adenylate kinase, it was not removed by further purification.

Hydrolysis of UDPαS-hexoses to UDPαS. Configurational assignments at P_α for UDPαS-hexoses were made by hydrolyzing them to UDPαS on which the configurational assignments were made by comparison of their ^{31}P NMR spectra with standard samples having known P_α configurations. The hydrolytic conditions did not interfere with the P_α configurations. The di(triethylammonium) salt of UDPαS-hexose (10 μmol) was dissolved in aqueous solution, adjusted to pH 2.5 with HCl, and heated at 100 °C for 15 min. The solution was neutralized with NaOH and applied to a 1.5 × 16 cm column of DEAE-Sephadex, and UDPαS was eluted with a 300-mL linear gradient of 0.1–0.85 M triethylammonium bicarbonate at pH 7.8 and 4 °C. UDPαS was eluted as a clean band at about 230 mL of the gradient volume in 75–80% yield. It was desalted by volatilization of the gradient buffer as described above.

Reaction of ATPγS,γ^{18}O with [adenine-8-^{14}C]ADP. [*adenine*-8-^{14}C]ATPγS,γ^{18}O was prepared in a 30-mL reaction mixture consisting of 50 mM Tris-HCl, pH 8.0, 50 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM (γR)ATPγS,^{18}O (Richard & Frey, 1978), 1.36 mM [*adenine*-8-^{14}C]ADP (1280 cpm/μmol), and 100 units of nucleoside diphosphate kinase. After 2 h at 25 °C the products were separated on a 2.2 × 45 cm column of DEAE-Sephadex A-25 which was eluted with a 3-L linear gradient of 0.3–0.7 M triethylammonium bicarbonate, and 25-mL fractions were collected. The major radioactive and A_{260} bands eluted corresponded to [^{14}C]ADP and [^{14}C]-ATPγS in fractions 41–49 and fractions 72–82, respectively. These were present in approximately equal amounts, and their specific activities were 980 cpm/μmol for [^{14}C]ADP and 520 cpm/μmol for [^{14}C]ATPγS. Minor bands of ^{14}C and A_{260} corresponding to AMP and ATP, and together corresponding to 12% of the total nucleotides eluted, also appeared in fractions 23–27 and 61–69. These could be attributed to the effects of the presence of a trace of residual myokinase activity in the nucleoside diphosphate kinase. This did not interfere with the stereochemical analysis. The recovery of ATPγS,γ^{18}O, now labeled with ^{14}C, was 26 μmol, or 87%. This was desalted by volatilization of the elution buffer.

Reaction of [^{14}C]ATPγS,γ^{18}O with AMP Catalyzed by Adenylate Kinase. In comparing the configurations at P_γ of (γR)ATPγS,^{18}O and [^{14}C]ATPγS, γ^{18}O prepared above we used both to phosphorylate AMP to ADPβS,β^{18}O catalyzed by adenylate kinase. The configurations of these products were then directly compared. The following procedure was used to prepare ADPβS,β^{18}O. The 130-mL reaction mixture consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.2 mM AMP, 1 mM dithiothreitol, 0.2 mM ATPγS,γ^{18}O, and 0.25 mg of rabbit muscle adenylate kinase at 25 °C. The progress of the reaction was followed by monitoring the production of ADP, which was measured by adding 0.5-mL aliquots of the reaction mixture to 0.5 mL of a solution containing 0.3 mM NADH, 2 mM phosphoenolpyruvate, 50 mM KCl, 0.2 μmol of P^1,P^5-diadenosine pentaphosphate, and 1 unit of lactate dehydrogenase. The A_{340} of this solution was monitored until it stabilized, and then 1 unit of pyruvate kinase was added and the rapid decrease in A_{340} reflecting the amount of ADP present was measured. The amount of ADP appearing in the reaction mixture stabilized at approximately 60% of the original ATPγS concentration within 1 h. At this point the products were separated and purified on a 3.0 × 30 cm column of DEAE-Sephadex A-25, which was eluted with a 3.0-L linear gradient of triethylammonium bicarbonate from 0.2 to 0.6 M. ADP and ADPβS were very well separated by this column, and the yield of ADPβS obtained after pooling fractions and volatilizing the gradient buffer by in vacuo rotary evaporation was 21 μmol, or 81% of the ATPγS originally present.

Phosphorylation of ADPβS,β^{18}O to ATPβS,β^{18}O. Acetyl phosphate and acetate kinase were used to phosphorylate ADPβS,β^{18}O to ATPβS,β^{18}O as described by Eckstein & Goody (1976).

Results

Stereochemical Course of UDP-glucose Pyrophosphorylase. In earlier work we showed that yeast UDP-glucose pyrophosphorylase accepts (αR)UTPαS as a good substrate and is inactive on (αS)UTPαS (Sheu & Frey, 1978). We also showed that in the presence of glucose-1-P this enzyme converts (αR)UTPαS to (αS)UDPαS-glucose, i.e., with inversion of configuration. We have further investigated the action of this enzyme on the synthetic mixture of (α-*R*+*S*)UDPαS-glucose.

Shown in Figure 1 are the progress curves taken from spectrophotometric tracings of the production of glucose-1-P from UDP-glucose and (α-*R*+*S*)UDPαS-glucose in the presence of UDP-glucose pyrophosphorylase and pyro-

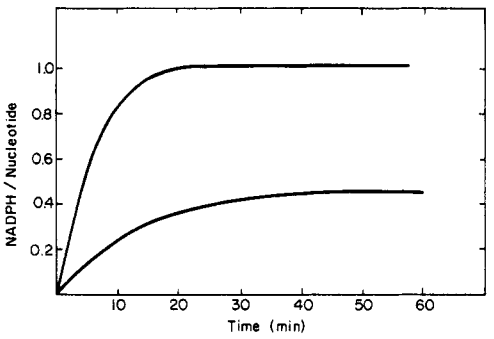


FIGURE 1: Synthetic (α-*R*+*S*)UDPαS-glucose as a substrate for UDP-glucose pyrophosphorylase. The reaction mixtures contained at 27 °C 2 mM sodium pyrophosphate, 1.5 mM cysteine, 2 mM $MgCl_2$, 0.3 mM NADP, 2 μM glucose 1,6-bisphosphate, 0.1 unit/mL of glucose-6-P dehydrogenase, and 50 mM Tris-HCl buffer at pH 7.6. Upper curve: the substrate was 0.05 mM UDP-glucose and 0.01 unit/mL of UDP-glucose pyrophosphorylase was present. Lower curve: the substrate was 0.065 mM synthetic (α-*R*+*S*)UDPαS-glucose and 0.05 unit/mL of UDP-glucose pyrophosphorylase was used. The solutions were monitored continuously at 340 nm for NADPH formation. The ordinate scale of A_{340} has been recalculated to reflect the ratio of NADPH produced to nucleotide present.

Table I: Stereochemistry of UDP-glucose Pyrophosphorylase with UDPαS-glucose

sample	origin	^{31}P NMR chemical shifts[a] (ppm) P_α	P_β	P_γ
A	UTPαS produced by UDP-glucose pyrophosphorylase	−42.62 (d)[b]	23.20 (dd)	6.62 (d)
B	A + (α*S*)UTPαS	−42.64 (d) −42.39 (d)	23.39 (dd)	6.75 (d)
C	UDPαS-glucose not accepted by UDP-glucose pyrophosphorylase	−43.43 (d)	13.74 (d)	
D	UDPαS from hydrolysis of C	−40.38 (d)	6.75 (d)	
E	D + (α*R*)UDPαS	−40.36 (d)	6.80 (d)	

[a] The negative signs refer to chemical shifts downfield from H_3PO_4. [b] The d refers to doublet and dd refers to doublet of doublets. The coupling constants (hertz) are as follows: for UTPαS, $J_{\alpha,\beta}$ = 28.65 and $J_{\beta,\gamma}$ = 20.75; for UDPαS-glucose, $J_{\alpha,\beta}$ = 29.30; for UDPαS, $J_{\alpha,\beta}$ = 32.28. The spectra contained no signals other than these and the external standard.

phosphate. The glucose-1-P formed was detected by conversion to 6-phosphogluconate and NADPH in the presence of phosphoglucomutase, glucose-6-P dehydrogenase, and NADP. The upper curve verifies that 1 mol of glucose-6-P is produced per mol of UDP-glucose under the conditions of Figure 1. The lower curve shows that only about 0.46 mol of glucose-1-P is produced per mol of (α-*R*+*S*)UDPαS-glucose, which is interpreted to mean that one of the two isomers is a substrate and the other is not. (α*S*)UDPαS-glucose is a reasonably good substrate with an apparent K_m of 2.2 times that for UDP-glucose and an apparent V about 9% that for UDP-glucose (Sheu & Frey, 1978), so it is expected that the unreacted isomer in Figure 1 is (α*R*)-UDPαS-glucose. Since the reaction proceeds in the opposite direction with inversion of configuration at P_α (Sheu & Frey, 1978), the expected nucleotide product is (α*R*)UTPαS.

This was shown to be the case by carrying out the reaction on a larger scale and purifying the products (see Experimental Procedures). The P_α chemical shifts in Table I were obtained. The spectrum of the UTPαS produced gave a P_α chemical shift that was consistent with that of (α*R*)UTPαS and, upon addition of authentic (α*S*)UTPαS, the P_β and P_γ signals were unchanged while there appeared two doublets for P_α. This spectrum was that of the mixture of isomers and confirmed that (α*R*)UTPαS was produced from (α-*R*+*S*)UDPαS-glucose. The spectrum of unreacted UDPαS-glucose consisted, as expected, of two doublets corresponding to one of the isomers. To assign the configuration at P_α, we subjected this sample to minimal acid hydrolytic conditions to cleave the sugar and produce UDPαS. As shown in Table I, the ^{31}P NMR spectrum of the hydrolysis product was consistent with that of (α*R*)UDPαS and, upon addition of an authentic sample of (α*R*)UDPαS, the spectrum was unchanged.

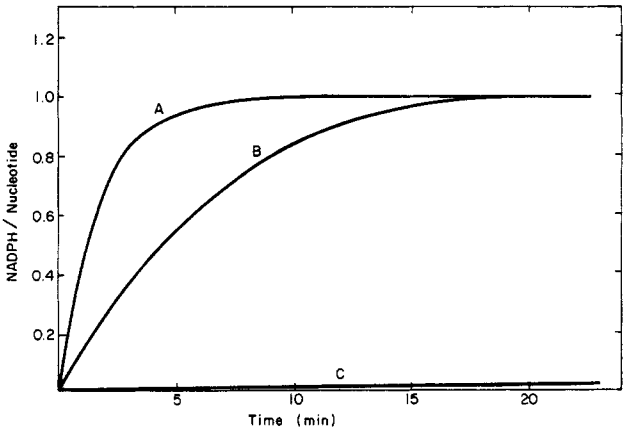


FIGURE 2: Time courses of galactose-1-P uridylyltransferase catalyzed reactions of UDP-glucose and its sulfur analogues with galactose-1-P. The reaction mixtures contained at 27 °C 0.5 mM galactose-1-P, 13 mM cysteine, 4 mM $MgCl_2$, 4 mM NaF, 1 mM NADP, 0.32 unit/mL of phosphoglucomutase, 0.1 unit/mL of glucose-6-P dehydrogenase, and 0.1 M sodium Bicinate buffer at pH 8.5. Curve A: the substrate was 40 μM UDP-glucose with 0.03 unit/mL of galactose-1-P uridylyltransferase. Curve B: the substrate was 40 μM (α*R*)UDPαS-glucose with 0.17 unit/mL of transferase. Curve C: the substrate was 40 μM (α*S*)UDPαS-glucose with 0.17 unit/mL of transferase. The solutions were monitored continuously at A_{340} as described in Figure 1.

Table I verifies that in the direction of UTPαS formation from UDPαS-glucose the substrate is (α*S*)UDPαS-glucose and the product is (α*R*)UTPαS. The reaction proceeds with inversion of configuration as we originally found in the direction of UDPαS-glucose formation (Sheu & Frey, 1978).

Because of the specificity of this enzyme for thionucleotide sugars, it can be used to prepare pure isomers of UTPαS and UDPαS-glucose. In the reaction of (α-*R*+*S*)UTPαS with glucose-1-P, (α*S*)UTPαS does not react and can be isolated pure (Sheu & Frey, 1978) while pure (α*S*)UDPαS-glucose is produced. In the reverse direction with (α-*R*+*S*)UDPαS-glucose as the substrate, (α*R*)UDPαS-glucose does not react and can be isolated free of (α*S*)UDPαS-glucose, while the product is pure (α*R*)UTPαS. Detailed procedures for effecting these resolutions are described under Experimental Procedures.

Stereochemical Course of UridylylαS Transfer by Galactose-1-P Uridylyltransferase. Using pure samples of UDPαS-glucose having the *R* or *S* configurations at P_α, prepared as above, we measured their relative effectiveness as substrates for galactose-1-P uridylyltransferase in Figure 2. At 40 μM nucleotide sugar, reaction progress curves A, B, and C were obtained for UDP-glucose, (α*R*)UDPαS-glucose, and (α*S*)UDPαS-glucose, respectively.

When corrected for the use of 5 times more enzyme in reaction B than in reaction A, the initial rate in reaction B is about one-fifteenth that in reaction A. This reflects both K_m and V effects which are not sorted out by this experiment, but in any case (α*R*)UDPαS-glucose is a very acceptable

Table II: Stereochemical Course of the Galactose-1-P Uridylyltransferase Reaction

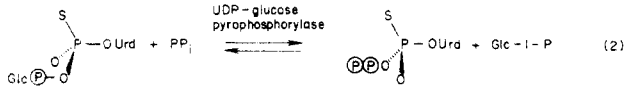
sample	origin	^{31}P NMR chemical shifts[a] (ppm) P_α	P_β
A	UDPαS-galactose produced from (αR)UDPαS-glucose	−43.48 (d)[b]	13.59 (d)
B	UDPαS from hydrolysis of A	−40.49 (d)	6.87 (d)
C	B + (αR)UDPαS	−40.39 (d)	6.80 (d)

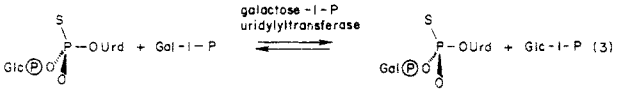
[a] The negative signs refer to chemical shifts downfield from H_3PO_4. [b] The d refers to a doublet. The coupling constants (hertz) are as follows: for UDPαS-galactose, $J_{\alpha,\beta} = 28.08$; for UDPαS, $J_{\alpha,\beta} = 32.28$. The spectra contained no signals other than these and the standard.

substrate. We detect no more than trace activity for (αS)-UDPαS-glucose.

UDPαS-galactose was prepared by the action of galactose-1-P uridylyltransferase on (αR)UDPαS-glucose. The ^{31}P NMR spectrum was the same as that of (αR)UDPαS-glucose; to make the configurational assignment, we hydrolyzed it under minimal acid conditions to UDPαS. The ^{31}P NMR spectrum of this product was found to be that of (αR)UDPαS. Upon admixture with a sample of authentic (αR)UDPαS, the spectrum, as given in Table II, remained that of (αR)UDPαS, with the P_α chemical shift fully 0.45 ppm upfield from that for (αS)UDPαS.

We conclude that uridylyl-S transfer catalyzed by galactose-1-P uridylyltransferase proceeds with net retention of configuration at P_α, in confirmation of the catalytic pathway of Scheme I. Equations 2 and 3 illustrate the stereochemical

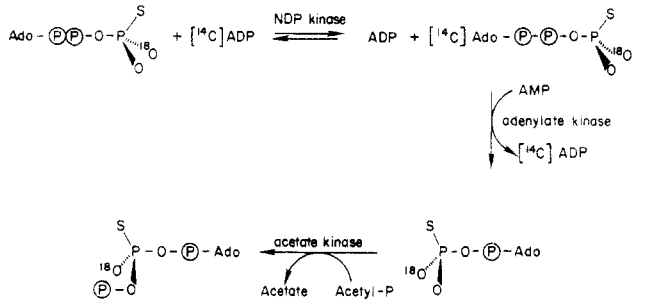




courses of uridylyl-S transfer catalyzed by UDP-glucose pyrophosphorylase (*inversion*) and galactose-1-P uridylyltransferase (*retention*). These are in chemical respects essentially the same reaction, i.e., the reversible transfer of a uridylyl-S group between phosphate acceptors. Since they occur with opposite stereochemical consequences, we conclude that irrespective of kinetic ambiguities they cannot proceed by analogous mechanisms of bond cleavage and formation.

Stereochemical Course of Thiophosphoryl Transfer by Nucleoside Diphosphate Kinase. Eckstein & Goody (1976) reported that ATPγS is a substrate for nucleoside diphosphate kinase. In our hands ATPγS reacted at somewhat less than 1% of the rate for ATP. We have proceeded according to Scheme III to determine the stereochemical course of this reaction. (γR)ATPγS,γ^{18}O was used as the donor substrate with [^{14}C]ADP as the acceptor to produce ADP and [^{14}C]-ATPγS,γ^{18}O. This exchange reaction is known to be catalyzed by nucleoside diphosphate kinase by the same mechanism as the phosphoryl transfer between any other donor–acceptor pair. The specific radioactivity of the [^{14}C]ATPγS,γ^{18}O produced showed that the reaction proceeded to 70% of equilibrium. The configuration of the [^{18}O]phosphorothioate group, represented as *R* in Scheme III, was determined by first using adenylate kinase to catalyze its transfer to AMP, producing ADPβS,β^{18}O. The [^{18}O]phosphorothioate group in

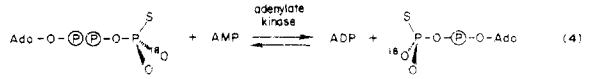
Scheme III

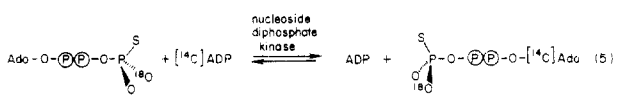


ADPβS,β^{18}O is represented in Scheme III as having the *S* configuration because adenylate kinase is known to catalyze this with 98% inversion of configuration (Richard & Frey, 1978). ADPβS,β^{18}O was then phosphorylated with acetyl phosphate catalyzed by acetate kinase, which is known to phosphorylate the *pro-R* oxygen at P_β of ADPβS (Richard et al., 1978; Jaffe & Cohn, 1978). The resulting (βR)ATP-βS,β^{18}O was finally degraded, first to tripoly([βS,β^{18}O]-phosphorothioate) and then to trimethyl phosphorothioate and trimethyl phosphate which were subjected to gas chromatographic–mass spectral analysis to determine the position of ^{18}O enrichment, i.e., whether the enrichment was in the bridging or nonbridging position, by the procedure described by Richard et al. (1978). In this procedure nonbridging ^{18}O is isolated exclusively in trimethyl phosphorothioate while bridging ^{18}O is about equally partitioned in trimethyl-phosphorothioate and trimethyl phosphate (Richard et al., 1978).

The [^{14}C]ATPγS,γ^{18}O from Scheme III was used to produce ADPβS,β^{18}O by adenylate kinase catalyzed thiophosphorylation of AMP. This was subjected to the foregoing degradation to measure the enrichment in ^{18}O at P_β. The mass analysis of the resulting trimethyl [^{18}O]phosphorothioate gave 89.9% of the M + 2 parent ion of mass 158. After stereospecific phosphorylation of the *pro-R* oxygen of P_β by acetyl phosphate catalyzed by acetate kinase, the resulting (βR)-ATPβS,β^{18}O was subjected to the same degradation. Mass analysis of the trimethyl [^{18}O]phosphorothioate gave 88.4% of the M + 2 parent ion, showing that 98.3% of the ^{18}O in (βR)ATPβS,β^{18}O was nonbridging. Therefore, the configuration at P_β of the ADPβS,β^{18}O must have been *S*, and, since the adenylate kinase reaction proceeds with inversion (Richard & Frey, 1978), the configuration at P_γ of [^{14}C]ATPγS,γ^{18}O must have been *R*.

We conclude that the configurations of P_γ in (γR)-ATPγS,γ^{18}O and [^{14}C]ATPγS,γ^{18}O are identical as shown in Scheme III, and, therefore, the nucleoside diphosphate kinase reaction proceeds with net *retention* of configuration. The stereochemical courses of the adenylate and nucleoside diphosphate kinase reactions are given in eq 4 and 5. These





reactions are in chemical respects essentially the same reaction, the reversible transfer of a thiophosphoryl group between acceptor phosphates. Since they occur with opposite stereochemical consequences, we again conclude that irrespective

Table III: Stereochemical Courses of Enzymatic Group Transfer

enzyme	group	stereochemistry	ref
UDP-glucose pyrophosphorylase	uridylyl	inversion	Sheu & Frey (1978) and this work
adenylate kinase	phosphoryl	inversion	Richard & Frey (1978)
galactose-1-P uridylyltransferase	uridylyl	retention	this work
nucleoside diphosphate kinase	phosphoryl	retention	this work
nucleoside phosphotransferase	phosphoryl	retention	Richard et al. (1979)
acetyl-CoA synthetase	adenylyl	inversion	Midelfort & Sarton-Miller (1978)
glycerokinase	phosphoryl	inversion	Blattler & Knowles (1979)
hexokinase	phosphoryl	inversion	*a*
pyruvate kinase	phosphoryl	inversion	*a*
acetate kinase	phosphoryl	inversion	*a*
alkaline phosphatase	phosphoryl	retention	Jones et al. (1978)
phosphoribosyl-PP synthetase	pyrophosphoryl	inversion	Li et al. (1978)
RNA polymerase	adenylyl	inversion	Burgers & Eckstein (1978)
terminal nucleotidyltransferase	adenylyl	inversion	Eckstein et al. (1977)
ribonuclease	phosphoester	inversion	Usher et al. (1972)

[a] J. R. Knowles and D. Pliura, private communication, and Orr et al. (1978).

of kinetic ambiguities they cannot proceed by analogous bond cleavage and formation mechanisms.

Discussion

The present work completes our stereochemical analysis of two chemically matched pairs of transferases, two nucleotidyltransferases and two phosphotransferases. In each pair of reactions we have studied, one of the two is known from other work to proceed by a double-displacement catalytic pathway involving a free, covalently bonded intermediate, either a nucleotidylenzyme or a phosphorylenzyme (Wong & Frey, 1974a,b; Mourad & Parks, 1966; Garces & Cleland, 1969). The second reaction of each pair is known to involve compulsory ternary complex formation and not to involve a free, covalently bonded enzyme–substrate intermediate (Tsuboi et al., 1969; Rhoads & Lowenstein, 1968; Noda, 1973). In each pair the double-displacement reaction proceeds with net *retention* of configuration at phosphorus and the other proceeds with net *inversion* of configuration at phosphorus. The stereochemical results constitute unequivocal proof that the bond cleavage/formation mechanisms for the two partners in each pair cannot be the same. The differing steady-state kinetic pathways accurately reflect differences in chemical reaction mechanisms. The ambiguities that plague other mechanistic studies of bisubstrate phospho- and nucleotidyltransferases which involve ternary complexes as compulsory intermediates evidently do not arise in stereochemical studies of these reactions.

In recent work a significant body of knowledge about the stereochemical courses of such transferases has accumulated. This has been brought together in Table III. In each case in which a large body of nonstereochemical evidence has failed to implicate a nucleotidyl- or phosphorylenzyme, the stereochemical course of the reaction has turned out to be *inversion* of configuration. In each case in which a large body of nonstereochemical evidence has established the involvement of a nucleotidyl- or phosphorylenzyme as a compulsory intermediate, the stereochemical course has turned out to be *retention* of configuration. The accumulated evidence implies that each enzymatic bond cleavage step at phosphorus in the reactions of biological phosphates so far studied occurs with inversion of configuration. This would result from an in-line displacement mechanism at phosphorus. If this is general for enzymatic reactions, it would result in net inversion of configuration at phosphorus whenever an odd number of displacements occur in an enzymatic mechanism and overall retention of configuration in a mechanism involving an even number of such displacements. It is emphasized that this interpretation is currently based on a limited body of stereochemical data. It should be noted in this connection that the stereochemical analysis of each step of an enzymatic double displacement has not yet been completed. Nevertheless, the pattern is developing consistently for a broad range of nucleotidyltransferases and phosphotransferases.

Analysis of the stereochemical courses of such reactions may be one of the most powerful indicators of mechanism, perhaps the most powerful *single* indicator. This type of study should be a high priority addition to the other more traditional mechanistic techniques for detailed mechanistic analysis of enzymatic and nonenzymatic reactions involving bond cleavage to phosphorus in phosphates.

Dissociative and associative mechanisms for displacement at phosphorus in phosphates have been established for nonenzymatic reactions (Westheimer, 1968; Benkovic & Schray, 1973). These reactions should proceed with net inversion, retention, or loss of stereochemical configuration at phosphorus, the stereochemical course being dependent upon which mechanism is followed. The current data indicating inversion at phosphorus at each bond cleavage step for enzymatic reactions is useful for the present purpose of defining the number of displacement steps; however, it does not bear significantly on the question of whether the enzymatic reactions occur by dissociative or associative mechanisms because enzyme–substrate binding interactions can be expected to prevent loss of configuration that would result from a dissociative mechanism operating in free solution.

Arguments have been advanced in support of the proposition that chemically similar enzymatic reactions can be expected to occur by identical bond cleavage/formation mechanisms, irrespective of differences in kinetics. This unifying concept has been invoked with specific reference to single- vs. double-displacement mechanisms of enzyme action; it was proposed that if one reaction is shown to occur by a mechanism involving a covalently bonded intermediate in a double-displacement mechanism, then other enzymatic reactions of the same chemical type can be expected to occur by the same mechanism involving a chemically similar intermediate (Spector, 1973). The present results contradict this proposition as applied to double- vs. single-displacement mechanisms by showing that for at least two pairs of chemically matched reactions the mechanisms cannot be the same. Current stereochemical data on nucleotidyl- and phosphotransferases offer no support for the dominance of single- or double-displacement mechanisms in such reactions. However, the data do indicate the possibility that each enzymatic bond cleavage step at phosphorus in phosphates may be found to proceed with in-

version of configuration. In this respect a unifying mechanism for individual displacement steps may well exist.

We should like to speculate on the evolution of single- and double-displacement mechanisms in bisubstrate transferase reactions. In the cases of the transferases considered here, it is evident that, when the group acceptor substrates are sterically and electrostatically similar, the mechanism that has evolved is the double-displacement mechanism involving a free, covalently bonded enzyme–substrate as a compulsory intermediate. This is the case for galactose-1-P uridylyltransferase and nucleoside diphosphate kinase. When the group acceptor substrates are sterically and electrostatically grossly dissimilar, as in the cases of UDP-glucose pyrophosphorylase and adenylate kinase, the mechanism that has evolved is a single displacement involving compulsory ternary complexes of the enzyme with both substrates.

It is easily conceded that efficiency in the evolution of binding sites should lead to the evolution of a common binding subsite for the group acceptors when they are sterically and electrostatically very similar and that it would be efficient for two such acceptors both to bind at this subsite in the course of catalysis. Necessarily they could not both be bound simultaneously at the same site. So it is essential for the acceptor generated by loss of the group being transferred from the group donor to dissociate from its subsite before the other acceptor binds at this site to accept the group. This could not be practical unless the bond energy to the group being transferred is preserved in some way during the changeover from one acceptor to the other. This becomes possible by the evolutionary appearance of an enzymatic nucleophilic acceptor group so positioned as to be able to form a covalent bond to the group being transferred. This could result from a point mutation. The double-displacement mechanisms for galactose-1-P uridylyltransferase and nucleoside disphosphate kinase can be understood to have evolved in this way.

In contrast, the group acceptors for UDP-glucose pyrophosphorylase (and those for adenylate kinase) are sterically and electrostatically so dissimilar that no single binding subsite could have reasonable affinity for both, so that these enzymes have binding subsites for both acceptor substrates.

In these cases ternary complexes of enzyme, donor substrate, and acceptor substrate are formed, with the donor spanning one of the acceptor subsites as well as the subsite involved in binding the group being transferred. In such complexes it is evidently efficient for the enzyme to promote the direct transfer of the group from one acceptor to the other without the formation of an intermediate. In these circumstances the intermediate is not needed to protect the bond energy of the group being transferred, and so the formation of such an intermediate offers no decisive catalytic advantage over a mechanism involving direct transfer between acceptors.

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