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Differential Inhibition of the Exchange Reactions Associated with Oxidative Phosphorylation†

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ABSTRACT: Two analogs of adenosine triphosphate (ATP), adenylyl imidodiphosphate (AMP-PNP) and adenylyl methylenediphosphonate (AMP-PCP), were used to investigate the ATP $\rightleftharpoons$ HOH exchange catalyzed by rat liver mitochondria and submitochondrial particles. Replacement of the terminal bridge oxygen of the triphosphate chain of ATP in these analogs prevents enzymatic cleavage by a wide variety of enzymes, including mitochondrial ATPase, while structural characteristics of ATP are retained (Dueè, E. D. and Vignais, P. V. (1968), *Biochem. Biophys. Res. Commun. 30,* 420; Yount *et al.* (1971a), *Biochemistry 10,* 2484). No incorporation of ^{18}O from water into AMP-PNP or AMP-PCP could be detected under conditions where the ATP $\rightleftharpoons$ HOH exchange was extensive. Both AMP-PNP and AMP-PCP inhibit ATPase activity in submitochondrial particles. The AMP-PNP and AMP-PCP analogs inhibit P_i $\rightleftharpoons$ ATP and P_i $\rightleftharpoons$ H_2O exchanges with or without ADP present but not the ATP $\rightleftharpoons$ H_2O exchange. This suggests there are separate sites for ATP in the facilitation of P_i binding and P_i $\rightleftharpoons$ H_2O exchange and for ATP directly involved in the ATP $\rightleftharpoons$ H_2O exchange.

Mitochondria catalyze a rapid exchange of water oxygen with the terminal phosphate oxygens of ATP (Cohn and Drysdale, 1955; Boyer *et al.,* 1956). This exchange is unique to the oxidative phosphorylation system and can exceed considerably the rates of the P_i $\rightleftharpoons$ ATP and ADP $\rightleftharpoons$ ATP exchanges (Boyer, 1967).

Difficulties encountered in explaining the very rapid rates of both the P_i $\rightleftharpoons$ HOH and ATP $\rightleftharpoons$ HOH exchange relative to the P_i $\rightleftharpoons$ ATP and ADP $\rightleftharpoons$ ATP exchanges may be overcome by proposing the existence of separate catalytic sites for the exchanges or by invoking the participation of covalent intermediates in ATP synthesis.

However, Boyer and his coworkers have suggested that all the exchanges could occur by the overall reversal of ATP formation and that the observed differences in exchange rates are consistent with a concerted mechanism for ATP synthesis and a single mode of entry of water oxygen, provided that substrate dissociation steps rather than covalent bond-forming and -breaking steps are rate limiting in the exchange (Mitchell *et al.,* 1967; Boyer, 1967; Boyer and Silverstein, 1963). Mitchell *et al.* (1967) have pointed out that the experimentally observed rates of P_i $\rightleftharpoons$ HOH and ATP $\rightleftharpoons$ HOH exchanges are simultaneously so rapid under some conditions that it is necessary to propose the existence of a reaction separate from the reversal of ATP synthesis to account for all of the observed exchange rates in terms of a single mode of entry of water oxygen. In support of this, Mitchell *et al.* (1967) found an uncoupler-resistant P_i $\rightleftharpoons$ HOH exchange in submitochondrial particles. Boyer *et al.* (1973) have confirmed and extended these findings and propose that the separate P_i $\rightleftharpoons$ HOH exchange may result from the reversible formation of very tightly bound ATP which is unable to exchange with ATP free in the medium.

An alternative explanation of the exchange reactions associated with oxidative phosphorylation has been advanced by Korman and McLick (1970, 1972, 1973). These authors also propose that ATP synthesis involves the direct union of ADP and phosphate to form ATP by a concerted mecha-

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nism, but the ATP ⇌ HOH and P_i ⇌ HOH exchanges are visualized as resulting from the reversible formation of a pentacovalent phosphorus intermediate by the addition of water to enzyme-bound ATP or phosphate. In this scheme, in contrast to that proposed by Boyer and coworkers, ATP ⇌ HOH and P_i ⇌ HOH exchanges could occur independent of the overall reversal of ATP synthesis.

Analogs of ATP have been described in which the terminal bridge oxygen of the triphosphate chain is replaced by a CH_2 group (Myers *et al.*, 1963) or an NH group (Yount *et al.*, 1971a,b). These compounds, adenylyl methylenediphosphonate (AMP-PCP) and adenylyl imidodiphosphate (AMP-PNP), are translocated into mitochondria but are not substrates for mitochondrial ATPase (Dueè and Vignais, 1968; Yount *et al.*, 1971a; Klingenberg, 1972).

If AMP-PCP and AMP-PNP can bind to the site at which ATP ⇌ HOH exchange occurs, and if the theory of Korman and McLick were valid, water oxygen might exchange with the terminal phosphate oxygens of these analogs even though the terminal phosphate cannot be enzymically cleaved.

In this publication it is demonstrated that no detectable exchange is catalyzed by mitochondria or submitochondrial particles between either of these analogs and water oxygen. It is also shown that AMP-PCP and AMP-PNP inhibit the ATPase activity, P_i ⇌ H_2O exchange, and P_i ⇌ ATP exchange reaction catalyzed by submitochondrial particles, while under similar conditions, the ATP ⇌ HOH exchange reaction is not significantly inhibited.

Materials and Methods

Preparation of Mitochondria and Submitochondrial Particles. Mitochondria were isolated from adult male rats (Sprague-Dawley, Madison, Wis.) as described by Johnson and Lardy (1967). Submitochondrial particles were obtained by sonication of intact mitochondria at 4 A for 45 sec (two times) using a Branson Sonifier. After removal of any remaining intact mitochondria, submitochondrial particles were obtained by centrifugation at 81,000*g* for 60 min and were resuspended in 0.25 M mannitol–0.07 M sucrose–5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–1 mM EGTA (pH 7.4).

P_i ⇌ ATP Exchange. This was measured as described by Pullman (1967). The total amount of ATP remaining at the end of the incubation was determined as described by Kornberg (1950). Exchange rates were expressed as the product of the specific activity of the ATP remaining and the average pool size of ATP throughout the incubation.

ATP ⇌ HOH and P_i ⇌ HOH Exchanges. These exchanges were measured as described by Boyer and Bryan (1967), using ^{18}O-enriched water, with modification for the determination of ^{18}O in AMP-PCP and AMP-PNP. For AMP-PCP, after adsorption of the nucleotide onto acid-washed charcoal (100 mg), the charcoal was heated at 100° in 2 ml of 2 N HCl for 30 min to cleave AMP-PCP to AMP and methylenediphosphonate. After removal of the charcoal by filtration the supernatant was repeatedly evaporated to near dryness by heating in a vacuum oven at 60°, until all traces of HCl were removed. The residual solution was taken to pH 4.1 with 0.1 N KOH, transferred to a break seal tube, and evaporated to dryness. The sample was then treated with guanidine hydrochloride, as described by Boyer and Bryan (1967), to convert the oxygens of methylenediphosphonate to CO_2. Samples of CO_2 obtained from ^{18}O-labeled ATP by this method had a similar specfic activity to duplicate samples obtained as described by Boyer and Bryan (1967). For AMP-PNP, acid hydrolysis of the nucleotide after adsorption onto charcoal was carried out for 60 min to ensure complete hydrolysis of the imido link between the β and γ phosphate. Rates of exchange were calculated as described by Boyer and Bryan (1967). The ATP remaining at the end of each incubation was determined enzymically (Kornberg, 1950) and rates of exchange were calculated using the average pool sizes of ATP present throughout the incubation. In incubations containing analog of ATP in addition to ATP, rates of ATP ⇌ HOH exchange were corrected for dilution of specific activity by phosphate derived from analog of ATP, by the use of appropriate control incubations where the analog of ATP was added after the incubation mixture had been treated with perchloric acid to terminate the reaction. Rates of P_i ⇌ HOH exchange were corrected for ^{18}O introduced into phosphate as a result of ATP hydrolysis.

TABLE 1: Lack of ^{18}O Exchange with AMP-PCP and AMP-PNP in Mitochondria.[a]

Additions	μAtoms Exchanged 60 min^{-1} mg^{-1} Adenine Nucleotide ⇌ H_2O	P_i ⇌ H_2O	Atom % Excess ^{18}O in Medium
1. AMP-PCP	0.016, 0.015 (2)	1.23	3.09
Carrier ATP	0.012, 0.007 (2)	1.49, 1.50 (2)	3.09
ATP	0.41, 0.27 (2)	9.43	0.74
2. AMP-PNP	0.0078, 0.011 (2)	1.96, 2.19 (2)	2.80
Carrier ATP	0.012, 0.0043 (2)	1.88	2.66
ATP	0.57	8.92, 8.92 (2)	2.80

[a] Incubations were for 60 min at pH 7.4 and 30° in a final volume of 2 ml. The reaction mixture contained 100 mM Tris-Cl^-, 25 mM potassium phosphate, 5 mM potassium succinate, 5 mM $MgCl_2$, mitochondria (18 mg of protein), and 10 mM AMP-PCP or 5 mM AMP-PNP (when present). For estimation of the normal rate of ATP ⇌ HOH exchange, ATP was added to the same concentration as that of the ATP analog. For the experiments marked "carrier ATP," the ATP was added to the reaction mixture only after reaction had been terminated with 0.6 M perchloric acid. The reaction was started by the addition of mitochondria.

ATPase Activity. [γ-^{32}P]ATP was prepared by the method of Penefsky (1967). Assays were run at 30° and pH 7.4 in a final volume of 2.0 ml of 100 mM Tris-Cl and 5 mM $MgCl_2$. The reaction was started with the appropriate amount of [γ-^{32}P]ATP solution and was terminated after 1 min by rapid mixing with 2 ml of cold 0.6 M perchloric acid. After removal of remaining [γ-^{32}P]ATP with acid-washed charcoal, an aliquot of the medium was counted in a Packard TriCarb spectrometer.

Chemicals. AMP-PCP was obtained from Miles Laboratories, Kankakee, Ill.; AMP-PNP was obtained from ICN, Irvine, Calif., and from P-L Biochemicals, Milwaukee, Wis. Oxygen-18 enriched water was obtained from Yeda Research and Development Co., Rehovot, Israel. All other chemicals and biochemicals were of the best commercial grade available.

Results

There was no incorporation of ^{18}O from water into AMP-

TABLE II: Lack of ^{18}O Exchange with AMP-PNP in Submitochondrial Particles.[a]

Additions	μAtoms Exchanged in 60 min mg of Protein^{-1} Adenine Nucleotide $\rightleftharpoons$ H_2O	$P_i \rightleftharpoons H_2O$	Atom % Excess ^{18}O in Medium
AMP-PNP	0.023, 0.023 (2)	0.16, 0.14 (2)	0.88
Carrier ATP	0.092, 0.035 (2)	1.50, 1.32 (2)	0.88
ATP	0.35, 0.16 (2)	2.54, 2.16 (2)	0.80
ATP-10-min incubation	3.48 ± 0.17 (11)	23.2 ± 1.1 (4)	0.80

[a] Incubations were for 60 min at pH 7.4 and 30° in a final volume of 2 ml. For the experiments labeled "ATP-10-min incubations," the rate of exchange is based on 10-min, not 60-min, incubations. The values stated for the 10-min incubation are mean ± standard deviations. The reaction mixture was identical with that in Table I, except that submitochondrial particles (8.2 mg of protein) were used.

PCP or AMP-PNP, after incubation for 60 min with intact mitochondria, above that observed in control experiments where ATP was added only after reaction had been terminated with perchloric acid (Table I). The very small apparent incorporation in control experiments may be due to exchange with endogenous mitochondrial ATP, nonenzymic ATP $\rightleftharpoons$ HOH exchange, or adsorption of a small quantity of ^{18}O-labeled phosphate onto the charcoal used to isolate adenine nucleotides from the incubation medium. Varying the concentration of phosphate from 5 to 40 mM did not affect incorporation into the analogs nor did varying the incubation time from 10 to 60 min. Similar results were obtained with submitochondrial particles as shown in Table II, demonstrating that lack of incorporation of ^{18}O into the analogs did not result from their failure to penetrate the inner membrane. The data in Table II show that submitochondrial particles incorporate less ^{18}O into the nucleotide triphosphate fraction with AMP-PNP present than in the control (carrier ATP) experiment. This implies that AMP-PNP is actually inhibiting the rate of exchange of endogenous ATP at low ATP concentrations. For comparison, the measured rates of ^{18}O exchange into ATP over the same 60-min period and with the same batches of mitochondria as used for the analogs are also shown in Tables I and II. These apparent rates are extensive compared to the rates obtained for the ATP analogs, although they are considerably lower than rates measured over shorter incubation periods (Tables I and II). Due to extensive hydrolysis of ATP by the action of mitochondrial ATPase, exchange of the ^{18}O-labeled terminal phosphate of ATP into the β phosphate of ADP by the action of myokinase will be measured during long incubations, rather than exchange into the γ phosphate of ATP.

The effect of AMP-PNP and AMP-PCP on the ATPase activity of submitochondrial particles was examined to determine whether these analogs can compete with ATP for the site of ATP hydrolysis. Both analogs inhibited ATPase activity but inhibition was not of a simple competitive nature. Nonlinear double reciprocal plots were obtained (Figure 1), indicating positive cooperativity. Curved double reciprocal plots are also obtained for AMP-PCP inhibition of purified rat liver mitochondrial ATPase (Lambeth and Lardy, 1971) and a Hill plot yields a Hill coefficient greater than unity (experiments conducted by R. Ebel).

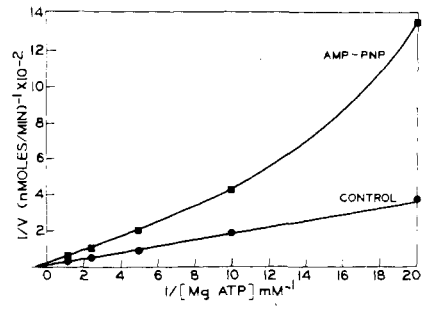

FIGURE 1: Inhibition of ATPase by AMP-PNP. Incubations were for 3 min at pH 7.4 and 30° in a final volume of 2.0 ml. The reaction mixture contained 100 mM Tris-Cl, 5 mM $MgCl_2$, 0.05–0.8 mM γ-labeled [^{32}P]ATP, and submitochondrial particles (0.6 mg of protein). When present, AMP-PNP was 20 μM. Incubations of 1 or 2 min yielded similar results.

Table III shows the effect of AMP-PNP and AMP-PCP on the $P_i \rightleftharpoons$ ATP, $P_i \rightleftharpoons H_2O$, and ATP $\rightleftharpoons H_2O$ exchange reactions catalyzed by submitochondrial particles. It must be emphasized that exchange rates have been calculated allowing for changes in the pool size of ATP during the incubation and that $P_i \rightleftharpoons$ HOH exchange rates have been corrected for ^{18}O incorporated into phosphate by ATP hydrolysis. The data demonstrate that under conditions where the $P_i \rightleftharpoons$ ATP exchange is inhibited up to 95% there is no detectable inhibition of the ATP $\rightleftharpoons H_2O$ exchange. Falcone and Witonsky (1964) have reported that some $P_i \rightleftharpoons$ ATP exchange not associated with oxidative phosphorylation can occur in mitochondria due to contamination with enzymes of the glycolytic sequence. However, the $P_i \rightleftharpoons$ ATP exchange in Table III was 98% inhibited by oligomycin, showing that it was almost exclusively associated with oxidative phosphorylation. The inhibition of the exchange reactions was also examined in the presence of ADP (Table III). The results show that inhibition is not caused by the lack of ADP following inhibition of ATPase activity by the analogs (Figure 1).

Discussion

Any proposed mechanism of ATP synthesis during oxidative phosphorylation must also explain the rate data available on the associated exchange reactions. A mechanism for ATP synthesis during oxidative phosphorylation has recently been proposed by Korman and McLick (1970,1972,1973) in which both ATP $\rightleftharpoons$ HOH and $P_i \rightleftharpoons$ HOH exchange may occur without dynamic reversal of ATP synthesis. It is proposed that the ATP $\rightleftharpoons$ HOH exchange results from the direct attack of a water molecule on enzyme-bound ATP to give an unstable trigonal bipyramidal reaction intermediate from which a water molecule is expelled in a type of Walden inversion reaction. Rapid equilibration of the terminal phosphate oxygens of ATP with water oxygen would thus be achieved without cleavage of the P–O bond formed during ATP synthesis. A similar mechanism is proposed for the $P_i \rightleftharpoons H_2O$ exchange with direct attack of water on enzyme-bound inorganic phosphate. The theory of Korman and McLick is consistent with the available data on relative exchange rates. A consequence of their proposed mechanism is that there should be incorpora-

TABLE III: Effect of AMP-PCP and AMP-PNP on the ATP ⇌ H_2O, P_i ⇌ H_2O, and P_i ⇌ ATP Exchanges.[a]

Reaction and Additions	μMoles Exchanged in 10 min/mg of Protein	% Inhibition
	P_i ⇌ ATP	
ATP	0.087 ± 0.035 (6)	
ATP + 5 mM AMP-PCP	0.087 ± 0.035 (6)	0
ATP + 10 mM AMP-PCP	0.019, 0.021 (2)	77
ATP + 5 mM AMP-PNP	0.05, 0.017 (6)	41
ATP + 10 mM AMP-PNP	0.004, 0.0004 (2)	95
ATP + 1 mM ADP	0.041, 0.037 (2)	55
ATP + 1 mM ADP + 10 mM AMP-PNP	0.019, 0.019 (2)	78 (51)
ATP + 1 mM ADP + 10 mM AMP-PCP	0.029, 0.021 (2)	71 (36)
ATP + 4 μg of oligomycin	0.0014, 0.0016 (2)	98
ATP + 0.2 mM DNP	0.005	94
	P_i ⇌ H_2O	
ATP	2.30 ± 0.34 (4)	
ATP + 10 mM AMP-PCP	1.65 ± 0.33 (4)	28
ATP + 10 mM AMP-PNP	0.59 ± 0.14 (3)	74
ATP + 1 mM ADP	2.01 ± 0.17 (3)	13
ATP + 1 mM ADP + 10 mM AMP-PCP	1.31 ± 0.40 (4)	44 (35)
ATP + 1 mM ADP + 10 mM AMP-PNP	0.51 ± 0.75 (2)	72 (69)
	ATP ⇌ H_2O	
ATP	0.49 ± 0.17 (6)	
ATP + 10 mM AMP-PCP	0.47 ± 0.18 (6)	
ATP + 10 mM AMP-PNP	0.55 ± 0.23 (5)	
ATP + 1 mM ADP	0.45 ± 0.18 (6)	
ATP + 1 mM ADP + 10 mM AMP-PCP	0.60, 0.70 (2)	
ATP + 1 mM ADP + 10 mM AMP-PNP	0.63 ± 0.21 (5)	

[a] Incubations were at pH 7.4 and 30° for 10 min in a final volume of 2 ml, or 1 ml in the presence of the AMP-PNP analog. The reaction mixture contained 100 mM Tris-Cl, 10 mM potassium phosphate, 5 mM potassium succinate, 15 mM $MgCl_2$, 5 mM ATP, and submitochondrial particles (1.5 mg of protein) and other additions as shown. Inhibition values in parentheses were calculated with respect to the control value in the presence of ADP. The reaction was started by the addition of submitochondrial particles. For measurement of ATP ⇌ HOH and P_i ⇌ HOH exchange rates, the incubation medium contained 0.92–1.88 atom % excess ^{18}O. Figures given are mean ± standard deviation, with the number of observations given in parentheses.

tion of water oxygen into ATP analogs in which the terminal phosphate cannot be enzymatically cleaved.

No significant incorporation of ^{18}O from water into AMP-PCP or AMP-PNP was detected in the present study, even on prolonged incubation of the analogs with mitochondria or submitochondrial particles. Any of the following explanations could account for this observation. (1) The ATP ⇌ HOH exchange can occur only when the terminal phosphate of ATP can be cleaved, *i.e.*, ATP ⇌ HOH exchange results from the dynamic reversal of ATP synthesis. (2) AMP-PCP and AMP-PNP may not bind at the site at which ATP ⇌ HOH exchange occurs. (3) AMP-PCP and AMP-PNP may bind at the site of ATP ⇌ HOH exchange but may not form the pentacovalent reaction intermediate postulated by Korman and McLick.

Table III shows there is inhibition of the P_i ⇌ ATP and P_i ⇌ HOH exchange reactions without concomitant inhibition of the ATP ⇌ HOH exchange. These results may be explained if it is assumed that the analogs are affecting the binding of P_i to the ATP synthetase. A requirement for ATP for the P_i ⇌ HOH exchange has been reported by Mitchell *et al.* (1967). These authors found that when ADP concentrations were maintained at low levels, by the addition of excess pyruvate kinase and phosphoenolpyruvate, the rate of P_i ⇌ HOH exchange was rapid and was proportional to the concentration of added ATP. Therefore, AMP-PCP and AMP-PNP may inhibit the P_i ⇌ HOH exchange by competition with ATP. Since the analogs do not inhibit the ATP ⇌ HOH exchange, this would imply there are separate sites for ATP involved in the facilitation of P_i binding and P_i ⇌ HOH exchange on one hand and for ATP involved directly in the ATP ⇌ HOH exchange on the other. Although direct competition of the analogs with P_i for binding to the site of exchange cannot be ruled out, it would seem less likely that ATP analogs would compete directly for a P_i binding site. Since the presence of the optimal concentration of ADP for stimulation of the P_i ⇌ HOH exchange (Jones and Boyer, 1969) does not relieve the strong inhibition of the P_i ⇌ HOH exchange by 10 mM AMP-PNP (Table III), inhibition is not the result simply of the lack of ADP following inhibition of ATPase activity by the analogs (Figure 1).

Since these analogs do not inhibit the ATP ⇌ H_2O exchange it is possible that these analogs also do not combine with the site where this exchange occurs. Therefore, these data do not invalidate the Korman and McLick hypothesis. They do point up the necessity of explaining these differential inhibitions in any proposed mechanism of ATP synthesis.

Acknowledgments

The authors thank Dr. F. E. Korman and Dr. J. McLick for many helpful discussions.

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Interaction of S-100 Protein with Cations and Liposomes†

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ABSTRACT: The interaction of the brain-specific protein S-100 with Ca^{2+}, K^+, and artificial lipid membranes (liposomes) was studied. The protein S-100 has two sets of Ca^{2+} binding sites with dissociation constants which, in 60 mM Tris-HCl buffer (pH 7.6 and 22°), are, respectively, $\simeq 5 \times 10^{-5}$ and 1×10^{-3} M. In the presence of K^+ the binding of Ca^{2+} to the high affinity sites induces a conformational change which causes an increase of the protein intrinsic fluorescence and makes the protein capable of interacting with 2-toluidinonaphthalene-6-sulfonic acid and with liposomes. The interaction with S-100 greatly facilitates the leak from liposomes of Rb^+ and Ca^{2+} but not of other solutes, such as D-glucose, γ-amino butyrate, and L-glutamate. It is proposed that when Ca^{2+} binds to the high affinity sites of S-100 it induces a conformational change which exposes some hydrophobic groups and thus makes the protein capable of interacting with liposomes and of changing their permeability to some cations.

The brain-specific protein S-100 has been recently shown to interact with lipid membranes (liposomes) and to induce an increase in their permeability to Rb^+ (Calissano and Bangham, 1971). Liposomes are liquid crystals formed by two or more lipid layers surrounding an aqueous phase (Bangham *et al.,* 1965). They have been used as a model of biological membranes to investigate the effect of several substances (antibiotics, proteins, etc.) on ion permeability. The effect induced by S-100 requires the presence of Ca^{2+} and varies with the composition of the lipid membrane, the largest effect being observed with negatively charged liposomes, consisting of phosphatidylserine and phosphatidylcholine. A simple hypothesis on the mechanism of this induced diffusion was based on the well-established interaction between S-100 and Ca^{2+} ions (Calissano *et al.,* 1969). The binding of this cation to the protein is accompanied by the exposure to the solvent of some hydrophobic amino acid residues. Ca^{2+} would facilitate the interaction between the negatively charged liposomes and the acidic protein S-100 both by electrostatic effects and by promoting the above-mentioned conformational changes which would make the protein more lipophilic.

The finding that a brain-specific protein, of still unknown function, exerts a calcium-dependent effect on membrane permeability seemed to deserve further study, in view of possible physiological implications.

Materials and Methods

Chemicals. Egg phosphatidylcholine and bovine brain phosphatidylserine, Na salts, were grade I products from Lipid Products Ltd., England, and used without further purification. The open ampoules were stored under nitrogen at −30° to minimize air oxidation. Stearlyamine was a kind gift from N. Miller (ARC, Babraham, England). 2-Toluidinonaphthalene-6-sulfonic acid, K salt, was purchased from Serva, Heidelberg, Germany. All common salts and solvents were reagent grade. KCl, $CaCl_2$, and RbCl for binding experiments and fluorescence studies were "Suprapur" reagents from Merck, Darmstadt, Germany. $^{86}RbCl$ and $^{45}CaCl_2$ were obtained from the Radiochemical Centre, Amersham, and had a specific activity of 2–10 mCi/mg.

S-100. The S-100 protein was prepared in pure form from beef brain as described by Moore (1965) and kept as a lyophilized powder at −30°. Protein concentration was measured by absorbancy at 280 nm assuming a molar extinction coefficient of 8260 mol^{-1} cm^{-1} (Calissano *et al.,* 1969). The protein was free of EDTA employed during the purification procedure (Levi *et al.,* 1974).

Liposome Preparation and Diffusion Assays. The method of liposome preparation was a modification of the original procedure of Johnson and Bangham (1969). Vesicles were prepared by adding 1.0 ml of 20 mM Tris-Cl buffer (pH 7.5), containing 60 mM KCl, 1 mM RbCl, plus 10–20

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