On the Mechanism of de Novo Polymerization by Form I Polynucleotide Phosphorylase of *Micrococcus luteus*†

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ABSTRACT: The diastereomers of adenosine 5′-*O*-(1-thiodiphosphate) (ADPαS) have been tested as substrates for the polymerization reaction of primer-independent polynucleotide phosphorylase from *Micrococcus luteus*. The preferred substrate is ADPαS(S_P), which has a similar K_m and a greatly reduced V_{max} when compared to the natural substrate ADP. The other diastereomer, ADPαS(R_P), is preferentially cleaved by a polyphosphate kinase activity (present with the phosphorylase) that may be responsible for the removal of the 5′-β-phosphate during de novo polymerization, leading to the observed 5′-phospho-poly(A). Inhibitor studies suggest that the kinase and de novo polymerization sites are not coincident. During de novo polymerization of the diastereomeric mixture, ADPαS(R_P) is selectively used to form 5′ termini, whereas ADPαS(S_P) serves to support the chain elongation. Thus there are two stereochemically distinct subsites for initiating polymerization. ADPβS functions as a substrate for polynucleotide phosphorylase with kinetic properties similar to those of ADP, indicating that removal of the β-phosphate (a thiophosphate) is not a kinetically important step and probably occurs after polymerization is complete. The average chain length of the polymeric product is considerably smaller for ADPαS vs. ADPβS or ADP, suggesting that the degree of processivity of the polymerization is determined by competition between the rate of polymerization and the rate of dissociation of the growing chain.

Primer-independent polynucleotide phosphorylase from *Micrococcus luteus* catalyzes the processive de novo polymerization of nucleoside diphosphates in a 5′ → 3′ direction. The product of polymerization is a high molecular weight polymer ($n \simeq 700$), which is terminated by a 5′-phosphate (Godefroy-Colburn & Grunberg-Manago, 1972). The polymerization reaction is only slightly stimulated by oligonucleotide primers, and incorporation of such primers is not readily observed (Moses & Singer, 1970).

Recently chiral thiophosphate analogues have been employed to study both the polymerization (Burgers & Eckstein, 1979) and the [^{32}P]P_i exchange (Marlier et al., 1981) reactions. The S_P diastereomer of ADPαS[1] is the preferred substrate for both reactions, but ADPαS(R_P) catalyzes the exchange of [^{32}P]P_i into ADPαS(S_P). A two-site model was proposed in which ADPαS(R_P) could be utilized only in the first step of de novo polymerization and therefore was incorporated into the 5′ terminus of the growing polymer. This paper presents evidence supporting this mechanism.

In addition, an enzymatic activity present with polynucleotide phosphorylase, previously shown to cleave the P_β–O bond of ADPαS(R_P) (Marlier et al., 1981), has now been examined in greater detail. The relationship of this activity to the overall polynucleotide phosphorylase mechanism is discussed.

Materials and Methods

All organic solvents were reagent grade and were used without further purification, with the following exceptions: (1) pyridine was dried over CaH_2 and distilled from the same; (2) triethyl phosphate was distilled under high vacuum; (3) dioxane was dried over sodium metal and distilled; (4) dimethylformamide was dried over CaH_2 and distilled under high vacuum. [2-^{3}H]Adenosine was from New England Nuclear and had a specific radioactivity of 18.6 Ci/mmol. ADPβS from Boehringer Mannheim was purified by DEAE-Sephadex chromatography prior to use. Unlabeled ADPαS was prepared as described previously (Marlier et al., 1981).

Polynucleotide phosphorylase (form I) was obtained from P-L Biochemicals and had a specific activity of 120 polymerization units/mg of protein. Adenylic acid deaminase (71 units/mg of protein), pyruvate kinase (1000 units/mg of protein), and alkaline phosphatase (29 units/mg of protein) were obtained from Sigma Chemical Co.

Whatman DEAE-cellulose (DE-52) was obtained from Reeve-Angel; Sephadex G-50 and DEAE-Sephadex A-25 were from Pharmacia. Liquiscint from National Diagnostics was employed as the liquid scintillation cocktail. Deionized water for all experiments was obtained from a Millipore Q filtration system.

Optical densities were measured on a Gilford UV-VIS spectrophotometer; UV spectra were run on a Cary 118 spectrophotometer. Radioactivity was measured on a Beckman Model 8100 liquid scintillation counter. HPLC was performed on an Altrex Model 100, equipped with a 254-nm UV detector. Two columns were used in these studies. A Whatman Partisil PXS 10/25 SAX column eluted with a linear gradient of 0.007 M KH_2PO_4, pH 4.0, to 0.25 M KH_2PO_4, 0.5 M KCl, at pH 4.5. The gradient was completed in 45 min, with a flow rate

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[1] Abbreviations: ADPαS(S_P) and ADPαS(R_P), diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ATPαS(S_P) and ATPαS(R_P), diastereomers of adenosine 5′-*O*-(1-thiotriphosphate); AMPS, adenosine 5′-*O*-thiophosphate; Ap(S)A(R_P) and Ap(S)A(S_P), diastereomers of adenylyl (3′–5′)adenylyl-*O*,*O*-phosphorothioate; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); NDP, nucleoside 5′-*O*-(diphosphate); ApA, adenylyl (3′–5′)adenylyl-*O*,*O*-phosphate; pAp(S)A, adenyl 5′-phosphate (3′–5′)adenyl-*O*,*O*-phosphorothioate; pp(S)Ap(S), adenosine 5′-*O*-(1-thiodiphosphate) 3′-*O*-(thiophosphate); p(S)Ap(S), adenosine 5′,3′-thiodiphosphate; p(S)Ap, adenosine 5′-thiophosphate 3′-phosphate; Ap(S), adenosine 2′- or 3′-thiophosphate; A>p(S), adenosine cyclic 2′,3′-thiophosphate; A>p, adenosine cyclic 2′,3′-phosphate; DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; PEP, phosphoenolpyruvate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; Mops, 4-morpholinepropanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography.

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of 1.5 mL/min. The second column was a Whatman PXS 10/25 ODS reverse-phase column, eluted with 0.1 M K_2HPO_4 (pH 6.0) at a flow rate of 1.5 mL/min. All ^{31}P NMR spectra were run on a Bruker WM360 at 145.805 MHz. Chemical shifts are from H_3PO_4.

Synthesis of [2-^{3}H]ADPαS(R_P). All 3 mCi of [2-^{3}H]-adenosine was lyophilized to dryness and 136 mg (500 μmol) of cold adenosine added. The synthesis of [2-^{3}H]AMPS was accomplished by the method of Murray & Atkinson (1968). The crude product was purified by DEAE-cellulose column chromatography (27 × 2 cm) eluted with a linear gradient of 0–0.4 M TEAB (500 mL each). Each fraction was 11 mL; [2-^{3}H]AMPS eluted in fractions 40–56. These fractions were pooled, and the buffer was removed by repeated evaporations. The yield was 46% (233 μmol) and the specific radioactivity was 5000 cpm/nmol.

The [2-^{3}H]AMPS was converted to ADPαS(R_P + S_P) by the method of Eckstein & Goody (1976) as modified by P. Frey (personal communication). The crude [2-^{3}H]ADPαS was purified by chromatography on a DEAE-cellulose column (25 × 2.5 cm), which was eluted by a linear 0–0.66 M TEAB gradient (750 mL each). Fractions of 14 mL were collected; [2-^{3}H]ADPαS(R_P + S_P) eluted in fractions 39–50. The buffer was removed by repeated evaporation. The overall yield from adenosine was 26% (131 μmol), and the specific radioactivity was 5000 cpm/nmol. The R_P and S_P diastereomers were separated by phosphorylation of [2-^{3}H]ADPαS(S_P) to [2-^{3}H]ATPαS(S_P) by pyruvate kinase (Sheu & Frey, 1977). The conditions were as follows: 1.23 mM [2-^{3}H]ADPαS(S_P + R_P), 49 mM Tris, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 5 mM PEP, and 100 units of pyruvate kinase in 20 mL of total reaction volume. Incubation was at room temperature for 19 h. Separation of [2-^{3}H]ADPαS(R_P) and [2-^{3}H]ATPαS(S_P) was accomplished on a DEAE-cellulose column (25 × 1.4 cm) eluted with a linear gradient of 0–0.3 M TEAB (500 mL each). Fractions of 11-mL volume were collected. [2-^{3}H]-ADPαS(R_P) eluted in fractions 30–44; [2-^{3}H]ATPαS(S_P) eluted in fractions 45–61. The [2-^{3}H]ADPαS(R_P) obtained in this manner did not contain [2-^{3}H]ADPαS(S_P) by HPLC (reverse-phase column) but was contaminated by 5–10% with [2-^{3}H]ATPαS(S_P).

Capping Experiments with [2-^{3}H]ADPαS(R_P). The reaction mixtures for these experiments contained 100 mM Tris, pH 7.9, 5 mM $MgCl_2$ 0.3 mM DTT, and 13.8 polymerization units of form I polynucleotide phosphorylase in a 0.5-mL total reaction volume. The concentrations of labeled and unlabeled ADPαS diastereomers are given in the tables. Incubation was at 37 °C for 96 h; the enzyme exhibited considerable activity over this long incubation time at these high protein concentrations (Marlier et al., 1981). The polymeric product was isolated by gel filtration on Sephadex G-50 (20 × 1 cm) and hydrolyzed in 0.3 M KOH at 37 °C for 20 h. The alkali was neutralized by passing the solution through a 5 × 1 cm Dowex 50 (H^+ form) column. The eluant was concentrated by evaporation under vacuum to a volume of ~1 mL and applied to a DEAE-Sephadex A-25 column (20 × 0.7 cm) that was prewashed with 100 mL of H_2O. The column was eluted with a linear gradient of 0.1–0.9 M TEAB (125 mL each). Fractions of 2.6 mL were collected and the optical density at 260 nm was determined for each fraction. The entire fraction was added to either 10 or 15 mL of scintillation cocktail, maintaining a homogeneous mixture. Each sample was counted twice for 5 min. Marker compounds were added to the DEAE-Sephadex column to identify the products of alkaline hydrolysis.

Polymerization Assays. The rate of polymerization was measured by two assays. The first measured the appearance of P_i by the colorimetric assay of Lanzetta et al. (1979). The second followed the formation of poly(adenylic acid) by gel filtration. For this assay, 50 μL of the reaction mixture was quickly passed through a Sephadex G-50 (29 × 0.6 cm) column preequilibrated with 10 mM NH_4OAc, and fractions of 0.65 mL were collected. The molar quantity of nucleoside diphosphate that had been incorporated into polymer was determined by utilizing a molar extinction coefficient of 10300 M^{-1} cm^{-1} at 257 nm per adenine incorporated (Blake & Fresco, 1973). This gel filtration assay was appropriate where ADPβS was employed as the substrate, and the thiophosphate produced could not be determined by the colorimetric assay. Control experiments showed good agreement between the two assays.

Polyphosphate Kinase Assay. The extent of ADPαS(R_P) conversion to AMPS was followed by the adenylic acid deaminase assay of Murray & Atkinson (1968). The buffer for the assay was 0.09 M potassium cacodylate, pH 6.5, containing 0.5 M KCl. The amount of AMPS produced was calculated by utilizing a ΔE of 8100 M^{-1} cm^{-1} at 265 nm (Murray & Atkinson, 1968).

Products of the Polyphosphate Kinase Activity. Reaction mixtures (100 μL) containing 2.0 mM ADPαS(R_P), 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, and 1.9 units of polynucleotide phosphorylase were incubated at 37 °C. The amounts of both expected products, AMPS and P_i, were measured simultaneously by the adenylic acid deaminase and colorimetric assays, respectively. Identical experiments were done with $NaHCO_3$–CO_2 buffer at pH 8.0. AMPS was produced with time, but no P_i could be detected. Treatment of the reaction mixture with 1.1 units of alkaline phosphatase slowly liberated the P_i, as determined by the colorimetric assay.

So that information regarding the relative molecular weight of the P_i-containing product could be obtained, a gel-filtration experiment was performed. A 100-μL solution containing 2.0 mM ADPαS(R_P), 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, and 1.6 units of polynucleotide phosphorylase was incubated at 37 °C until greater than 80% of the ADPαS(R_P) was converted to AMPS (adenylic acid deaminase assay). This reaction mixture was added to a Sephadex G-50 column (29 × 0.9 cm) and eluted with 10 mM NH_4OAc. Fractions of 0.65-mL volume were collected. Two 50-μL aliquots were withdrawn from each fraction, and 2 μL of 1 M Tris, pH 8.0, was added to each aliquot. Alkaline phosphatase (1.1 units) was added to one of the two aliquots, and both were allowed to react at room temperature for 1 h. The amount of P_i in each fraction was then determined for each aliquot by the colorimetric assay.

Structural identification of this P_i-containing product was accomplished by ^{31}P NMR. A 500-μL sample containing 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, 2 mM ADPαS(R_P), and 7 units of polynucleotide phosphorylase was incubated at 37 °C until greater than 85% of the ADPαS(R_P) had been converted to AMPS. To this solution was added 1.35 mL of D_2O, 0.2 mL of 0.02 M EDTA, and 0.01 mL of 25 mM K_2HPO_4 (as an internal standard). The ^{31}P NMR analysis was performed on this solution.

Kinetic Studies on the Polyphosphate Kinase. The initial rates of conversion of ADPαS(R_P) to AMPS were measured by the adenylic acid deaminase assay. In addition to various levels of ADPαS(R_P), the reaction mixtures (100 μL) contained 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, and 1.87 polymerization units of polynucleotide phosphorylase at 37 °C. All values for K_m and V_{max} were determined by a Lineweaver–Burk

Table I: Rate of ADPαS(S_P) Exchange and Polymerization in the Presence and Absence of ApA and ADPαS(R_P)

expt	substrate(s)	concn (mM)	rate of polymerization[a]	rate of exchange[b]
1	ADPαS(S_P)	1.0	0.61	0.08
2	ADPαS(S_P)	1.0	0.59	0.25
	ADPαS(R_P)	1.0		
3	ADPαS(S_P)	1.0	0.63	0.26
	ApA	0.1		

[a] In nanomoles of ADPαS polymerized per hour per unit. Measured as described under Materials and Methods. [b] Estimated from previous data (Marlier et al., 1981). In nanomoles of [β-^{32}P]ADPαS formed per hour per unit.

double-reciprocal plots, and the data are presented in the various tables.

The specificity of the polyphosphate kinase activity was investigated by utilizing ADPαS(S_P), ADPαS(R_P), AMP, ATPαS(S_P), and ATPαS(R_P) as possible substrates. The 100-μL reaction mixtures contained 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, 1.8 polymerization units of polynucleotide phosphorylase, and 2.0 mM of one of the above substrates. Incubation was at 37 °C. The ADPαS(R_P) and ADPαS(S_P) reactions were monitored by the adenylic acid deaminase assay, the AMP reaction was followed by the adenosine deaminase assay, and the reactions of ATPαS(R_P) and ATPαS(S_P) were followed by HPLC (SAX column).

A dinucleotide, pAp(S)A, was tested as an inhibitor of the polyphosphate kinase activity. The 100-μL reaction mixtures contained 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.44 mM ADPαS(R_P), and 1.43 polymerization units of polynucleotide phosphorylase. The levels of dinucleotide are given in the tables. Incubation was at 37 °C for 1.5 h; the extent of reaction was measured by the adenylate deaminase assay.

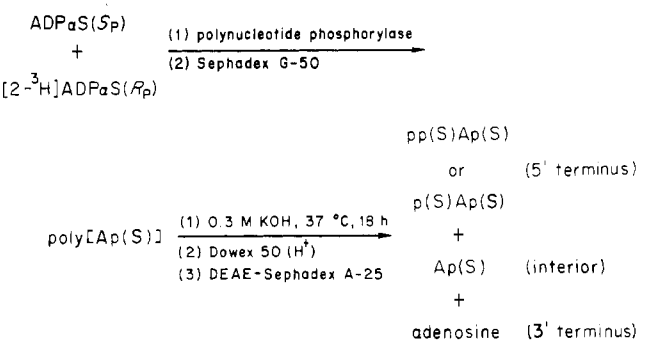
Kinetic Studies of Polymerization by Polynucleotide Phosphorylase. Kinetic parameters of ADPαS, ADPβS, and ADP polymerization were determined. The 100-μL reaction mixtures contained 100 mM Tris, pH 7.9, 14 mM $MgCl_2$, 0.1 mM DTT, 0.062 unit of polynucleotide phosphorylase, and various levels of the substrate. The rate of reaction was measured by either the colorimetric or gel-filtration assays.

Molecular Size of the Polymeric Product. Polymerization mixtures (100 μL) contained 100 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.01 mM DTT, and 5.0 mM either ADP, ADPαS(S_P), ADPαS(S_P + R_P), or ADPβS. Reaction mixtures containing ADP or ADPβS had 0.43 polymerization unit of polynucleotide phosphorylase and were incubated at 37 °C for 3 h; those with ADPαS contained 1.8 polymerization units and were incubated at 37 °C for 24 h. The polymeric products were isolated by gel filtration on a Sephadex G-50 column (29 × 0.6 cm) preequilibrated and eluted with 10 mM NH_4OAc. Fractions of 0.65 mL were collected. The polymer, contained in fractions 6–8, was pooled, lyophilized, and redissolved in 50 μL of H_2O. Horizontal gel electrophoresis was run with 1.8% agarose. The running buffer was 20 mM Mops, pH 7.2, 1 mM EDTA, and 50 mM NaOAc. Electrophoresis was at 3 V/cm for 12 h. Gels were stained with 8 μg of acridine orange in 250 mL of distilled H_2O (McMaster & Carmichael, 1977). The gel was destained for 5 h in distilled H_2O (several rinses) and visualized under a UV lamp (254 nm).

Results

Capping Experiments. Although ADPαS(R_P) catalyzes the [^{32}P]P_i exchange into ADPαS(S_P), it does not catalyze the polymerization reaction. Incubation of either 1.0 mM ADPαS(S_P) alone or 1.0 mM ADPαS(S_P) plus 1.0 mM ADPαS(R_P) with polynucleotide phosphorylase gave nearly identical rates (Table I). Furthermore, dinucleotides, which activated the [^{32}P]P_i exchange reaction, do not catalyze the polymerization reaction (Table I). These data suggested that ADPαS(R_P) may be incorporated into the 5′ terminus of the first dinucleotide formed during de novo polymerization. The experimental protocol that was utilized to test this hypothesis is outlined in Scheme I and is discussed in detail below.

Scheme I: End-Group Analysis of Poly(A) from ADPαS Polymerization by Primer-Independent Polynucleotide Phosphorylase



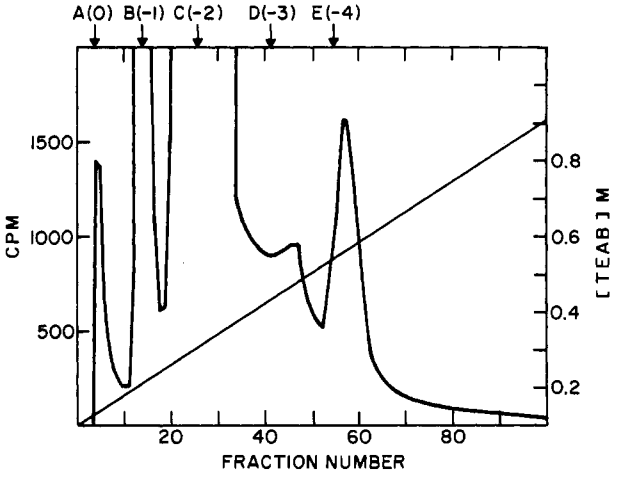


FIGURE 1: DEAE-Sephadex A-25 column chromatography of the hydrolysis products formed according to Scheme I. Conditions are described under Material and Methods. Marker compounds: (A) adenosine; (B) 2′,3′-cAMP; (C) AMPS; (D) ADPαS; (E) p(S)Ap. The formal charge of each marker compound is given in parentheses next to each letter.

To accomplish the capping experiment outlined in Scheme I, it was first necessary to determine the nature of the 5′-end group of the polymer and to determine whether the experimental protocol outlined in Scheme I will yield a 1:1 ratio of 5′ terminus to 3′ terminus. So that this information could be obtained equimolar quantities of [2-^{3}H]ADPαS(S_P) and [2-^{3}H]ADPα(R_P) of identical specific radioactivity were incubated with the enzyme at 37 °C for 96 h and then subjected to the procedure outlined in Scheme I.

The radioactive peaks from the DEAE-Sephadex A-25 column, including the 5′ terminus, were identified by coelution of unlabeled marker compounds (Figure 1). These cold marker compounds eluted in the order adenosine, 2′,3′-cAMP, 5′-AMPS, and p(S)Ap, as expected from their ionic charge. The last radioactive peak eluted from the DEAE-Sephadex G-25 column nearly coincident with authentic p(S)Ap and is due to p(S)Ap(S), which is derived from the 5′-thiophosphate terminus. Control experiments with ADPαS supported by reports in the literature (Harvey & Grunberg-Manago, 1970)

Table II: Capping of the 5′ Terminus of Poly(A) by ADPαS(R_P)

expt	substrates	initial concn (mM)	cpm adenosine	cpm interior[a]	cpm p(S)Ap(S)[b]	5′ end/3′ end	% capping[c]
1	[2-^{3}H]ADPαS(S_P)	2.3	36 000	1 500 000	29 400	0.8	
	[2-^{3}H]ADPαS(R_P)	2.3					
2	ADPαS(S_P)	2.4	3 000	180 000	9 000	3.0	25
	[2-^{3}H]ADPαS(R_P)	2.4					
3	ADPαS(S_P)	2.4	3 900	320 000	13 300	3.4	63
	[2-^{3}H]ADPαS(R_P)	4.7					
4	ADPαS(S_P)	2.3	5 300	390 000	21 000	4.0	89
	[2-^{3}H]ADPαS(R_P)	7.0					
5	ADPαS(S_P)	2.5	2 600	340 000	950	0.37	
	ADPαS(R_P)	5.0					
	[2-^{3}H]ATPαS(S_P)	0.8					

[a] Includes cpm for 2′,3′-cAMPS (peak 2 in Figure 1) plus cpm for 2′- and 3′-AMPS (peak 3 in Figure 1). [b] Includes counts from incompletely hydrolyzed p(S)A>p(S) (peak 4 in Figure 1). The amount of this material was estimated from the relative amounts of Ap(S) and A>p(S) arising from the interior chain (peaks 2 and 3 in Figure 2). [c] The percent capping represents the amount of the 5′ terminus derived from [2-^{3}H]ADPαS(R_P). See the text for a discussion of how this value was calculated.

have demonstrated that had a 5′-pyrophosphate group been formed, it would be stable to this analytical procedure. The molecule derived from the pyrothiophosphate end group, pp(S)Ap(S), would have eluted in a fraction greater than 60 and would have been detected if present at levels 10–20% that of the observed p(S)Ap(S). Likewise only p(S)Ap(S) was found when [2-^{3}H]ADPαS(S_P) was the polymer precursor.

The radioactivity that elutes with cold authentic A>p is due to A>p(S). This cyclic nucleotide (peak B, Figure 1) is an intermediate in the alkaline hydrolysis and originates from the interior nucleotides of the poly(A)chain. In all cases, the amount of the cyclic nucleotide was only ~10% that of Ap(S) (the ring-opened nucleotide). The relative amount of this cyclic nucleotide was used to correct for the amount of cyclic nucleotide resulting from the 5′ terminus [i.e., p(S)A>p(S)]. In our control the ratio of p(S)Ap(S) to adenosine (5′ terminus/3′ terminus) was found to be 0.8 (Table II), close to the expected value of 1.0 and within the experimental precision of the analytical procedure.

The actual capping experiments were done with various levels of [2-^{3}H]ADPαS(R_P) and 2.5 mM ADPαS(S_P). Alkaline hydrolysis and analysis of the polymer demonstrated appreciable label in the 3′ terminus and interior nucleotides, as well as a higher level of radioactivity in the 5′ terminus (Table II). It seemed unlikely that all the radioactivity in the 3′-terminal and interior nucleotides would be due to incorporation of [2-^{3}H]ADPαS(R_P), because Burgers & Eckstein (1979) found the R_P diastereomer to be at least 50 times less reactive than the S_P diastereomer and because we could not detect [^{32}P]P_i exchange with the R_P diastereomer (Marlier et al., 1981).

The source of the radioactive label in the 3′-terminal and interior nucleotides was discovered in a separate control experiment. [2-^{3}H]ATPαS(S_P), which is a contaminant of [2-^{3}H]ADPαS(R_P) (see Materials and Methods), was incubated with cold ADPαS(S_P) and ADPαS(R_P) in the presence of polynucleotide phosphorylase. Analysis of the polymeric product demonstrated (Table II) incorporation of label into the 3′-terminal, interior, and 5′-terminal nucleotides. Analysis of the unreacted substrates also showed label incorporation into ADPαS and AMPS [the latter derived from polyphosphate kinase action on the ADPαS(R_P)]. This is consistent with a low level of contamination of polynucleotide phosphorylase by adenylate kinase. Such contamination has been widely observed, even in highly purified polynucleotide phosphorylase (Craine & Klee, 1976). It is further known that adenylate kinase is highly specific for the S_P diastereomers of ADPαS and ATPαS (Sheu & Frey, 1977). Therefore, only ADPαS(S_P) becomes labeled by adenylate kinase action.[2]

Because of the contaminating adenylate kinase activity, the capping of the 5′ end of the polymer by [2-^{3}H]ADPαS(R_P) was measured by determining the ratio of the radioactivity of the 5′ terminus to that of the 3′ terminus. No capping would result in a 1:1 ratio; capping would result in a ratio greater than 1. Experiments 2–4 in Table II clearly demonstrate that the 5′ terminus contains more radioactivity than the 3′ terminus and this ratio is dependent on the amount of [2-^{3}H]-ADPαS(R_P) initially present. As expected, the control experiment where only ATPαS(S_P) was labeled (Table II, experiment 5) showed that the 5′ terminus of the polymer does not have as much radioactivity as the 3′ terminus. This is because only ADPαS(S_P) can become labeled by the adenylate kinase.

The efficiency of capping of [2-^{3}H]ADPαS(R_P) (Table I) was finally calculated by assuming that all the radioactivity in the 3′ terminus and interior of the polymer was derived from [2-^{3}H]ADPαS(S_P). The final specific radioactivity of the [2-^{3}H]ADPαS(S_P) was determined from the optical density and radioactivity of the Ap(S) fractions from the DEAE-Sephadex column. The percent of capping of [2-^{3}H]-ADPαS(R_P) was then calculated from the known specific radioactivities of the two diastereomers of [2-^{3}H]ADPαS and the total amount of radioactivity incorporated into the 5′ terminus and the 3′ terminus. Two features inherent in this calculation should be emphasized: (1) if the rates of equilibration of label into the ADPαS(S_P) diastereomer and of polymerization are comparable rather than equilibration being more rapid, the number of chains determined by the amount of label in the 3′ terminus will be overestimated so that the actual percent capping would be greater; (2) the number of polymers labeled at the 5′ end with the S_P diastereomer, which were not explicitly included in the method of calculation, must be less than 10%, based on the control (experiment 5, 950 cpm/10 000–20 000 cpm).

Since [2-^{3}H]ADPαS(R_P) is hydrolyzed during the course of polymerization, a control experiment was performed to determine the amount of ADPαS(R_P) remaining with time for each of the three initial concentrations of [2-^{3}H]ADPαS-(R_P) utilized in the capping experiments. These data are presented in Table III. When the initial concentration of [2-^{3}H]ADPαS(R_P) was 2.5 mM (experiment 2, Table II),

[2] Suppression of the adenylate kinase activity with the inhibitor Ap_5A was not attempted because this inhibitor activated [^{32}P]P_i exchange (J. F. Marlier and S. J. Benkovic, unpublished results) and potentially could cap the 5′ end of the polymer.

Table III: Rate of Hydrolysis of ADPαS(R_P) and Polymerization of ADPαS(S_P)

expt[a]	time (h)	[ADPαS(R_P)] (mM)[b]	[P_i] (mM)[c]
1	20	0.98	0.35
	42	0.32	0.96
	64	0	1.17
2	20	2.81	0.58
	42	1.87	1.01
	64	0.73	1.11
3	20	5.31	0.40
	42	2.82	1.00
	64	1.28	1.11

[a] Initial concentration of ADPαS(R_P) was 2.5 (experiment 1), 5.0 (experiment 2), and 7.5 mM (experiment 3); ADPαS(S_P) was 2.5 mM in all cases. [b] Determined by adenylic acid deaminase assay described under Materials and Methods. [c] Determined by colorimetric assay described under Materials and Methods.

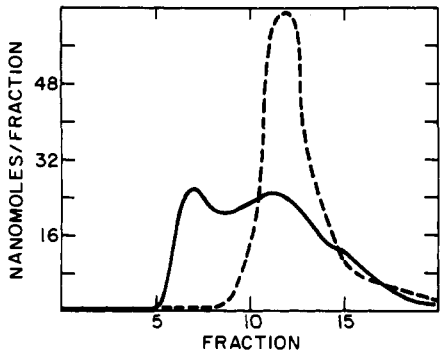


FIGURE 2: Sephadex G-50 column chromatography of the products of ADPαS(R_P) hydrolysis by the polyphosphate kinase activity. Conditions are described under Materials and Methods. AMPS and unreacted ADPαS(R_P) (--); inorganic phosphate (—) after treatment with 1.1 units of alkaline phosphatase as described under Materials and Methods. No inorganic phosphate was present before treatment with alkaline phosphatase.

better than 60% of this substrate is hydrolyzed during the first 20 h of incubation. Since AMPS is neither a substrate nor an activator of the exchange reaction (Marlier et al., 1981), this competing hydrolysis leads to the low efficiency of [2-^{3}H]ADPαS(R_P) incorporation at this level of nucleotide. Higher initial levels of [2-^{3}H]ADPαS(R_P) result in higher efficiency of incorporation (experiments 3 and 4, Table II).

Polyphosphate Kinase Activity. An activity associated with form I polynucleotide phosphorylase cleaves the P_β–O bond of ADPαS(R_P) but does not release free P_i into solution (Marlier et al., 1981). Hydrolysis of 2 mM ADPαS(R_P) in both Hepes and bicarbonate buffers at pH 8, which also does not result in P_i formation, eliminates transphosphorylation of the buffer as an explanation for the missing P_i. However, the missing P_i could be liberated by treatment with alkaline phosphatase. A solution of ADPαS(R_P) that had been almost completely hydrolyzed was passed through a Sephadex G-50 column. The amount of the phosphatase-liberated P_i was measured for each fraction from the column, and most of the P_i was found in the high molecular weight portion of the elution profile (Figure 2).

Identification of the high molecular weight product was accomplished by ^{31}P NMR. ADPαS(R_P) was hydrolyzed by polynucleotide phophorylase and the ^{31}P NMR spectra were measured at pH 8.0. Two resonances were present; one at 43.1 ppm agrees with a chemical shift of 43.3 ppm reported for AMPS (Jaffe & Cohn, 1978), and the other at −22.6 ppm agrees with the value (−22.6 ppm) reported for the internal phosphorus atoms of inorganic polyphosphate (Salhany et al., 1975). The broad elution profile on Sephadex G-50 (Figure 2) indicates a rather wide range of molecular weights for the polyphosphate chain. However, the terminal phosphates were not detectable in the ^{31}P NMR, and therefore the average chain length is quite high (probably $n > 10$). This is consistent with the known ability of *M. luteus* to store high-energy phosphates in the form of polyphosphate (Glonek et al., 1971). Therefore the activity is a polyphosphate kinase, and the overall reaction is shown in eq 1.

$$\text{ADP}\alpha\text{S}(R_P) \rightleftharpoons \text{AMPS} + P_i \quad (1)$$

Table IV: Specificity of the Polyphosphate Kinase

substrate	concn (mM)	time (h)	% reaction
AMP	2.0	18	~0[a]
		42	~0
ADPαS(S_P)	2.0	22	3[b]
		46	7
ADPαS(R_P)	2.0	22	69[b]
		44	88
ATPαS(S_P)	2.0	24	3[c]
ATPαS(R_P)	2.0	24	3[c]
		48	4

[a] Measured by adenosine deaminase assay; assay conditions are as described under Materials and Methods. [b] Measured by adenylic acid deaminase assay. [c] Measured by HPLC.

Table V: Inhibition Studies with pAp(S)A[a]

[ADPαS(R_P)] (mM)	[pAp(S)A] (mM)	rate of reaction
0.44	0.10	3.0 ± 0.3
0.44	0.50	2.6 ± 0.3
0.44		3.0 ± 0.3

[a] Measured by the adenylic acid deaminase assay; assay conditions are as described under Materials and Methods.

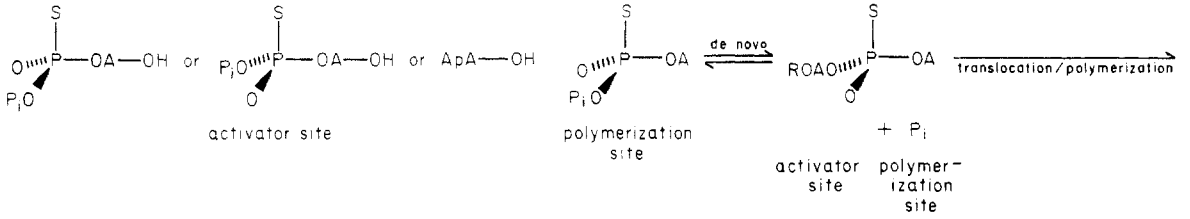
The specificity of the polyphosphate kinase was tested with a variety of substrates. The results are summarized in Table IV. AMP, ATPαS(S_P + R_P), and ADPαS(S_P) are poor substrates; ADPαS(R_P) on the other hand is a good substrate. Therefore a pyrophosphate group is required for substrate activity, and a preference is shown for the R_P stereochemistry at the α phosphorus.

The hydrolysis of ADPαS(R_P) is not significantly inhibited by pAp(S)A (Table V). These types of dinucleotides are known to activate exchange and therefore are expected to bind at the active site with activation constants of ~50 μM. The V_{max} (4.9 nmol h^{-1} unit^{-1}) and K_m (330 μM) for the hydrolysis were first measured in the absence of inhibitor to determine an optimum concentration of ADPαS(R_P) for inhibition experiments. The observed lack of inhibition argues against the polyphosphate kinase activity residing at the active site of polynucleotide phosphorylase.

ADPβS as a Substrate for Polynucleotide Phosphorylase. ADPβS was tested as a substrate for the polymerization reaction of polynucleotide phosphorylase, and the measured kinetic parameters were compared to those for ADP and ADPαS(S_P). The data are given in Table VI. ADPβS is nearly as good a substrate as ADP under the conditions studied. This indicates that removal of the β-thiophosphate group, which is expected to be slow for this analogue, is not a crucial step in the overall polymerization process.

Relative Molecular Weight of Poly(A) Products. The relative mobility of the poly(A) formed from polymerization of ADP, ADPβS, and ADPαS was determined on agarose gel electrophoresis. In each case rather diffuse bands were ob-

Scheme II: Mechanism of Polymerization and [^{32}P]P_i Exchange Catalyzed by Polynucleotide Phosphorylase[a]



[a] [^{32}P]P_i exchange may actually occur at polymer lengths greater than $n = 2$.

Table VI: Kinetic Constants and Relative Polymer Mobility for Various Substrates of Polynucleotide Phosphorylase

substrate	K_m (mM)[a]	V_{max} (nmol h^{-1} unit^{-1})[a]	rel polymer mobility[b]
ADP	1.4 ± 0.5	212 ± 50	0.3
ADPβS	1.2 ± 0.5	110 ± 30	0.3
ADPαS(S_P)	1.8 ± 0.5	2.1 ± 0.4	1.5
ADPαS(S_P + R_P)			1.5

[a] Rates measured by assays described under Materials and Methods. Kinetic parameters were determined by Lineweaver-Burk double-reciprocal plots. [b] The relative mobilities were determined by measuring the location of the most dense area of the band on the gel and then normalizing to the rate per voltage gradient, given in arbitrary units. Electrophoresis conditions and polymerization conditions are given under Materials and Methods.

tained, indicating a wide range of molecular weights for the homopolymers. The relative mobilities of each poly(A) species are given in Table VI. The polymers formed by polymerization of ADP or ADPβS are nearly identical in mobility and therefore have similar molecular weights. Literature values for the average length of polymers formed from ADP polymerization are $n \simeq 700$ (Moses & Singer, 1970). We have determined this value under our conditions (results not reported) and find a similar average chain length for polymer from ADPβS. The polymer from ADPαS polymerization is more mobile on the agarose gels and therefore has a lower molecular weight. Capping experiments indicate an average chain length of 40–80. This indicates a direct relationship between rate of polymerization (V_{max}) and molecular size (relative mobility).

Discussion

A plausible mechanism for the reactions of polynucleotide phosphorylase must account for the following experimental observations: (1) ADPαS(S_P) is the preferred substrate for the polymerization reaction, which proceeds with net inversion of configuration (Burgers & Eckstein, 1979); (2) ADPαS(S_P) is the preferred substrate for the [^{32}P]P_i exchange reaction, which proceeds with overall retention of configuration (Marlier et al., 1981); (3) at low levels of ADPαS(S_P) the exchange reaction is activated by ADPαS(R_P) and dinucleotides but not by AMPS; (4) the activation of exchange by ADPαS(R_P) or dinucleotides is inhibited by high levels (>2 mM) of ADPαS(S_P); (5) ADPαS(R_P) and dinucleotides do not activate the de novo polymerization reaction (Table I); (6) [2-^{3}H]-ADPαS(R_P) is incorporated into the 5′ terminus of a polymer formed de novo (Table II).

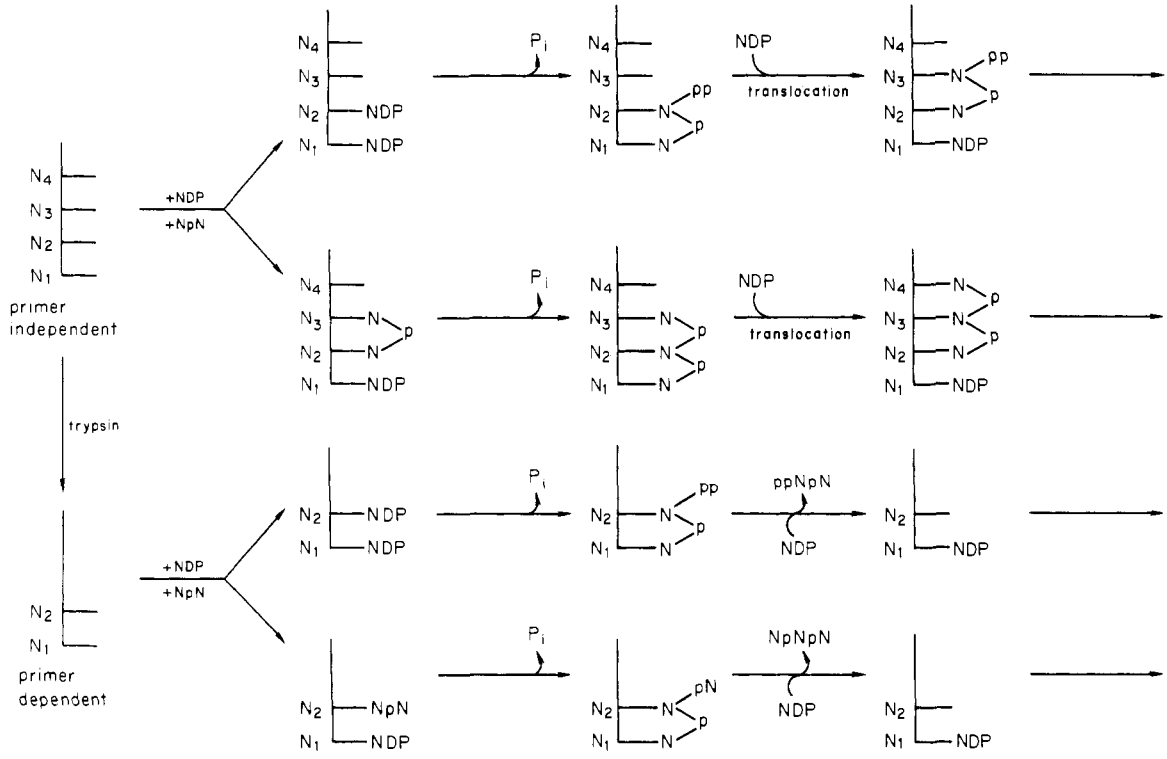
A mechanism that accommodates all of the above experimental observations is outlined in Scheme II. In this mechanism polynucleotide phosphorylase has at least two binding sites at the active site. The first, or activator site, binds molecules that will activate the exchange reaction [ADPαS-(R_P) or dinucleotides] as well as ADPαS(S_P). The second, or polymerization site, is selective for ADPαS(S_P), since this is the preferred substrate for both the exchange and polymerization reactions. The first internucleotide bond is formed when the 3′-hydroxyl of a molecule bound at the activator site attacks the α phosphorus of ADPαS(S_P) bound at the polymerization site. The internucleotide bond formed in this process has the R_P (or inverted) stereochemistry; reversal of this reaction by [^{32}P]P_i yields [β-^{32}P]ADPαS(S_P) (retention of configuration).

The above information can also aid in understanding the observed differences between primer-independent and primer-dependent polynucleotide phosphorylase. The primer-independent enzyme catalyzes a de novo processive polymerization reaction. Oligonucleotide primers neither stimulate this reaction nor are they readily incorporated into the 5′ terminus of a newly synthesized polymer (Moses & Singer, 1970). On the other hand, polymerization catalyzed by the primer-dependent enzyme is nonprocessive and occurs via elongation of oligonucleotide primers (Moses & Singer, 1970).

Our experiments, which demonstrate that high levels of ADPαS(S_P) can inhibit activation of exchange by dinucleotides, suggest that both elongation of a primer and de novo polymerization could occur with the primer-independent enzyme. However, because NDP can also bind tightly at the activator site, these molecules competitively inhibit the binding of dinucleotides and decrease the amount of primer elongation. Experiments done to test oligonucleotide incorporation into poly(A) by the primer-independent enzyme typically used very high NDP levels (20 mM) and low (1 mM) oligonucleotide (Moses & Singer, 1970). If a primer elongation pathway exists for this enzyme, the high levels of NDP relative to oligonucleotide may have obscured the incorporation of the primer. Since polymerization by the primer-independent enzyme is processive, once the first dinucleotide is formed, it can then translocate (by an unknown mechanism) and another NDP can be polymerized onto the 3′ end. Once this process is started, oligonucleotides are completely excluded from the activator site until the polymer disassociates from the enzyme. This mechanism is depicted in Scheme III. The scheme does not take into account the fate of the expected 5′-pyrophosphate group, which is discussed in detail later.

For the primer-dependent enzyme, activation of exchange by dinucleotides can also be inhibited by high levels of ADPαS(S_P). Therefore, de novo formation of dinucleotides is probably available to the primer-dependent enzyme as well. However, because the polymerization is nonprocessive, any dinucleotides formed de novo must first disassociate from the enzyme and mix with an external pool of primer molecules prior to subsequent elongation. Thus an external pool of oligonucleotides will activate the polymerization and incorporate into the 5′ end of the polymer. A plausible explanation of the nonprocessive mechanism assumes that trypsinolysis destroys or alters oligonucleotide binding sites near the activator and polymerization sites. These sites (N_3 and N_4, Scheme III) would presumably be important for the translo-

Scheme III[a]



[a] N_1, N_2, N_3, and N_4 represent nucleotide binding sites on the enzymes. N_1 binds the NDP that undergoes nucleophilic attack (polymerization site). N_2 is the activator site (see text).

cation step, and alteration would either slow or stop this process. The details of this model are also shown in Scheme III.

An activity is present with primer-independent polynucleotide phosphorylase, which cleaves the P_β–O bond of ADPαS(R_P) (Marlier et al., 1981). The products of this reaction are AMPS and a high molecular weight polyphosphate, as determined by ^{31}P NMR. Therefore, the activity is a polyphosphate kinase activity. Studies on the specificity of this enzyme showed a requirement for a 5′-pyrophosphate and a preference for the R_P stereochemistry at the α phosphorus. We undertook several experiments to ascertain whether or not this activity is responsible for the observed formation of a 5′-phosphate (or thiophosphate) end group during de novo polymerization and whether or not it is an important activity for the overall functioning of polynucleotide phosphorylase.

First, dinucleotides are known to bind tightly to the active site of polynucleotide phosphorylase and activate the [^{32}P]P_i exchange reaction with K_a values of ~50 μM (Marlier et al., 1981). Such activation occurs, presumably, by elongation of the 3′-hydroxyl of the dinucleotide, followed by phosphorolysis in the presence of [^{32}P]P_i. A dinucleotide might be expected to be a potent inhibitor of hydrolysis by the polyphosphate kinase activity, if such an activity resided at the active site near the origin of the 5′ terminus of the polymer chain. No significant inhibition was observed with pAp(S)A as an inhibitor (Table V). The anticipated inhibition is ca. 80% if pAp(S)A were a competitive inhibitor of ADPαS(R_P) hydrolysis where K_i = 50 μM and K_m = 330 μM, respectively.

Second, ADPβS was tested as a substrate for the polymerization reaction. Because of the presence of the unreactive β-thiophosphate group, this substrate would be expected to be a less reactive substrate, if the breaking of the P_β–O bond is a required step in de novo polymerization. ADPβS was found, however, to be nearly as good a substrate as ADP for polymerization (Table VI). Furthermore, the polymeric product is of the same average chain length as that formed by ADP polymerization (Table VI).

The results of the above experiments and the fact that polyphosphate kinases have been isolated in a relatively pure form from many different sources (Kulaev, 1979) mitigate against a critical role for the polyphosphate kinase in the functioning of polynucleotide phosphorylase. It is most likely that the polyphosphate kinase is a separated protein that copurifies with polynucleotide phosphorylase.

The polyphosphate kinase activity could still be responsible for the observed formation of the 5′-phosphate (or thiophosphate) end group, if the measured rate of hydrolysis of both ADPαS(S_P) and ADPαS(R_P) (Table IV) is sufficient to account for the formation of one pyrophosphate end group per large polymer molecule ($n \geq 50$). The most likely mode of attack would be on the polymer after it has been released from the enzyme. As of yet, however, no evidence exists to either support or rule against the requirement that the polyphosphate kinase be active toward oligonucleotide and polynucleotide 5′-pyrophosphates.

Some restrictions can be placed on the rate-limiting steps in this process. It is qualitatively apparent that polymer length or processivity depends on the rate of polymerization vs. dissociation of the growing chain from the enzyme. That ADPαS produces an RNA of lower molecular weight (vs. ADPβS and ADP) is in accord with an intrinsically lower rate of polymerization resulting from the less reactive thiophosphoryl center. Although the R_P diastereomer can preferentially cap the 5′ end of the RNA chain, the rate of polymerization is not stimulated. This is in contrast to its catalysis of $P_i \rightleftharpoons$ ADPαS(S_P) exchange. Since the latter results from P_i attack on an olignucleotide of $n \geq 3$, the rate-limiting step(s) in the polymerization process might be the translocation on the enzyme and/or elongation of the oligonucleotide chain after priming lengths have been exceeded.

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Proteoliposome Interaction with Human Erythrocyte Membranes. Functional Implantation of γ-Glutamyl Transpeptidase†

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ABSTRACT: The transfer of detergent solubilized and purified γ-glutamyl transpeptidase (γ-GTase), of hog kidney cortex, from proteoliposomes into human erythrocyte ghost membranes has been studied. The transfer of γ-glutamyl transpeptidase was observed upon incubation of γ-GTase incorporated dipalmitoylphosphatidylcholine vesicles with erythrocyte ghost membranes at 37 °C for 12 h. The extent of transfer was dependent upon the fluidity of donor proteoliposomes, being more when dipalmitoylphosphatidylcholine proteoliposomes were used compared to dimyristoylphosphatidylcholine, and intermediate values were observed when binary mixtures of DMPC and DPPC were used. Moreover, the transfer of γ-GTase was facilitated when rigid basic phospholipid proteoliposomes were used as donor. The transfer of γ-GTase has been observed to be associated with the removal of intrinsic membrane proteins and lipids from erythrocytes, mainly acetylcholinesterase, sphingomyelin, and cholesterol. An enhancement in the fluorescence due to resonance energy transfer was observed when ghost membranes containing fluorescent donor probe were incubated with proteoliposomes containing fluorescent acceptor probe, indicating that fusion but not adsorption of vesicles occurs during the transfer process. However, the inability of entrapped [^{14}C]-sucrose delivery from proteoliposomes into ghost membrane vesicles suggests that fusion per se is not primarily involved in the transfer process. It appears that the transfer of γ-glutamyl transpeptidase occurs by a collisional transfer process resulting in intermembrane protein transfer. The γ-glutamyl transpeptidase implanted ghost membranes exhibited the uptake of L-glutamate which was inhibited by serine–borate, an inhibitor of transpeptidase activity. In addition, the uptake of L-glutamate was inhibited by the dipeptide γ-glutamyl-L-glutamate, thus supporting the proposed role of γ-glutamyl transpeptidase in the uptake of amino acids in biological membranes.

The interaction of phospholipid vesicles with cells for the introduction of new materials, e.g., drugs and enzymes into mammalian cells, has received considerable attention in recent years (Pagano & Weinstein, 1978; Kimelberg & Mayhew, 1978). Studies have shown that several membrane-associated proteins can undergo transfer spontaneously between artificial phospholipid vesicles (Roseman et al., 1977; Enoch et al., 1977) and between cells and phospholipid vesicles (Bouma et al., 1977; Cook et al., 1980). Roseman et al. (1977) showed that cytochrome b_5 bound to egg phosphatidylcholine vesicles when incubated with protein-free phospholipid vesicles underwent exchange between lipid bilayers. Subsequently Enoch et al. (1977, 1979) demonstrated that cytochrome b_5 reductase bound to dimyristoylphosphatidylcholine vesicles could be transferred into another artificial membrane, and this exchange did not involve vesicle fusion. Bouma et al. (1977) have shown that several membrane-associated proteins from intact human erythrocyte membrane were transferred to dimyristoylphosphatidylcholine vesicles when erythrocytes were incubated with liposomes. It was further shown by Cook et al. (1980) that transfer of protein from erythrocyte membrane to phospholipid vesicles was facilitated when the recipient phospholipid vesicles were more fluid than the donor erythrocyte membranes.

Recent studies from our laboratory (Kalra et al., 1981) have shown that detergent solubilized γ-glutamyl transpeptidase when incorporated into dipalmitoylphosphatidylcholine vesicles followed by incubation with erythrocytes resulted in the transfer of γ-glutamyl transpeptidase into erythrocyte membrane. The extent of transfer of γ-glutamyl transpeptidase into erythrocytes was relatively low when dimyristoylphosphatidylcholine vesicles were used as compared to DPPC[1]

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