between nucleotide transport systems.

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Registry No. ATP translocase, 9068-80-8; azido-AMP, 60731-47-7; azido-ATP, 53696-59-6; ATP, 56-65-5; AMP, 61-19-8.

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Phosphorus-31 Nuclear Magnetic Resonance Investigation of Membrane Vesicles from *Escherichia coli*†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance studies of isolated membrane vesicles prepared from *Escherichia coli* PSM116 as described by Hunt and Hong [Hunt, A. G., & Hong, J.-S. (1981) *J. Biol. Chem. 256*, 11988–11991; Hunt, A. G., & Hong, J.-S. (1983) *Biochemistry 22*, 844–850] are detailed here. This strain harbored a recombinant plasmid containing the phosphoglycerate transport system from *Salmonella typhimurium* (pJH7). Evidence indicating a surprising metabolic diversity, such as the presence of the enzymes enolase and phosphoglycerate mutase, is presented. The nature of the energization of these membrane vesicles for transport as described by Hugenholtz et al. [Hugenholtz, J., Hong, J.-S., & Kaback, H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3446–3449] is also discussed. Membrane vesicles prepared from the PSM116 strain do not form a transmembrane pH gradient when phosphoenolpyruvate is added. The present results show that phosphorus-31 nuclear magnetic resonance spectroscopy is an excellent tool to investigate the metabolism of membrane vesicles.

In recent years, nuclear magnetic resonance (NMR)[1] spectroscopy has proven to be a useful technique for studying bioenergetics and metabolism in intact cells and tissues [for example, see Lam et al. (1979), Burt et al. (1979), Shulman

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[1] Abbreviations: NMR, nuclear magnetic resonance; PEP, phosphoenolpyruvate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 2-PGA, 2-phosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; pgt, phosphoglycerate transport; EDTA, ethylenediaminetetraacetic acid; MDPA, methylenediphosphonic acid; DCCD, *N,N′*-dicyclohexylcarbodiimide; PMS, phenazine methosulfate.

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et al. (1979), Ugurbil et al. (1979), Hollis (1980), Gadian (1982), and Tehrani et al. (1982)]. Of special interest to the present work are ^{13}C and ^{31}P NMR studies of *Escherichia coli*. By using ^{31}P NMR spectroscopy, Shulman and his co-workers have verified several conclusions drawn by other investigators using different methods concerning the roles of the Mg-ATPase and the respiratory chains of *E. coli* in the energetics of this organism (Navon et al., 1977; Brown et al., 1977; Ugurbil et al., 1978a; Ogawa et al., 1978). ^{13}C NMR studies by Shulman and his colleagues as well as by other investigators have contributed substantially toward understanding the importance of several metabolic pathways involved in the carbon flux in *E. coli* (Ugurbil et al., 1978b; Ogino et al., 1982). For recent reviews on ^{31}P and ^{13}C NMR studies of intact cells, refer to Shulman et al. (1979), Ugurbil et al. (1979), Scott & Baxter (1981), and Gadian (1982).

In principle, the ^{31}P NMR technique should also be a useful one for studying similar processes in isolated membrane vesicles from *E. coli*. Here, we describe the first such studies of isolated membrane vesicles from *E. coli* using ^{31}P NMR spectroscopy. Using this technique, we have characterized the utilization of phosphoenolpyruvate (PEP) by membrane vesicles capable of transporting this compound. Furthermore, we have investigated the means of energization of membrane vesicles, under essentially anaerobic conditions, by the addition of PEP. This system has the distinct advantage of eliminating various experimental difficulties in supplying sufficient amounts of O_2 to very dense *E. coli* cells used by other earlier investigators.

Materials and Methods

Materials. PEP, AMP, ADP, ATP, glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), 2-phosphoglyceric acid (2-PGA), 3-phosphoglyceric acid (3-PGA), lysozyme, and 3-(*N*-morpholino)propanesulfonic acid (Mops) were obtained from Sigma. All other chemicals were reagent grade and obtained from commercial sources.

Bacterial Strain. The strain of *E. coli* K12 used in these experiments was PSM116 (thi, metC, glnP$_o$, glnP) (Masters & Hong, 1981). This strain harbored a recombinant plasmid containing the phosphoglycerate transport (pgt) system from *Salmonella typhimurium*) (pJH7). The pgt gene of this plasmid is expressed constitutively (J.-S. Hong, unpublished results). Cultures were grown in minimal medium E (Vogel & Bonner, 1956) containing 0.5% glycerol, 0.4 mM methionine, 40 μM vitamin B_1, and 25 μg/mL ampicillin.

Preparation of Isolated Membrane Vesicles. Membrane vesicles were prepared from stationary-phase cells as described previously (Kaback, 1971), except that spheroplasts were lysed in 50 mM Mops, pH 7.0 with KOH, instead of phosphate buffer as detailed by Hunt & Hong (1981, 1983). ADP was present in the lysis buffer at a concentration of 10 mM. After removal of cell debris and washing, membrane vesicles were stored at −80 °C in 0.1 M Mops, pH 7.0 with KOH, containing 10 mM ethylenediaminetetraacetic acid (EDTA). The protein content of these suspensions was 40–80 mg/mL.

Preparation and Analysis of Extracts for Identification of ^{31}P Resonances. Extracts of membrane vesicles were prepared by adding $HClO_4$ to 0.3 M to membrane vesicles prepared and incubated as described for NMR measurements. Twenty minutes after the addition of PEP, membrane vesicles were extracted with $HClO_4$ and the resulting precipitates removed by centrifugation. The extracts were carefully neutralized with KOH, and the $KClO_4$ precipitates were removed by centrifugation. The supernatants were stored at −20 °C. Extracts were diluted 3-fold into 0.1 M Mops, pH 7.0, plus 1 mM EDTA before ^{31}P NMR analysis. Signals present in extracts were identified by the addition of small amounts of known compounds to the extracts, thus facilitating the assignment of the ^{31}P resonances of phosphorylated metabolites inside the membrane vesicles.

NMR Measurements of Membrane Vesicles. ^{31}P NMR spectra were obtained at 121.5 MHz on a Bruker WH-300 high-resolution spectrometer operated in the Fourier-transform mode, using quadrature detection, 60° pulses of 15 μs, a repetition rate of 0.54 s, and a spectral width of 7575 Hz. Time-dependent studies were done by automatically storing on disks blocks of 332 scans, corresponding to 3 min of signal accumulation. A 2-mm capillary containing 0.3 M methylenediphosphonic acid (MDPA) (obtained from Aldrich) in D_2O was inserted into each of the 10-mm sample tubes. MDPA was used as the ^{31}P chemical shift reference, and D_2O was used for the field locking of the spectrometer. The ^{31}P chemical shift of MDPA is 17.18 ppm downfield from that of 85% H_3PO_4 at 30 °C, the temperature of the probe. The ^{31}P chemical shift scale is presently defined as positive in the low-field direction with respect to the standard reference signal of 85% H_3PO_4. The accuracy of our chemical shift measurements is ±0.02 ppm.

Spectra of membrane vesicles were obtained by using 3-mL samples, at 40–80 mg of protein/mL, and incubating at 30 °C without any aeration. Under these conditions, the dissolved O_2 concentration is probably very low [see, for example, Navon et al. (1977)]. Any additions were made as described in the figure legends.

Results

^{31}P NMR spectra of membrane vesicles before and at various times after the addition of PEP are shown in Figure 1. Before the addition of PEP, there is only one clearly discernible signal, that of inorganic phosphate (P_i), and two almost undetectable signals in the regions expected for sugar phosphates, AMP, and the α-phosphates of ADP and ATP (Figure 1A). Immediately after the addition of PEP, a modest increase in the P_i signal is apparent, due to the presence of a small amount of P_i in the PEP solution (Figure 1B). Moreover, a new signal, that of 3-PGA, appears immediately after the addition of PEP. After 4 min or so, another signal, that of 2-PGA, appears and continues to grow until it becomes the major peak, besides that of P_i and PEP, in the spectrum. The 2-PGA signal grows until the PEP signal decreases to about 30% of its original intensity and then decays gradually. The 3-PGA signal, on the other hand, decays continuously until the PEP and 2-PGA signals disappear. Throughout the course of the experiment, the P_i signal grows except for the first 9 min after the addition of PEP (Figure 1B).

Other, much smaller, signals can also be detected in the course of such an experiment (results not shown). Three signals, corresponding to G6P, F6P, and AMP, appear after the addition of PEP. These signals are moderately stable, remaining long after the PEP and 3-PGA signals are gone. ATP can also be detected. However, like the 2-PGA signal, the ATP signals are transient and disappear shortly after the consumption of the 3-PGA. The signal corresponding to the α-phosphate of ADP is more stable and remains for the duration of the experiment.

The appearance of 2- and 3-PGA upon the addition of PEP to membrane vesicles suggests that the enzymes enolase and phosphoglycerate mutase are present in these membrane vesicles. This was verified by investigating the effect of fluoride on the appearance of these compounds. The addition of fluoride totally abolished the appearance of 2- and 3-PGA (see

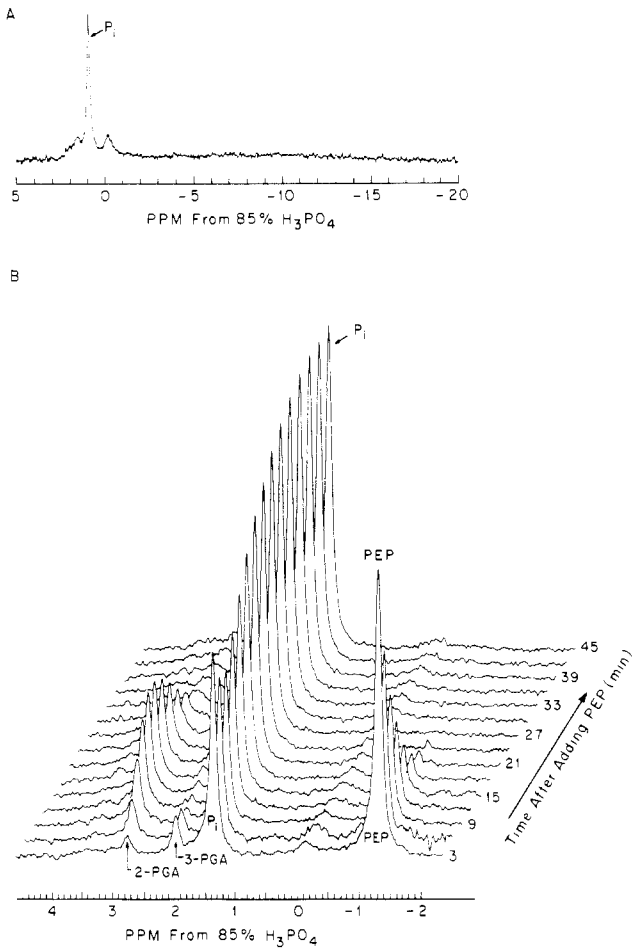

FIGURE 1: 121.5-MHz ^{31}P NMR investigation of the effect of phosphoenolpyruvate on membrane vesicles from *E. coli* PSM116 containing a plasmid with the phosphoglycerate transport system from *S. typhimurium* (pJH7) at 30 °C: (A) membrane vesicles in the absence of PEP; (B) membrane vesicles in the presence of 20 mM PEP. In (B), the spectra, with the exception of the bottom one, are displaced slightly to the right to facilitate comparison.

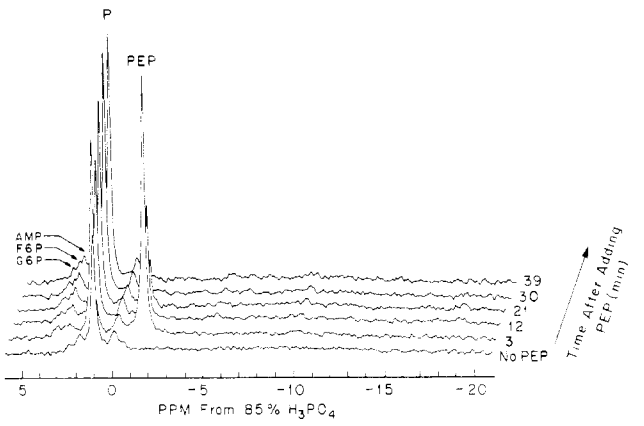

FIGURE 2: 121.5-MHz ^{31}P NMR investigation of the effect of 30 mM NaF on the utilization of phosphoenolpyruvate by membrane vesicles from *E. coli* PSM116 containing a plasmid with the phosphoglycerate transport system from *S. typhimurium* (pJH7) at 30 °C. The spectra, with the exception of the bottom one, are each displaced slightly to the right to facilitate comparison.

Figure 2). However, the appearance of the hexose phosphates and of AMP, ADP, and ATP is not affected by fluoride. The rate with which PEP disappears (90% consumed in 24 min) is also not affected by this inhibitor.

The effect of *N,N'*-dicyclohexylcarbodiimide (DCCD) on the utilization of PEP by vesicles is shown in Figure 3. The

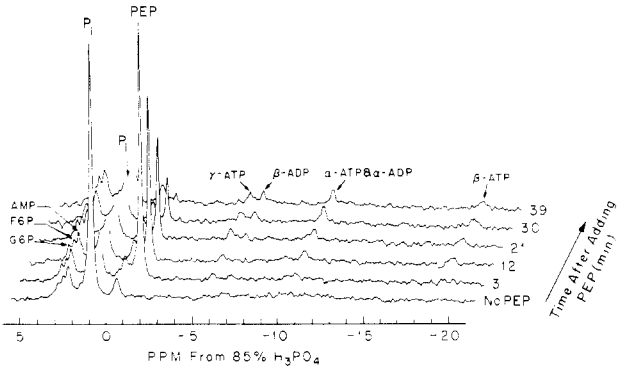

FIGURE 3: 121.5-MHz ^{31}P NMR investigation of the effect of 100 μM *N,N'*-dicyclohexylcarbodiimide on the utilization of phosphoenolpyruvate by membrane vesicles from *E. coli* PSM116 containing a plasmid with the phosphoglycerate transport system from *S. typhimurium* (pJH7) at 30 °C. The spectra, with the exception of the bottom one, are each displaced slightly to the right to facilitate comparison. The P_i peaks are omitted for clarity.

addition of DCCD has little effect on the signals seen; ATP and ADP are readily visible, and the rate at which PEP disappears in the experiment is somewhat smaller (71% consumed in 24 min).

In the presence of fluoride, DCCD has much more profound effects. The combined presence of these two inhibitors slows considerably the decrease in the intensity of the PEP signal seen in the above experiments (only 31% consumed in 24 min) (results not shown). Furthermore, the signals corresponding to the various phosphate groups of ADP and ATP are significantly enhanced. As expected, the phosphoglyceric acids are not formed under these conditions.

Hugenholtz et al. (1981) have shown that the membrane vesicles prepared from strains capable of transporting PEP can use this compound to drive proline transport, provided that ADP and pyruvate kinase are incorporated into these vesicles. This result strongly suggests that such membrane vesicles are capable of forming an electrochemical gradient of protons ($\Delta\mu_{H^+}$) in the presence of PEP.

In the experiments presented above, there is no indication of the presence of a pH gradient across the membrane since only one inorganic phosphate signal is seen. This is not due to our inability to detect the internal inorganic phosphate in these experiments since this signal can readily be seen if the external phosphate is broadened by the addition of paramagnetic Mn^{2+} ions to the membrane vesicle suspensions (Figure 4). Rather, the position of this internal phosphate signal indicates that no pH gradient exists in these membrane vesicles under such conditions.

Discussion

Two basic conclusions that can be drawn from the results are summarized as follows: (i) it is apparent that the membrane vesicle preparations used in these experiments possess sufficient metabolic capabilities to form large amounts of 2- and 3-phosphoglyceric acids from PEP; and (ii) under the experimental conditions used here, membrane vesicles do not form a transmembrane pH gradient when PEP is added.

Recently, Hunt & Hong (1981) described membrane vesicle preparations capable of performing binding-protein-dependent transport of glutamine. A thorough biochemical characterization of such preparations has indicated the presence of substantial metabolic capabilities in these membrane vesicles (Hunt & Hong, 1983). A partial list of the enzymatic activities possessed by such membrane vesicles is presented in Table I. The results described in this paper confirm the

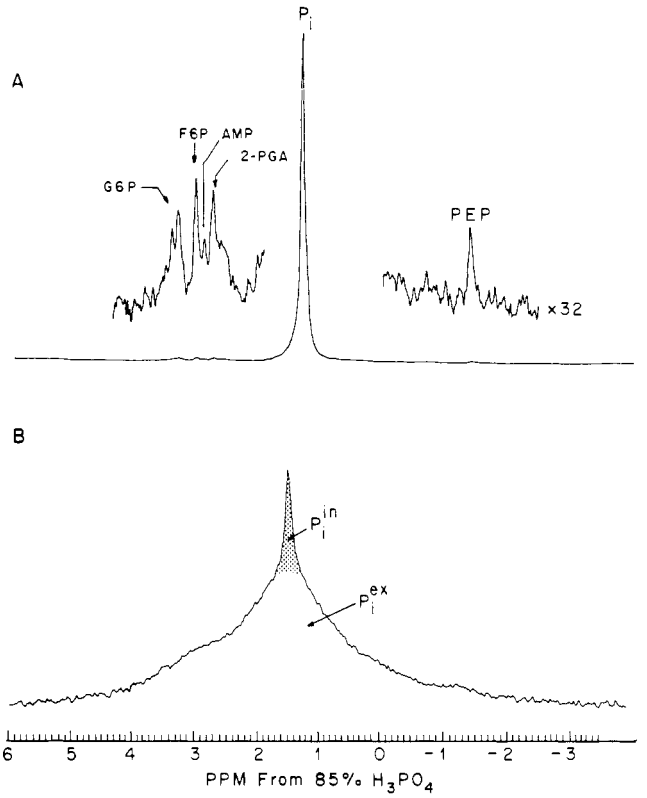

FIGURE 4: 121.5-MHz ^{31}P NMR investigation of the effect of $MnCl_2$ on the ^{31}P resonance of inorganic phosphate from membrane vesicles of *E. coli* PSM116 containing a plasmid with the phosphoglycerate transport system from *S. typhimurium* (pJH7) at 30 °C in the presence of phosphoenolpyruvate: (A) in the absence of $MnCl_2$ (the inserted spectra are a 32-fold expansion); (B) in the presence of 1 mM $MnCl_2$.

Table I: Enzymatic Activities Exhibited by *E. coli* Membrane Vesicles

from previous work[a]	from this work
adenylate kinase	adenylate kinase
pyruvate kinase	pyruvate kinase
Mg^{2+}-ATPase	Mg^{2+}-ATPase
pyruvate dehydrogenase	
phosphotransacetylase	
acetate kinase	
adenosine kinase	
nucleoside kinase	
nucleosidediphosphate kinase	
	enolase
	phosphoglycerate mutase

[a] Taken from Hunt & Hong (1983).

presence of pyruvate kinase, adenylate kinase, and the energy-transducing ATPase in membrane vesicles. (Pyruvate kinase must be present in order for the membrane vesicles to form ATP from PEP; adenylate kinase must be present to enable the membrane vesicles to form AMP; the presence of active Mg-ATPase is indicated by the effect that DCCD has on the rate of metabolism of PEP and on the magnitude of the ATP signals in the presence of fluoride.) In addition, our data indicate that our membrane vesicle preparations contain the enzymes enolase and phosphoglycerate mutase (appearance of 2-PGA and 3-PGA on addition of PEP and inhibition by fluoride). Thus, these membrane vesicle preparations are much more versatile metabolically than we might have otherwise assumed [see, for example, Kaback (1971)]. The question of whether the enzymes detected in membrane vesicles are present because of some association with the inner membrane of *E. coli*, or instead are simply trapped at the same concentration that they attain in the lysis buffer during the preparation of vesicles, awaits further study.

The absence of a transmembrane pH gradient in these experiments is, at first glance, surprising. Hugenholtz et al. (1981) have shown that similar membrane vesicle preparations are capable of using PEP to drive proline transport, even in the presence of cyanide or under anoxic conditions, similar to those in our experiments. The membrane vesicle preparations described here are also capable of using PEP to drive proline transport (unpublished results). At the external pHs used in these experiments, ΔpH was expected to be a major component of $\Delta\mu_{H^+}$, the driving force for proline transport in membrane vesicles (Hugenholtz et al., 1981). Indeed, on the basis of the results of Ramos & Kaback (1977a), we expected to see a ΔpH of approximately 1.5 units and a corresponding internal phosphate signal at 2.7 ppm from 85% H_3PO_4. Instead, we see overlapping internal and external P_i signals. On the basis of pH calibration curves performed in various buffers (i.e., "external" buffer, lysis buffer, "internal" buffer) and in the presence or absence of thick suspensions of membrane vesicles, we conclude that there can be no more than a 0.2 pH unit difference across the membrane vesicles in our experiments (results not shown).

This finding is, however, not surprising when viewed in the context of the results of Ramos & Kaback (1977b). These authors observed that the addition of substrate transported by proton–substrate symport caused a substantial diminution of the D-lactate-generated or ascorbate–PMS-generated pH gradient in membrane vesicles. Since PEP is apparently transported in membrane vesicles, such as those described here, in symport with protons (Hugenholtz et al., 1981), it is expected that the principal component of $\Delta\mu_{H^+}$ in these membrane vesicles, at any external pH, will be $\Delta\psi$. Thus, ΔpH should be small or nonexistent.

The studies described here are the first ^{31}P NMR studies conducted with isolated *E. coli* membrane vesicles. Here, we have established, or verified, the presence of a number of enzymatic activities in such membrane vesicles. Furthermore, we have corroborated conclusions drawn by other investigators concerning the effect of transport substrates on ΔpH in vesicles (Ramos & Kaback, 1977a,b). Further studies using this technique should considerably advance our understanding of the biochemistry of isolated bacterial membrane vesicles.

Registry No. NaF, 7681-49-4; $MnCl_2$, 7773-01-5; ATPase, 9000-83-3; phosphoenolpyruvate, 138-08-9; *N,N'*-dicyclohexylcarbodiimide, 538-75-0; adenylate kinase, 9013-02-9; pyruvate kinase, 9001-59-6; enolase, 9014-08-8; phosphoglycerate mutase, 9032-62-6; 2-phosphoglyceric acid, 2553-59-5; 3-phosphoglyceric acid, 820-11-1.

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Polyamines as Modulators of Membrane Fusion: Aggregation and Fusion of Liposomes†

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ABSTRACT: We have studied the effect of the polyamines (spermine, spermidine, and putrescine) on the aggregation and fusion of large (approximately 100 nm in diameter) unilamellar liposomes in the presence of 100 mM NaCl, pH 7.4. Liposome fusion was monitored by the Tb/dipicolinic acid fluorescence assay for the intermixing of internal aqueous contents, and the release of contents was followed by carboxyfluorescein fluorescence. Spermine and spermidine at physiological concentrations aggregated liposomes composed of pure phosphatidylserine (PS) or phosphatidate (PA) and mixtures of PA with phosphatidylcholine (PC) but did not induce any fusion. However, liposomes composed of mixtures of acidic phospholipids, cholesterol, and a high mole fraction of phosphatidylethanolamine could be induced to fuse by spermine and spermidine in the absence of divalent cations. Putrescine alone in the physiological concentration range was ineffective for both aggregation and fusion of these liposomes. Liposomes made of pure PC did not aggregate in the presence of polyamines. Addition of aggregating concentrations of spermine caused a drastic increase in the rate of Ca^{2+}-induced fusion of PA liposomes and a large decrease in the threshold Ca^{2+} concentration required for fusion. This effect was less pronounced in the case of PS or PA/PC vesicles. Preincubation of PA vesicles with spermine before the addition of Ca^{2+} resulted in a 30-fold increase in the initial rate of fusion. We propose that polyamines may be involved in the regulation of membrane fusion phenomena accompanying cell growth, cell division, exocytosis, and fertilization.

The naturally occurring polyamines (putrescine, spermidine, and spermine) are ubiquitous components of living material (Cohen, 1971; Bachrach, 1973). Their biosynthesis is exquisitely controlled, and major changes in intracellular polyamine concentrations occur after stimulation of cell growth and division and at certain phases of the cell cycle (Jänne et al., 1978; Heby & Jänne, 1981). The requirement of polyamines for cellular events such as division was demonstrated by the use of polyamine biosynthesis inhibitors (Mamont et al., 1976, 1978; Heby & Jänne, 1981).

At physiological pH, polyamines are polycations, and an important part of their function in the biosynthesis of nucleic acids and proteins has been ascribed to their properties as counterions of polynucleotides [reviewed in Cohen (1971, 1978), Caldarera et al. (1976), and Algranati & Goldemberg (1977)]. Because of their cationic properties, polyamines are also expected to interact with acidic phospholipids in biomembranes. Some effects of polyamines on membrane properties have been described, including membrane stabilization against osmotic stress (Tabor, 1962; Harold, 1964), changes in membrane fluidity (Spisni et al., 1976), and effects on electrokinetic properties of red blood cells (Chun et al., 1976). However, few studies have tried to delineate the possible involvement of polyamines in physiological functions of biomembranes. Among such functions, membrane fusion is particularly important since it is a prerequisite in exocytosis (secretion, neurotransmission, cell growth), endocytosis, formation of secondary lysosomes, and cell division (Poste & Allison, 1973).

We have studied the effect of polyamines on the aggregation and fusion of phospholipid vesicles (liposomes) of different composition in order to understand the interaction of polyamines with different components of biological membranes. The fusion of liposomes containing acidic phospholipids has been extensively investigated (Papahadjopoulos et al., 1979;

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