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Sulfuryl Transfer Catalyzed by Pyruvate Kinase†

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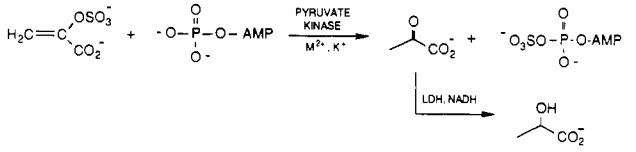
ABSTRACT: Sulfoenolpyruvate, the analogue of phosphoenolpyruvate in which the phosphate ester has been replaced by a sulfate ester, has been synthesized in three chemical steps from ethyl bromopyruvate in 40% overall yield. This compound is a substrate for pyruvate kinase, producing pyruvate and adenosine 5′-sulfatopyrophosphate. The latter compound has been identified by NMR spectroscopy and by comparison with an authentic sample. Sulfuryl transfer from sulfoenolpyruvate is 250–600-fold slower than phosphate transfer from phosphoenolpyruvate under identical conditions. Sulfoenolpyruvate is not a substrate for phosphoenolpyruvate carboxylase. Kinetic studies reveal that it does not bind to the active site; instead, it binds to the site normally occupied by glucose 6-phosphate and activates the enzyme in a manner similar to that shown by glucose 6-phosphate.

Mechanisms of enzyme-catalyzed phosphoryl group transfers continue to be studied by a variety of methods. Substrate analogues in which the phosphate group has been replaced by another anionic group (thiophosphates, phosphonates, etc.) have played an important role in this effort (Eckstein, 1983; Engel, 1977). Sulfate esters are structurally and electronically similar to phosphate esters, and the two families share much chemistry. Although sulfate esters have served as analogues of phosphate esters in a number of connections, we are aware of no reports of sulfuryl transfer catalyzed by enzymes that ordinarily catalyze phosphoryl transfer. We report here the first example of such a reaction.

Because of the central role of phosphoenolpyruvate (PEP)[1] in metabolism (Davies, 1979), this compound has been the target of substantial enzymatic and chemical experimentation. Most of the analogues, such as 3-bromophosphoenolpyruvate, 3-fluorophosphoenolpyruvate (Diaz et al., 1988; Stubbe & Kenyon, 1972; Gonzalez & Andreo, 1988), 1-carboxyallenyl phosphate (Wirsching & O'Leary, 1988), phosphoenol-α-ketobutyrate (Stubbe & Kenyon, 1971; Duffy et al., 1982), and others, involve variations distal to the phosphate group. Work done with phosphoenolthiopyruvate (Sikkema & O'Leary, 1988), thiophosphoenolpyruvate (Orr et al., 1978; Hansen & Knowles, 1982), α-(dihydroxyphosphinylmethyl)-acrylic acid (Stubbe & Kenyon, 1972), and methylphosphoenolpyruvate (Lazarus et al., 1979) represent the fewer cases utilizing variations in the phosphate functionality of PEP. These analogues show little or no activity and low affinity for enzyme active sites. To date, the only catalytically active analogues of PEP in which the phosphate is replaced by another group are the thiophosphates, in which an oxygen of the phosphate has been replaced by sulfur (Hansen & Knowles, 1982; Sikkema & O'Leary, 1988).

Scheme I



This paper describes the synthesis and study of sulfoenolpyruvate (SEP), a sulfate analogue of PEP. We present evidence that pyruvate kinase catalyzes a sulfuryl transfer to ADP to form adenosine 5′-sulfatopyrophosphate (Scheme I).

Materials and Methods

Materials

Sodium cyanoborohydride (Aldrich), DL-lactic acid (Aldrich), sulfur trioxide pyridine complex (Aldrich), sodium pyruvate (Sigma), oxalic acid (Mallinckrodt), tricyclohexylammonium phosphoglycolate (Sigma), disodium NADH (Sigma), and potassium ADP (Sigma) were used as supplied.

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[1] Abbreviations: SEP, sulfoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; THF, tetrahydrofuran; AP_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate.

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Trilithium AP_5A was purchased from Boehringer Mannheim. Ethyl bromopyruvate (Aldrich) was distilled under anhydrous conditions at reduced pressure and stored at 3 °C. HEPES (free acid) was purchased from U.S. Biochemical. Tetrahydrofuran (Aldrich Gold Label) was distilled from sodium–benzophenone ketyl. Chelex 100 chelating resin was purchased from Bio-Rad in the sodium form and washed with 1 M NaOH and water before use. Dowex 50X8-200 (H^+) ion-exchange resin was obtained from Aldrich Chemical Co. and was cleaned according to the method of Dunaway-Mariano and Cleland (1980) before use. Conversion to the potassium form was accomplished by rinsing with 4–5 column volumes of 1 M KOH and then with water. Water was purified with a Millipore Super Q water purification system.

Pyruvate kinase (rabbit muscle, type III), lactate dehydrogenase (rabbit muscle), and malate dehydrogenase (bovine heart) were purchased from Sigma Chemical Co. PEP carboxylase was isolated from maize as described by O'Leary, et al. (1981) and had a specific activity of 10 units/mg.

Methods

Proton nuclear magnetic resonance spectra were recorded at 200.13 MHz on a Bruker WP-200 Fourier transform spectrometer. Carbon NMR spectra were obtained at 90.6 MHz on a Bruker AM-360 or at 50.1 MHz on a JEOL FX-200 Fourier transform spectrometer. Chemical shifts are given relative to tetramethylsilane or sodium 3-(trimethylsilyl)-propionate for proton and carbon spectra. Dioxane or $CHCl_3$ was used as an internal standard for carbon spectra.

^{31}P spectra were recorded at 202.42 MHz on a Bruker AM-500 NMR spectrometer equipped with a 5-mm broad-band probe. All spectra were recorded at room temperature. A flip angle of 30° and a repetition rate of 5 s were employed. Chemical shifts are expressed relative to 85% H_3PO_4 as an external standard with the lock signal provided by D_2O; negative values are upfield from the standard.

IR spectra were recorded on a Beckman Acculab-7 or a Mattson Polaris FT-IR spectrometer. UV spectra and kinetics were measured on a Cary 118 or Cary 2200 spectrophotometer equipped with a thermostated cell compartment. Enzyme rate data were analyzed by using the computer programs of Cleland (1979).

For syntheses, all glassware and syringes were oven dried and cooled under nitrogen. Unless noted, all reactions were carried out under nitrogen. Moisture-sensitive reagents were weighed in a glovebag.

Synthesis. (*a*) *Pyridinium Methyl Sulfolactate.* A 10-mL round-bottom flask containing a magnetic stirring bar was charged with sulfur trioxide/pyridine complex (3.4 mmol, 524 mg) and 5 mL of dry THF. A 313 mg (3.0 mmol) sample of *dl*-methyl lactate was dissolved in 3 mL of THF and transferred by cannula to the stirring SO_3/pyridine solution over a period of 5 min. The reaction proceeded for 3 h at room temperature. During this time, product fell out as a brown/yellow oil.

The THF layer was carefully removed with a Pasteur pipet, and unreacted sulfur trioxide complex was precipitated by dissolving the oil in 20 mL of chloroform and refrigerating at 3 °C for 2 h. After separation of the precipitate by centrifugation, the chloroform was removed under reduced pressure to give a 97% yield (2.9 mmol, 722 mg) of pyridinium methyl sulfolactate. The salt decomposed to starting material rapidly at room temperature but could be stored overnight at 3 °C without substantial decomposition.

^1H NMR ($CDCl_3$, 200 MHz): δ 1.55 (3 H, d, J = 7.0 Hz), 3.75 (3 H, s), 5.02 (1 H, q, J = 7.0 Hz), 8.03 (2 H, br t, J = 7 Hz), 8.50 (1 H, m), 9.07 (2 H, br d, J = 5.2 Hz). ^{13}C NMR ($CDCl_3$, 90.6 MHz): δ 18.6, 52.1, 71.9, 127.4, 142.1, 146.5, 172.8. IR (neat): 3400 (br), 2980 (s), 2950 (s), 1750 (s), 1260 (s), 1140 (s) cm^{-1}.

(*b*) *Dipotassium Sulfolactate.* Pyridinium methyl sulfolactate was converted to the monopotassium salt by batchwise application of Dowex 50 (K^+). The slurry was filtered through paper to remove the Dowex, frozen, and lyophilized to yield a white, fluffy solid. Recovery was typically 90–95%.

A 575-mg sample (2.6 mmol) of potassium methyl sulfolactate was dissolved in a minimum of water, and 7 mL (2.5 equiv) of 1 M KOH was added with stirring. The solution immediately turned a fluorescent yellow. The mixture was stirred at room temperature for 1–2 h and then transferred to an Erlenmeyer flask and treated with 2–3 equiv of Dowex 50 (H^+) until the pH tested <1 by pH paper. The resin was filtered off, and the filtrate was treated with 3-4 equiv of Dowex 50 (K^+), gently stirred, and then filtered. The resulting solution was frozen and lyophilized to yield a yellow solid.

The crude product was recrystallized from methanol by dissolving the solid in boiling solvent, filtering out the solid matter, and cooling to room temperature. Anhydrous ether was added slowly to the methanol solution until it remained slightly cloudy. The mixture was kept at 3 °C for several hours, and the resulting white precipitate was isolated by centrifugation. Residual solvent was removed under vacuum to yield 195 mg of hygroscopic dipotassium sulfolactate.

^1H NMR (D_2O, 200 MHz): δ 1.43 (3 H, d, J = 6.9 Hz), 4.65 (1 H, q, J = 6.9 Hz). ^{13}C NMR (D_2O, 90.6 MHz): δ 10.2, 58.2, 170.3.

(*c*) *Ethyl 2-Hydroxy-3-bromopropanoate.* Caution: Reagents should be weighed and the reaction executed in a fume hood since HCN is liberated as a byproduct. The reduction was carried out at room temperature by a variation of the general method of Borch (1971). Ethyl 3-bromopyruvate (14 mmol, 2.8 g) dissolved in 4 mL of methanol was added in one portion to a 25-mL round-bottom flask containing a stirring solution of sodium cyanoborohydride (6.2 mmol, 388 mg), methanol (4 mL), and a trace of bromocresol green. The resulting mixture bubbled vigorously and turned dark blue, and 1 N methanolic HCl was added dropwise to restore a yellow color. The solution was maintained at pH 4 in this manner for 90 min.

Methanol was removed under a stream of nitrogen while heating at 45 °C with a water bath, and the resulting slurry was dissolved in 10 mL of saturated ammonium sulfate. The aqueous solution was extracted with 5 × 20 mL portions of ether, and the organic layers were combined and dried ($MgSO_4$). The volume was reduced to 10 mL and then placed at −78 °C for 1 h to precipitate ethyl 2-hydroxy-3-bromopropanoate. The solid was collected by centrifugation at 0 °C and blown dry with nitrogen to afford 1.98 g (10 mmol, 70% from ester) of pure product as a white powder, mp = 48–50 °C (lit. 49–50 °C; Bartlett & Chouinard, 1983).

^1H NMR ($CDCl_3$, 200 MHz): δ 1.34 (3 H, t, J = 7.2 Hz), 3.21 (1 H, d, J = 6.5 Hz); (ABX system) 3.69 (1 H, ^3J = 3.34 Hz, ^2J = 10.71 Hz), 3.73 (1 H, ^3J = 3.30 Hz, ^2J = 10.71 Hz), 4.51 (1 H, $^3J_{H-OH}$ = 6.5 Hz, ^3J = 3.34, 3.30 Hz); 4.32 [2 H, q of ABq, J(ABq) = 11.3 Hz, J(q) = 7.2 Hz]. ^{13}C NMR ($CDCl_3$, 50.1 MHz): δ 14.0, 34.9, 62.2, 69.7, 171.3.

(*d*) *Ethyl 3-Bromopropanoate 2-Sulfate Pyridinium Salt.* A 673-mg (3.4-mmol) sample of ethyl 2-hydroxy-3-bromopropanoate was dissolved in 4 mL of dry THF and transferred by cannula to a stirring solution of sulfur trioxide/pyridine complex (3.7 mmol, 590 mg) in 6 mL of THF. The reaction

was allowed to proceed at room temperature for 48 h, after which the THF was removed by using a stream of dry nitrogen and a warm water bath. The cream-colored residue was dissolved in 10 mL of $CHCl_3$ and placed at −15 °C for 2 h. The resulting precipitate (unreacted SO_3/pyridine) was removed by centrifugation, and the solution was decanted though a glass wool plug. The $CHCl_3$ was removed by using a nitrogen stream and an oil bath (60 °C) to afford the pyridinium salt of ethyl 3-bromopropanoate 2-sulfate in 91% yield (3.1 mmol, 1.11 g). ^1H NMR indicated purity of approximately 96%. The oil could be stored at −15 °C for 1–2 days without decomposition.

^1H NMR ($CDCl_3$, 200 MHz): δ 1.31 (3 H, t, J = 7.2 Hz); (ABX system) 3.81 (1 H, ^3J = 3.66 Hz, ^2J = 10.9 Hz), 3.92 (1 H, ^3J = 4.32 Hz, ^2J = 10.9 Hz), 5.27 (1 H, ^3J = 3.66, 4.32 Hz); 4.27 [2 H, q of ABq, J(ABq) = 11.3 Hz, J(q) = 7.2 Hz], 8.07 (2 H, br t, J = 6.7 Hz), 8.53 (1 H, tt, J = 7.8, 1.5 Hz), 9.10 (2 H, br d, J = 6.7 Hz). IR (neat): 3300 (s), 3100 (s), 2900 (s), 2100 (w), 1750 (s), 1250 (s), 1200 (s), 1100 (s), 750 (s) cm^{-1}.

(*e*) *Dipotassium Sulfoenolpyruvate.* To a stirring solution of the pyridinium salt of ethyl 3-bromopropanoate 2-sulfate (3.1 mmol, 1.1 g) in 1 mL of water was added 10 mL of 1 M KOH (10 mequiv of OH^-) in 1-mL portions over the course of 20 min. The final mixture was allowed to stir at room temperature for 60 min.

The resulting yellow solution was loaded onto an 11 × 1.2 cm column of Dowex 50 (H^+) and eluted with water into a flask cooled to 0 °C until the effluent tested pH 6 with paper. This acidic solution was immediately titrated with saturated $Ba(OH)_2$ until neutral. The water was removed under reduced pressure. The resulting white/yellow solid was dissolved in 50 mL of water and filtered. The barium salt of SEP was selectively precipitated by the addition of 400 mL of absolute ethanol to the filtrate. The ethanolic solution was placed at 3 °C overnight, and the solid was collected by centrifugation and washed with 2 × 10 mL of absolute ethanol and then 3 × 10 mL portions of anhydrous ether. Residual solvent was removed from the white solid under vacuum to yield 637 mg (2.1 mmol, 70% from pyridinium salt) of barium SEP.

The barium salt was dissolved in a minimum of water (approximately 20 mL), loaded onto a 10.5 × 1.2 cm column of Dowex 50 (K^+), and eluted with 3 column volumes of water. The solution was lyophilized to yield the desired dipotassium salt in 88% yield (1.8 mmol, 482 mg). No precipitate was observed upon addition of $AgNO_3$, $BaCl_2$, or K_2SO_4 to an aqueous solution of this product, indicating that the sample was free of Br^-, SO_4^{2-}, and Ba^{2+} impurities.

^1H NMR (D_2O, 200 MHz): δ 5.48 (1 H, d, J = 1.99 Hz), 5.73 (1 H, d, J = 1.99 Hz). ^{13}C NMR (D_2O, 125.7 MHz): δ 107.9 (dd, $^1J_{C-H}$ = 169 Hz), 149.9 (dd, $^2J_{C-H}$ = 6.6, 4.0 Hz), 169.9 (dd, $^3J_{C-H}$ = 8.6, 3.1 Hz).

(*f*) *Hydrolysis of Sulfoenolpyruvate.* The rate of hydrolysis of SEP was studied under acidic conditions by using a method similar to that used by Benkovic and Schray (1968) for PEP. The buffers utilized were HCl (pH 0–2.1), formate (pH 3), acetate (pH 4–5.1), MES (pH 6), and HEPES (pH 7.2). All buffers contained 0.2 M KCl and 1.7 mM EDTA, and buffer pH was adjusted at 75 °C. All kinetic runs were carried out with 5 mM SEP in screw-top vials at 75 ± 0.5 °C in a Haake FT water bath. Aliquots were removed at intervals and quenched with $NaHCO_3$ at 0 °C, and the reaction was assayed with lactate dehydrogenase.

As an additional test for purity and extent of hydration in the final product, a carefully weighed sample of SEP was dissolved in 0.1 M HCl and hydrolyzed to pyruvate by heating at 85 °C for 3 h. This procedure indicated that sample purity was greater than 92%. The remaining 8% was assumed to be water of hydration.

(*g*) *Adenosine 5'-Sulfatopyrophosphate.* An NMR scale reaction was run based on the preparation by Ikehara et al. (1961). A 5-mL round-bottom flask containing a stirring bar was charged with $NaHCO_3$ (100 mg) and ADP (16 mg), 1 mL of D_2O was added, and the solution was stirred until all the solid dissolved. To this mixture, 67 mg of SO_3/pyridine was added in one portion. The solution expelled CO_2 vigorously, and the smell of pyridine was noted. This reaction mixture was heated with stirring at 45 °C for 40 min and then filtered through a glass wool plug into an NMR tube.

Enzyme Studies. All solutions were prepared fresh for each experiment and kept on ice throughout. PEP carboxylase was dialyzed against 50 mM HEPES buffer at pH 7.5 for approximately 12 h. Kinetic studies were run in 1-mL total volume in 1-cm cuvettes. Enzyme solutions were assayed periodically throughout the experiments to assure that no loss of activity occurred.

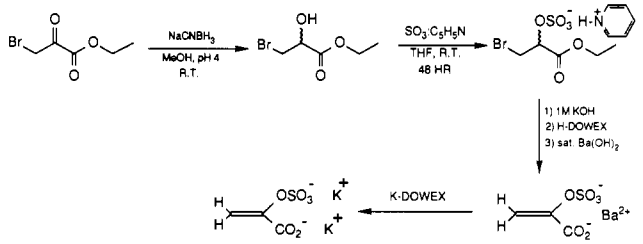
(*a*) *Pyruvate Kinase Kinetics.* Unless noted, all assays contained 0.1 M HEPES buffer, pH 7.5, 100 mM KCl, 3 mM ADP, 0.2 mM NADH, 20 units of lactate dehydrogenase, and 3 mM $MnCl_2$ or 5 mM $MgCl_2$ and were performed at 37 °C. Reactions were initiated by addition of pyruvate kinase.

For studies of the pyruvate kinase concentration dependence of SEP substrate activity, assays were conducted in the presence of 20 mM SEP. The reaction was initiated by the addition of various amounts of pyruvate kinase ranging from 0 to 90 μg/mL. The same assay was performed in the absence of nucleotide, with identical enzyme concentrations. The pyruvate kinase used was tested for nonspecific phosphatases by assaying an ADP-deficient solution containing 5 mM PEP. As a third control, SEP was withheld from the assay solution with subsequent addition of pyruvate kinase as described above.

(*b*) *^{31}P NMR Sample Preparation.* Reaction mixtures were composed of 0.1 M HEPES buffer (650 μL), pH 7.5, 3 mM $MnCl_2$, 100 mM KCl, 10 mM ADP, 300 μM AP_5A, 0.1% bovine serum albumin, 65 mM SEP, and 3–5 mg of pyruvate kinase. A control was run in parallel containing all components except SEP. Mixtures were incubated at 25 °C for 5 h, after which the samples were loaded onto separate 1 × 8 cm columns of Chelex 100 (Na^+) at 4 °C and eluted with approximately 20 mL of water. These solutions were lyophilized, and the resulting solid was dissolved in 650 μL of HEPES buffer, pH 8.0, containing 0.3 mM EDTA and 10% D_2O (for field-frequency lock).

(*c*) *Partitioning of SEP between Hydrolysis and Sulfuryl Transfer.* To study the partitioning of SEP between a sulfuryl transfer to ADP and hydrolysis, the experiment outlined in Scheme III was performed. To 1 mL of a solution containing 0.1 M HEPES buffer, pH 7.5, 100 mM KCl, 4 mM $MnCl_2$, 0.1 % BSA, 200 μM AP_5A, 5 mM ADP, and 35 mM SEP was added approximately 4 mg of pyruvate kinase. This mixture was incubated at 25 °C, and 20-μL aliquots were withdrawn at intervals and assayed for pyruvate formed and ADP remaining in the reaction mixture. The 50-fold dilution of the reaction mixture essentially eliminated the further reaction of SEP with pyruvate kinase, allowing for accurate determination of pyruvate and ADP. The aliquot was injected into a 1-mL cuvette containing 40 units of lactate dehydrogenase and 0.2 mM NADH. Calculations based on the absorbance change indicated the amount of pyruvate produced. Excess PEP was then added to the same cuvette, and a second ab-

Scheme II



sorbance change due to the reaction of PEP with the remaining ADP was recorded. This change should reflect the amount of ADP present.

(*d*) *Phosphoenolpyruvate Carboxylase.* PEP carboxylase was assayed by observing the disappearance of NADH at 340 nm in the presence of 0.1 M HEPES, pH 8.0, 5 mM $NaHCO_3$, 0.2 M NADH, 1 mM $MnCl_2$ or 5 mM $MgCl_2$, and 20 units of malate dehydrogenase at 25 °C. Reactions were initiated by the addition of enzyme.

SEP was tested as a substrate for PEP carboxylase by using malate dehydrogenase or lactate dehydrogenase coupling assays. Assays contained 20 mM SEP and 20 units of malate dehydrogenase or lactate dehydrogenase. The same experiment was run in parallel with 5 mM PEP. Sixty times as much enzyme was used with SEP as with PEP in these experiments.

PEP carboxylase activation by glucose 6-phosphate was studied in 0.1 mM EDTA, 0.1% bovine serum albumin, 1 mM PEP, and 0–20 mM glucose 6-phosphate. Activation by SEP was studied by using the same assay except in the presence of 0–20 mM SEP. The combined effect of SEP and glucose 6-phosphate on PEP carboxylase was assayed by varying glucose 6-phosphate from 0 to 20 mM while varying SEP over a range of 0–10 mM.

Results

Synthesis of 2-Sulfolactate. 2-Sulfolactate was synthesized by the sulfation of methyl lactate with sulfur trioxide/pyridine complex. Hydrolysis of the methyl ester with aqueous base, ion-exchange chromatography, and recrystallization from methanol yielded the desired product.

Synthesis of Sulfoenolpyruvate. The synthetic sequence is given in Scheme II. Commercially available ethyl bromopyruvate was reduced to the corresponding lactate derivative. To avoid formation of ethyl glycidate, the reduction was carried out under acidic conditions with cyanoborohydride (Borch et al., 1971). Subsequent sulfation with the pyridine complex of sulfur trioxide in tetrahydrofuran yielded the β-bromo sulfate ester in good yield. Attempts to perform the sulfation step in dimethylformamide gave poor yields and substantial decomposition even with long reaction times or elevated temperatures. Aqueous base-catalyzed dehalogenation and deesterification of the β-bromo sulfate ester yielded the desired enol sulfate and bromide ion. Precipitation and cation exchange yielded the potassium salt of SEP in approximately 40% overall yield. The cyclohexylammonium salts proved to be difficult to handle and were not easily purified.

Hydrolysis of Sulfoenolpyruvate. The acid-catalyzed hydrolysis of SEP was monitored by enzymatic assay of pyruvate production vs time. Little or no detectable hydrolysis of SEP occurred above pH 2 at 75 °C. At pH <2, pseudo-first-order kinetics was observed for at least 2–3 half-lives and hydrolysis occurred rapidly with rate constants of 0.05 min^{-1} at pH 1 and 0.15 min^{-1} at pH 0.2. This is nearly an order of magnitude faster that the hydrolysis of PEP under the same conditions (Benkovic & Schray, 1968).

Table I: Inhibition of Pyruvate Kinase by PEP Analogues at pH 7.5 in the Presence of Mn^{2+} or Mg^{2+} [a]

analogue	K_i (Mn^{2+}) (mM)	K_i (Mg^{2+}) (mM)
L-phospholactate[b]	0.383 ± 0.056	
D-phospholactate[b]	0.021 ± 0.005	
DL-sulfolactate	2.2 ± 0.3	5.2 ± 0.6
phosphoglycolate[c]		3.5 ± 0.6
sulfoglycolate[c]		16
sulfoenolpyruvate	3.1 ± 0.5	5.8 ± 0.3

[a] Reactions are coupled to lactate dehydrogenase in the presence of ADP, K^+, and NADH. [b] Nowak & Mildvan, 1970. [c] Dougherty & Cleland, 1987.

Table II: Inhibition of PEP Carboxylase by PEP Analogues at pH 7.5 in the Presence of Mn^{2+} or Mg^{2+} [a]

analogue	K_i (Mn^{2+}) (mM)	K_i (Mg^{2+}) (mM)
L-phospholactate[b]	0.0011	0.10
DL-sulfolactate[c]	8.5 ± 1.3	>20

[a] Reactions are coupled to malate dehydrogenase in the presence of HCO_3^- and NADH. [b] O'Leary, 1982. [c] There was a very small intercept effect seen; K_i listed is K_{is}.

Table III: Kinetic Parameters for Interaction of Pyruvate Kinase with Sulfoenolpyruvate[a]

metal	substrate	K_m (mM)	V_{max} (units/mg)	V/K (min^{-1} mg)
Mn^{2+}	PEP	0.040 ± 0.005	60 ± 2	1500 ± 120
	SEP[b]	7.2 ± 0.7	0.10 ± 0.01	0.014 ± 0.001
Co^{2+}	PEP	0.025 ± 0.001	74 ± 1	2960 ± 120
	SEP	8.7 ± 0.6	0.29 ± 0.01	0.033 ± 0.002

[a] At pH 7.5, 37 °C, in the presence of lactate dehydrogenase, ADP, K^+, and NADH. [b] K_m for ADP with this compound is 0.27 ± 0.03 mM.

Inhibition of Pyruvate Kinase by 2-Sulfolactate. When tested against PEP, 2-sulfolactate showed linear competitive inhibition in the presence of either Mn^{2+} or Mg^{2+}. The inhibition constants for various sulfate and phosphate analogues of PEP are given in Table I.

Inhibition of Phosphoenolpyruvate Carboxylase by 2-Sulfolactate. 2-Sulfolactate showed noncompetitive inhibition of PEP carboxylase when tested against PEP in the presence of Mn^{2+} (Table II). The intercept effect observed was small (K_{ii} = 37 mM). The reason for this small effect is not known. Only slight inhibition was observed even at 20 mM inhibitor with Mg^{2+} as the metal cofactor.

Reaction of Sulfoenolpyruvate with Pyruvate Kinase. SEP showed linear competitive inhibition of pyruvate kinase when tested against PEP in the presence of Mn^{2+} or Mg^{2+} at pH 7.5 (Table I).

When SEP was treated with pyruvate kinase at pH 7.5 in the presence of ADP, lactate dehydrogenase, NADH, and Mn^{2+} or Co^{2+} at pH 7.5, a decrease in absorbance at 340 nm was observed due to the generation of pyruvate and consequent oxidation of NADH.

Conversion of SEP to pyruvate showed saturation kinetics with pyruvate kinase, and activity was linear with enzyme concentration over the entire concentration range used. No activity was observed in the absence of nucleotide or metal cofactor, and nonspecific phosphatases were not detected in ADP-deficient solutions containing PEP, lactate dehydrogenase, NADH, and pyruvate kinase. Likewise, assay solutions lacking SEP showed no change in absorbance at 340 nm upon addition of pyruvate kinase.

Initial velocity studies at pH 7.5 in the presence of Mn^{2+} indicate that the kinetic mechanism is sequential since the double-reciprocal plots show characteristic intersecting patterns

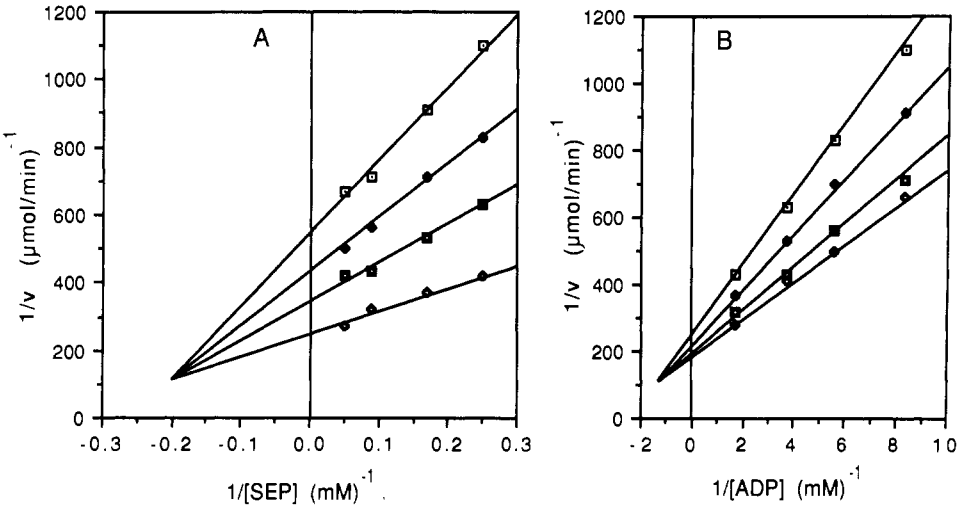


FIGURE 1: (A) Reciprocal plot of initial velocity vs SEP concentration for the reaction of pyruvate kinase with SEP and ADP at 37 °C, pH 7.5, with 0.12 mM ADP (⊡), 0.18 mM ADP (♦), 0.27 mM ADP (□), and 0.60 mM ADP (◇). (B) Reciprocal plot of initial velocity vs ADP concentration with SEP concentration of 4 mM (⊡), 6 mM (♦), 11 mM (□), and 20 mM (◇).

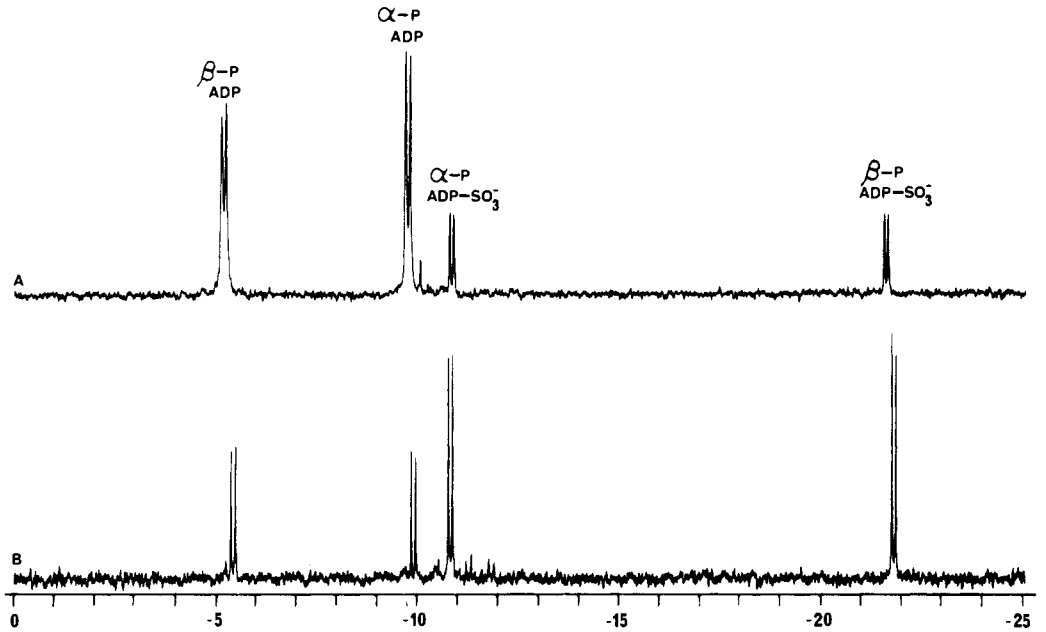


FIGURE 2: ^{31}P NMR spectra (202 MHz) of (A) synthetic adenosine 5′-sulfatopyrophosphate, pH 7.5, and (B) the product generated by reaction of SEP with ADP and pyruvate kinase at pH 8.0. The reaction mixture contained 3 mM $MnCl_2$, 100 mM AP_5A, 0.1 mM bovine serum albumin, 65 mM SEP, and 5 mg/mL pyruvate kinase. Metals were removed before the spectra were run.

with linear replots (Figure 1). Kinetic constants are given in Table III. Saturation kinetics was seen with all metals except Ni^{2+}, where no activity was detected under the assay conditions. When Mg^{2+} was used as the metal activator, activity was less than half that seen with Mn^{2+}. On the basis of maximal velocities, the effectiveness of the cations decreased in the order $Co^{2+} > Mn^{2+} > Mg^{2+} > Ni^{2+}$ (no activity). The order of effectiveness for PEP is $Mg^{2+} = Co^{2+} > Mn^{2+} > Ni^{2+}$ (Baek & Nowak, 1982).

Two competitive inhibitors against PEP, phosphoglycolate and oxalate, are also competitive inhibitors against SEP at pH 7.5 in the presence of Mn^{2+} at 37 °C. The calculated K_i values were 4.3 ± 1.2 μM for oxalate, in good agreement with the value of 6.0 ± 1.5 μM against PEP (Reed & Morgan, 1974), and 37 ± 2 μM for phosphoglycolate.

Product Analysis. In an attempt to determine whether pyruvate kinase catalyzes sulfuryl transfer to ADP or simply the hydrolysis of SEP, ^{31}P NMR spectra were obtained following the reaction (Figure 2). The phosphorus chemical shifts and coupling constants for the product generated with pyruvate kinase match those of synthetic adenosine 5′-sulfatopyrophosphate. The slight difference in chemical shifts arises from the difference in sample composition and pH (see Figure 2). The presence of ADP in the spectrum is due to the occurrence of a small amount of hydrolysis of ADP sulfate during the workup. Thus, these spectra indicate that pyruvate kinase catalyzes the transfer of the sulfuryl group of SEP to the β-phosphate of ADP. The control containing pyruvate kinase but no SEP showed a small amount of ATP formation due to contaminating myokinase activity but no indication of ADP sulfate.

To assign the α- and β-phosphates of adenosine 5′-sulfatopyrophosphate, the proton-coupled spectrum was acquired, and this spectrum clearly showed that the −10.8 ppm resonance is coupled to the 5′-methylene hydrogens and thus corresponds to the α-phosphate. Thus, under these conditions, the α-phosphate resonance comes at −10.8 ppm and the β-phosphate at −21.8 ppm, with J_{pp} = 20.0 Hz. Under identical conditions,

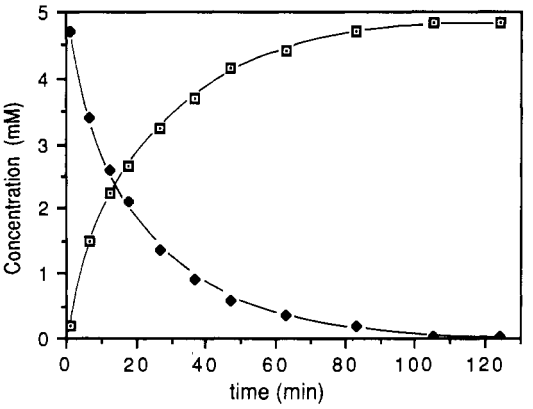


FIGURE 3: Time course of pyruvate formation and ADP depletion in the pyruvate kinase catalyzed sulfuryl transfer from SEP to ADP at pH 7.5, 25 °C: (□) pyruvate; (♦) ADP.

ADP gave peaks at −5.4 and −9.9 ppm, J_{pp} = 21.9 Hz, for the β- and α-phosphates, respectively (Figure 2).

Yield of ADP Sulfate. The procedure outlined in Scheme III was used to measure the appearance of pyruvate (product) and the amount of unreacted ADP (substrate) vs time. Figure 3 clearly shows a 1:1 stoichiometry between pyruvate formation and ADP depletion over the entire reaction, indicating that SEP transfers a sulfuryl group to ADP *exclusively*, with no pyruvate kinase catalyzed hydrolysis of SEP.

Interaction of Sulfoenolpyruvate with Phosphoenolpyruvate Carboxylase. Many PEP analogues give corresponding pyruvate analogues with PEP carboxylase via an enzyme-catalyzed hydrolysis pathway (Diaz et al., 1988). For this reason, product formation with SEP was monitored with lactate dehydrogenase (hydrolysis) as well as malate dehydrogenase (carboxylation). SEP was neither hydrolyzed nor carboxylated in the presence of $NaHCO_3$, Mn^{2+} or Mg^{2+}, and PEP carboxylase even at high levels of enzyme.

Surprisingly, SEP did not appear to inhibit PEP carboxylase; instead, activation was observed. When PEP concentration was varied at various concentrations of SEP, the kinetics indicated that SEP activates by lowering the apparent K_m for PEP with little or no detectable effect on V_{max}. Little activation was observed with Mg^{2+} as the metal activator except at high concentrations of SEP (>10 mM). With Mn^{2+} as the metal cofactor, SEP activation showed saturation kinetics. The activating effect was much larger in the case of Mn^{2+}: In the presence of 1 mM PEP, a total activation of 2.4-fold could be achieved, and half of this activation was attained at approximately 8 mM SEP.

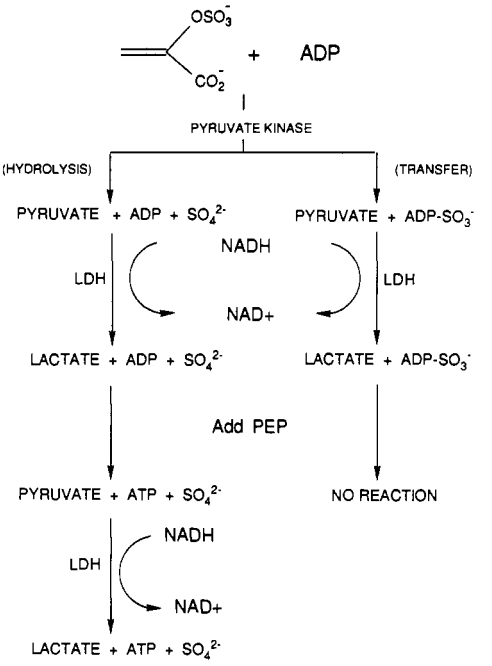
At pH 8.0, glucose 6-phosphate activates PEP carboxylase in the presence of Mg^{2+} (Coombs et al., 1973; Wong & Davies, 1973; Gonzalez et al., 1984). Our studies indicate that a similar and more pronounced effect is seen when Mn^{2+} is used as the activating metal. In the presence of 1 mM PEP, 8 mM glucose 6-phosphate, and 1 mM Mn^{2+}, enzyme activity was 301% that of nonactivated enzyme. In the presence of 5 mM Mg^{2+}, activity was 205% of the control.

The presence of high concentrations of glucose 6-phosphate reduces the activating ability of SEP. At 20 mM glucose 6-phosphate, the activating effect of SEP is essentially eliminated.

Discussion

When SEP is treated with pyruvate kinase in the presence of a divalent metal, K^+, and ADP, pyruvate and adenosine 5′-sulfatopyrophosphate are formed (Scheme I). The latter compound results from sulfuryl transfer from SEP to ADP.

Scheme III



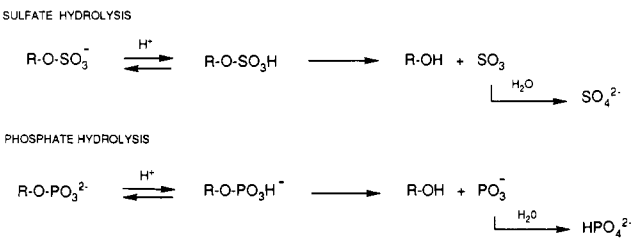
The reaction rate is 600 (Mn^{2+}) or 250 (Co^{2+}) time slower than that observed with PEP under identical conditions (Table III). To our knowledge, this is the first example of sulfuryl transfer in a kinase reaction.

Both products have been conclusively identified. Pyruvate was identified by its reaction with NADH in the presence of lactate dehydrogenase. Adenosine 5′-sulfatopyrophosphate was identified by its NMR spectrum (Figure 2). The lack of a doublet resonance at −4.5 ppm characteristic of the γ-phosphate of ATP clearly indicates that the nucleotide formed is not ATP. Also, the upfield resonance at −21.8 ppm in Figure 2 is a doublet, whereas that in ATP is a triplet due to coupling to the α- and γ-phosphorus atoms. Synthesis of authentic adenosine 5′-sulfatopyrophosphate by the literature procedure (Ikehara et al., 1961) provided material that was identical in every respect with that obtained in the pyruvate kinase reaction. Conclusive assignment of the phosphorus resonances of adenosine 5′-sulfatopyrophosphate was based on proton-coupled ^{31}P NMR (not shown), where the signal at −10.8 ppm was clearly coupled to two protons, whereas the resonance at −21.8 ppm was unaffected. Thus, in analogy to ATP, we assign the signal at −10.8 ppm to the α-phosphate of adenosine 5′-sulfatopyrophosphate.

Kinetics of Sulfate Transfer to ADP. To examine the transfer mechanism more closely, we performed a variety of kinetic experiments. When initial velocity data were plotted as reciprocal plots, a linear intersecting pattern was obtained (Figure 1), indicating that the kinetic mechanism of the reaction is sequential. This result is consistent with the sequential kinetics observed for the reaction of PEP (Ainsworth & Macfarlane, 1973). The fact that the reaction is ADP dependent and is inhibited by known competitive inhibitors of pyruvate kinase further confirms that the reaction occurs at the enzyme active site. Pyruvate kinase catalyzes the transfer of a sulfuryl group from SEP to ADP without catalyzing the simple hydrolysis of SEP to pyruvate.

Interestingly, metal dependences are very different for phosphate transfer and sulfate transfer by pyruvate kinase. With PEP as substrate, the relative effectiveness of divalent metals is $Mg^{2+} = Co^{2+} > Mn^{2+} > Ni^{2+}$ (Baek & Nowak, 1982). In the case of SEP, Mn^{2+} is much more effective than

Scheme IV



Mg^{2+} on the basis of maximal velocities. Furthermore, it appears that the difference in metal activation is not due to tighter binding of SEP since the K_i values for inhibition against PEP are similar (Table I). The variation in metal preference might be due to a change in the amount of nonproductive substrate binding experienced and/or to the relative abilities of phosphate and sulfate groups to form inner-sphere complexes with these metals.

Interaction of Sulfoenolpyruvate with Phosphoenolpyruvate Carboxylase. The alternate substrates tested to date with PEP carboxylase turn over via either a carboxylation or hydrolysis pathway (Diaz et al., 1988). SEP was originally designed as a possible inhibitor or substrate for PEP carboxylase, but this compound is neither hydrolyzed nor carboxylated by the enzyme. Instead, this compound shows kinetics characteristic of enzyme activation and appears to affect the K_m for PEP while leaving V_{max} unaffected. A similar effect is seen with glucose 6-phosphate, where most of the effect is due to the lowering of K_m for PEP (Ting & Osmond, 1973), but there may also be a small increase in V_{max} (Coombs et al., 1974).

In an attempt to determine the nature of the SEP activation, we studied the effect of added glucose 6-phosphate on this process. The results suggest that as the concentration of glucose 6-phosphate is increased, the ability of SEP to activate diminishes. This result is most simply consistent with the two compounds binding at a common binding site. Furthermore, the data indicate that the principal difference between SEP and glucose 6-phosphate is the strengths of their binding at the allosteric site, not their ability to activate the enzyme, since saturation with either compound increases the maximum velocity to the same extent. Why SEP binds at the possible allosteric site is not known. Both glucose 6-phosphate and SEP are dianions, and this common feature may be related to their common binding mechanisms. Recently, Jenkins et al. (1986) have seen similar behavior with the dianion methyl 2-(dihydroxyphosphinylmethyl)-2-propenoate.

Inhibitory Properties of Sulfate and Phosphate Esters. Tables I and II list K_i values for a variety of phosphate and sulfate esters designed to inhibit PEP-utilizing enzymes. Since both pyruvate kinase (Dougherty & Cleland, 1985) and PEP carboxylase (O'Leary et al., 1981) prefer to bind PEP as the trianion, some loss of affinity for the enzyme is expected with the dianionic sulfate analogues. With pyruvate kinase, a decrease of 5-fold and 10-fold in binding is observed in the glycolate and lactate pairs, respectively (Table I).

PEP carboxylase is much more discriminating between the di- and trianionic species, with sulfolactate binding nearly 2 orders of magnitude less tightly than phospholactate. If metal–phosphate coordination is the primary binding mechanism in PEP carboxylase, perturbation of the charge by substitution with sulfate is expected to be felt strongly. In the case of pyruvate kinase, two divalent metals are required for catalysis, one enzyme bound and the other required to complex the nucleotide substrate, as well as a monovalent cation. The large number of potential metal–substrate interactions in this enzyme could attenuate the binding effects due to the loss of charge in substituting sulfate for phosphate. Steric effects are unlikely to be important in the phosphate/sulfate difference because the typical bond angles and lengths of phosphate and sulfate esters are quite similar.

Reactivity of Sulfate Esters. The normal mode of basic hydrolysis of sulfate esters involves sulfate displacement via carbon–oxygen bond cleavage (Calhoun & Burwell, 1955). SEP, having an sp^2-hybridized carbon at C-2, is therefore expected to be stable to base. Indeed, SEP appears stable in 1 M KOH (see synthesis) for up to an hour without noticeable decomposition. No decomposition under normal assay conditions (pH 7–8) was detectable over several hours.

On the other hand, SEP is easily hydrolyzed to pyruvate and sulfate in 1–2 h at pH 1, 75 °C. Under strongly acid conditions, sulfate monoesters are thought to hydrolyze via an A-1 mechanism that involves preequilibrium proton transfer, followed by rate-determining unimolecular decomposition to eliminate SO_3 (Benkovic, 1972; Douglas, 1976). The pH dependence of the hydrolysis of SEP is consistent with this explanation. This mechanism is much like the acid-catalyzed reactions of phosphate monoester monoanions, which eliminate metaphosphate after a preequilibrium proton transfer (Scheme IV; Kaiser et al., 1962). Interestingly, SEP hydrolysis is about an order of magnitude faster than PEP hydrolysis at low pH.

The facility with which pyruvate kinase catalyzes sulfuryl transfer from SEP to form ADP sulfate suggests that the reaction occurs by a dissociative mechanism, as associative mechanisms for sulfuryl transfer are rare or unknown. In turn, this suggests that pyruvate kinase might also operate on PEP itself by a dissociative mechanism.

Elucidation of the kinetics and mechanism of other potential sulfuryl-transferring kinases is under current investigation.

Registry No. PEP, 138-08-9; SEP, 118319-52-1; ADP, 58-64-0; Mn, 7439-96-5; Co, 7440-48-4; pyruvate kinase, 9001-59-6; adenosine 5′-sulfatopyrophosphate, 4241-77-4; ethyl 3-bromopyruvate, 70-23-5; PEP carboxylase, 9067-77-0; pyridinium methyl sulfolactate, 118334-52-4; *dl*-methyl lactate, 2155-30-8; dipotassium sulfolactate, 118334-53-5; potassium methyl sulfolactate, 118334-57-9; ethyl 2-hydroxy-3-bromopropanoate, 92234-23-6; ethyl 3-bromopropanoate-2-sulfate pyridinum salt, 118334-55-7; dipotassium sulfoenolpyruvate, 118334-58-0; DL-sulfolactic acid, 93713-87-2.

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Characterization of Fucosyltransferase Activity during Mouse Spermatogenesis: Evidence for a Cell Surface Fucosyltransferase†

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ABSTRACT: Fucosyltransferase activity was quantified in mouse germ cells at different stages of spermatogenesis. Specifically, fucosyltransferase activities of pachytene spermatocytes, round spermatids, and cauda epididymal sperm were compared. Fucosyltransferase activity of mixed germ cells displayed an apparent V_{max} of 17 pmol (mg of protein)$^{-1}$ min^{-1} and an apparent K_m of approximately 13 μM for GDP-L-[^{14}C]fucose in the presence of saturating amounts of asialofetuin at 33 °C. Under these conditions, cellular fucosyltransferase activity was found to increase during spermatogenesis. In agreement with assays of intact cells, examination of subcellular fractions indicated that a large fraction of fucosyltransferase activity was associated with the cell surface. The fraction of fucosyltransferase activity that was associated with the cell surface progressively increased throughout spermatogenesis and epididymal maturation so that nearly all of the fucosyltransferase in epididymal sperm was on the cell surface. Specifically, by comparison of activities in the presence and absence of the detergent NP-40, the fraction of fucosyltransferase activity that was associated with the cell surface in pachytene spermatocytes, round spermatids, and epididymal sperm was 0.36, 0.5, and 0.85, respectively. These results suggest that a cell surface fucosyltransferase may be important during differentiation of spermatogenic cells in the testis as well as during epididymal maturation and fertilization.

Spermatogenesis involves complex morphological and biochemical events leading to the development of mature spermatozoa from proliferating germ cells. The development of a mature sperm from its stem cell, the spermatogonium, can be divided into four phases: (1) mitotic proliferation of stem cells, (2) meiosis, (3) the formation of highly specialized spermatozoa (spermiogenesis), and (4) the release of sperm into the lumen of the seminiferous tubule (spermiation). Further maturational changes of spermatozoa occur during epididymal transit. Although relatively little is known about the biochemical events that regulate spermatogenesis, macromolecules at the cell surface, many of which are glycosylated, likely play a prominent role in the development of germ cells.

Glycosylation of proteins occurs via specific glycosyltransferases, and, with few exceptions, the Golgi apparatus appears to be the site of the oligosaccharide elongation reactions. Developing spermatogenic cells represent an interesting situation since after meiosis the Golgi apparatus of the newly formed round spermatid undergoes impressive morphological

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