BBA 75212

PROPERTIES OF AN ATP-BINDING PROTEIN ISOLATED FROM MEMBRANES OF NERVE ENDINGS

L. G. ABOOD AND A. MATSUBARA

Center for Brain Research and Department of Biochemistry, University of Rochester, Rochester, N.Y. 14627 (U.S.A.)

(Received August 23rd, 1968)

SUMMARY

Sonicated membranes derived from isolated nerve-ending particles of rat brain are able to adsorb ATP and other triphosphate nucleotides but not diphosphates or monophosphates. The interaction of ATP was determined by measuring the adsorption of [^{14}C]ATP to a surface film of the sonicated material and by surface-potential changes. The material responsible for adsorption is a protein, and although adsorption is facilitated by lipids, they are not directly involved. From pH and other studies it appears that the ATP is electrostatically bound to the amino groups of such amino acids as glutamine and glutamate. Divalent cations are not involved, and the adsorption does not appear to be directly related to ATPase or other ATP-linked enzymic activity.

INTRODUCTION

In a previous study[1] it had been demonstrated that ATP is concentrated in the membranous components of rat brain, particularly those derived from nerve-ending particles. Furthermore, the ATP, and other nucleotides, were strongly adsorbed to the membranous components and could not be readily removed by repeated washing with aqueous solutions, hypotonic shock, or even ultrasonic disintegration. On the basis of other studies demonstrating an interaction between phospholipids, Ca^{2+}, and ATP[2,3], it was inferred that the ATP may in part be associated with the membrane phospholipids. One of the chief difficulties with this inference was the fact that the Ca^{2+}, but not the adenine nucleotides, could be extracted from the membranes with organic solvents that completely removed the phospholipids.

The present study was originally undertaken with the purpose in mind of examining the characteristics and nature of the ATP adsorption to membranous fragments derived largely from nerve-ending particles of brain tissue. One significant and unexpected observation was that ATP is adsorbed to specific proteins derived from the membranes and that the role of phospholipids was a secondary one. A study has been made of the distribution of the protein in various tissues and the subcellular fractions of rat brain. Some of the physical and chemical properties of the protein have been examined, and a preliminary attempt at purification is described.

METHODS AND MATERIALS

Radiotracer measurement of [^{14}C]ATP adsorption

The surface adsorption of [^{14}C]ATP was measured by the radiotracer technique of ANIASSON AND LAMM[4]. Briefly, the technique consists of determining the radioactivity of a sample in a planchet before and after the application of the surface-active material to which the radiotracer is adsorbed. The planchets, which were made of Teflon, were 2 cm in diameter and 0.6 cm high. Radioactivity was measured in 1.2 ml solution containing 0.1 mM Na_2ATP, 5 mM HCl and 0.05 μC [^{14}C]ATP, utilizing a Geiger–Müller tube with a mica end window (thickness, 1 mg/cm^2). (Greater sensitivity could be obtained using a windowless gas-flow Geiger–Müller detector, Nuclear-Chicago, model D-47K2, and a "Q" gas-flow pressure of 15 lb·inch^{-2}.) After the initial radioactivity was determined, a 20-μl sample (50 μg protein) of sonicated material or beef brain protein Pr-2 (50 μg protein) was gently applied to the surface of the solution and radioactivity again measured. Since ATP adsorption became saturated within 6–8 min and remained reasonably constant, radioactivity was determined 10 min after application of the surface film.

The amount of radioisotope adsorbed to the surface film was calculated according to the formula proposed by NILSSON[5]:

$$I' = \frac{I_m - I}{S \cdot A}$$

where I is the radioactivity (counts/min) without the surface film, I_m the radioactivity with the film, S the specific activity (counts/min per mole) of the radiotracer, and A the surface area (cm^2) of the Teflon planchet.

Surface-potential measurements

The technique for the measurement of surface potential was that of YAMINS AND ZISMAN[6] as modified by KINLOCH AND MCMULLEN[7]. Details of the experimental procedure are described elsewhere[3]. The Langmuir trough, machined from Teflon, had a capacity of 50 ml and dimensions of 12 cm × 7 cm × 0.5 cm. After an optimal quantity of sonicated microsomes or purified protein was applied to the surface, the potential was measured as a function of time or after surface compression.

Subcellular fractionation

The procedure employed for subcellular fractionation of whole rat brain has been described in detail elsewhere[1,8]. Briefly it involves the use of discontinuous density-gradient centrifugation employing concentrations of Ficoll ranging from 2.5 to 30%. Preparation of the surrounding membranes of nerve-ending particles was accomplished after hypotonic shock and recentrifugation in a discontinuous sucrose gradient, as described elsewhere[1]. All the samples were resuspended in 0.25 M sucrose and centrifuged at 100000 × g for 30 min prior to use. The "microsomal" (small membrane) fraction of rat tissue was that fraction obtained by centrifugation of a 0.25 M sucrose homogenate at 80000 × g for 1 h, following the removal of the heavy pellet by centrifugation at 12000 × g for 20 min.

Preparation of ATP-adsorptive protein from beef brain

Approx. 200 g of gray matter from fresh beef brain were homogenized (Waring blendor) in 3 l of acetone chilled to —20°. After 30 min at 0° and decantation, the suspension was centrifuged at 15000 × *g* for 20 min. The supernatant was discarded. The residue was then homogenized in 1.5 l of chloroform–methanol (2:1, by vol.) at 5° and centrifuged at 15000 × *g* for 15 min. After the residue was again extracted with 1.5 l of chloroform–methanol (2:1, by vol.), it was recentrifuged and resuspended in 200 ml of ethyl ether. This suspension was dried by filtration *in vacuo* in a sintered-glass funnel. The residue, which was designated Pr-1, was stored in a desiccator at —20° and employed as the stock material for further purification. Approx. 20 g (dry wt.) were obtained with a lipid content of less than 0.1 %.

Pr-1 was further purified by homogenizing 10 g in 200 ml of 0.1 M citric acid, which, after remaining at 0° for 60 min, was centrifuged at 10000 × *g* for 20 min. The yellowish supernatant was then carefully adjusted to pH 3.8 with NaOH, allowed to remain at 0° for 30 min and then centrifuged at 10000 × *g* for 20 min. ATP-adsorptive activity was present in both the supernatant and residue at this stage. The residue was rehomogenized in 200 ml of citric acid and the suspension centrifuged at 15000 × *g* for 30 min. The clear, yellow supernatant was then dialyzed for 24 h against running distilled water and finally lyophilized to yield Pr-2. Pr-2 was stored *in vacuo* at —20°.

The subcellular fractions were disrupted by sonication, employing a Branson cell disrupter with a power density of 460 $W \cdot inch^{-2}$ and an operating frequency of 20 kcycles/sec. Sufficient disintegration of most fractions required 3–4 min, although the myelin fraction required up to 8 min. The samples were immersed in an ice bath during the procedure.

The procedures for chemical alteration of the protein were adapted from FRAENKEL-CONRAT[9]. 2-Hydroxy-5-nitrobenzylbromide[10] and dansyl chloride[11] were dissolved in acetone and added to the sonicated suspension to a final concentration of 5 %. Other procedures are described in Table VI.

All the special reagents including the crystalline enzymes and proteins were products of either Sigma Chemical or Nutritional Biochemical. The [8-^{14}C]ATP (specific activity, 20 mC/mmole) was obtained, in the form of tetrasodium salt, from New England Nuclear. It was established, both electrophoretically and by thin-layer chromatography[12], that the [^{14}C]ATP was over 95 % pure. Further purification was carried out with some material employing Dowex-1 column chromatography[13], but the results obtained with ATP adsorption were essentially similar.

RESULTS

Cytoplasmic distribution of ATP-adsorptive activity

Various cytoplasmic fractions of rat brain were tested for their ability to adsorb ATP after sonication (Table I). The highest activity was observed in the nerve-ending fractions. The next highest activity resided in the small membrane ("microsomal") fraction. Some activity was also observed in the mitochondrial and myelin-axonal fractions. By further fractionation of the nerve endings[1] it could be shown that the major activity resided in the surrounding membranes. Slight activity was observed in the soluble protein fraction, which was concentrated either by precipitation with $(NH_4)_2SO_4$ or dilute HCl.

TABLE I

DISTRIBUTION OF ATP-ADSORPTIVE ACTIVITY IN VARIOUS SUBCELLULAR FRACTIONS OF RAT BRAIN

The results are expressed as ATP adsorbed/mg protein at saturation (10 min following application of sonicated material). Values are averages of 3 experiments agreeing within 7%.

Cell fraction	*ATP adsorbed (nmoles/mg protein)*
Nerve-ending membranes	60
Nerve endings (large)	56
Nerve endings (small)	17
Myelin + processes	6
Membrane fragments*	32
Mitochondria	4
Soluble	3
Ribosomes	2

* Membrane fragments include disintegrated synaptic membranes, vesicles, endoplasmic reticulum, neuronal-glial processes, and mitochondrial membranes.

TABLE II

ATP-ADSORPTIVE ACTIVITY IN SONICATED "MICROSOMAL" FRACTIONS OF VARIOUS RAT TISSUES

The values are the averages of 3 separate experiments and agree within 8%.

Tissue	*ATP adsorbed (nmoles/mg protein)*
Brain	64
Skeletal muscle	31
Heart	25
Kidney	29
Liver	13
Duodenum	9
Serum	2

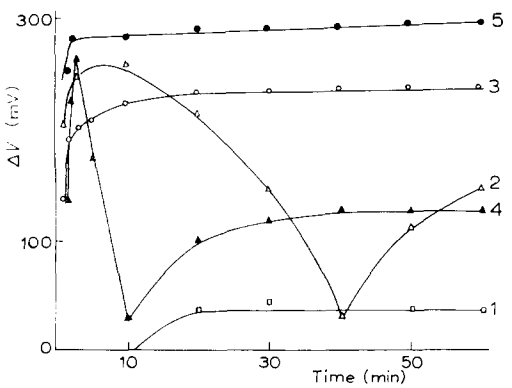

Fig. 1. Surface-potential (ΔV) changes of sonicated membranes in the presence of Ca^{2+} and ATP *vs.* time. The pH of all solutions was 3.0, adjusted with HCl. Curve 1, control; Curve 2, 0.1 mM ATP; Curve 3, 0.01 M $CaCl_2$; Curve 4, 0.1 mM ATP, 0.1 mM $CaCl_2$; Curve 5, 0.1 mM ATP, 0.01 M $CaCl_2$. Time is after the application of the sonicated nerve-ending membranes.

Distribution of activity in various tissues

The ATP-adsorptive activity was examined in the "microsomal" (small membrane) fraction of various rat tissues (Table II). Of the tissues studied, brain had by far the greatest activity. Less than half of the brain activity was observed in skeletal muscle, heart, and kidney. Still lower activity was found in liver and duodenum, while a trace was observable in serum.

The application of the sonicated suspension on a water surface resulted in no significant potential change while that on the solutions containing Ca^{2+} led to the formation of surface films, having a high and consistent surface potential (Fig. 1). On the other hand, the surface films formed on the solutions containing ATP behaved quite differently. They had a high potential immediately after application of the suspension, followed first by a sudden decrease and then a gradual increase. This inconsistency of the surface potential was repeatedly observed with solutions containing ATP, although the shapes of curves were not necessarily the same. These variations are due to such factors as the diffusion of the suspension particles, the inhomogeneity of the surface film, and desorption. Similar results were obtained with the sonicated suspensions of beef brain membrane fragments (Fig. 2).

A comparison was made of the surface-potential changes produced by AMP, ADP, and ATP utilizing the sonicated membrane preparation (Fig. 2). In order to make the data more reproducible, it was desirable to inject an excess of sonicated material into the subsolution, namely, approx. 3 mg protein. Mixing was accomplished by repeated aspiration employing a 5-ml pipette. The differences at the time of the saturated surface-potential values ranged between 200 and 300 mV. The effect of ADP on the potential was considerably less than that of ATP, while AMP had almost no effect. With ATP the change in ΔV was almost instantaneous, while with ADP the rate of change was considerably slower and linear.

The same experiment was carried out on sonicated suspensions of other rat organs. In this case, all samples were pretreated with NaOH and diluted to the

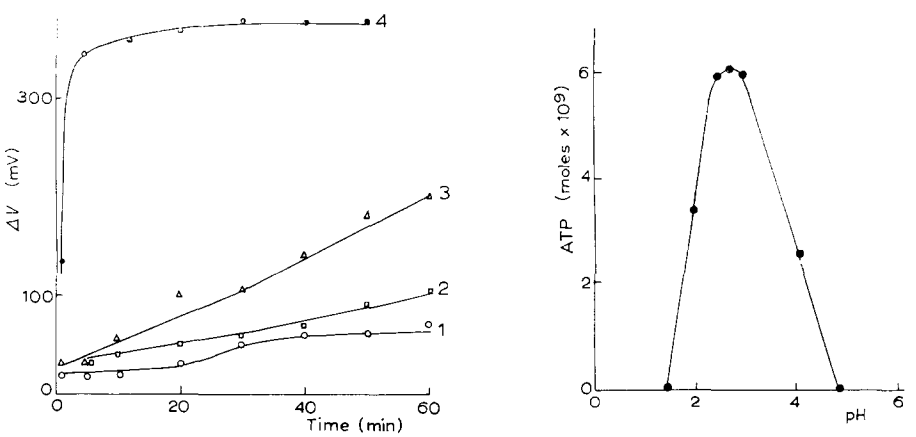

Fig. 2. Surface-potential (ΔV) changes of sonicated membranes in the presence of AMP, ADP, and ATP. Concentrations of all nucleotides were 0.1 mM; pH 3.0. Curve 1, control; Curve 2, AMP; Curve 3, ADP; Curve 4, ATP.

Fig. 3. pH curve for ATP-adsorptive activity of protein fraction Pr-2.

appropriate concentration. The only tissues other than brain where a potential change occurred in the presence of ATP was liver and, to a lesser extent, kidney. With the protein fraction Pr-2 similar results were obtained in the surface-potential studies.

Effect of proteolytic enzymes

The pH optimum for ATP-adsorptive activity was in the range 2.5–3.0 (Fig. 3). Activity decreased precipitously on either side of the optimum, being 0 at pH 1.5 and 5.0.

TABLE III

EFFECT OF VARIOUS INORGANIC AND ORGANIC ELECTROLYTES ON ATP ADSORPTION TO SONICATED NERVE ENDINGS AND BEEF BRAIN PROTEIN Pr-2

Activity is expressed in terms of mg protein. Each value is an average of 3 determinations which agreed within 7%.

Electrolytes	*Concn. (mM)*	*Sonicated nerve endings*		*Pr-2*	
		ATP (nmoles/mg)	*Decrease (%)*	*ATP (nmoles/mg)*	*Decrease (%)*
Control		50	—	8.2	—
NaCl	10	33	34	5.1	38
NaCl	100	11	78	—	—
KCl	10	33	34	5.2	36
KCl	100	10	80	—	—
Na_2SO_4	1	29	42	4.6	44
K_2SO_4	1	28	44		
K_2SO_4	10	3	94	—	—
NaH_2PO_4	1	30	40	—	—
NaH_2PO_4	10	20	60	5.0	39
NaI	10	32	36	5.2	36
NaF	10	32	36	5.1	38
$NaNO_3$	1	30	40	5.2	36
HCl	100	0	100	0.5	96
Citric acid	10	34	32	—	—
Glutamate	10	35	30	—	—
Tetraethylammonium·HCl	10	33	34	5.2	36
Sucrose	100	8	84	—	—
Aspartate	10	56	0	7.9	4

TABLE IV

EFFECT OF VARIOUS PROTEOLYTIC ENZYMES ON ATP ADSORPTION BY BEEF BRAIN PROTEIN, Pr-2

Data expressed as % original activity remaining after incubation of protein with enzyme (2 mg crystalline proteolytic enzyme/10 mg brain protein).

Proteolytic enzyme	*% Original activity after*	
	30 min	*2 h*
Pronase	60	24
Papain	50	25
Trypsin	70	40
Chymotrypsin	80	34
Pepsin	95	85
Ficin	100	90

A variety of anions were tested for their effect on ATP adsorption to both the sonicated material and Pr-2 (Table III). At a concentration of 0.01 M various sodium halides produced a comparable decrease of about 35 %. Both KCl and NaCl at 0.1 M resulted in an 80 % decrease. SO_4^{2-} at 1 mM produced over 40 % inhibition and at 0.01 M almost complete inhibition. The inhibitory effect of $H_2PO_4^-$ was intermediate between the halides and SO_4^{2-}. Citrate, glutamate, and tetraethylammonium at 0.01 M resulted in about 30 % inhibition. Sucrose at 0.1 M was almost completely inhibitory. Aspartate was unique in that it produced no inhibitory effect at 0.01 M. EDTA at 1 mM had only a small inhibitory effect.

The effect of a number of crystalline proteolytic enzymes was tested for their ability to modify the ATP-adsorptive activity of the partially purified brain protein (Table IV). Pronase and papain had the greatest destructive effect, accounting for a 75 % loss in activity after 2 h incubation. Both chymotrypsin and trypsin were also destructive to an almost comparable degree. Pepsin and ficin, on the other hand, produced only a small loss in activity.

Effect of other nucleotides

In the presence of low concentrations (0.1 mM) of other nucleotide triphosphates the adsorption of ATP to sonicated nerve endings was inhibited (Table V). Diphosphate and monophosphate nucleotides were not inhibitory; neither were nucleosides or the free bases (data not presented).

TABLE V

EFFECT OF VARIOUS NUCLEOTIDES ON ATP ADSORPTION OF SONICATED NERVE ENDINGS

Each planchet contained [^{14}C]ATP (0.1 μM) and the final concentration of each nucleotide was 0.1 mM. Each experiment is an average of 3 determinations agreeing within 7 %.

Nucleotide	*nmoles/mg protein*	*Decrease (%)*
ATP	50	—
ADP	44	12
AMP	50	0
UTP	2	96
UMP	49	2
GTP	3	94
CTP	3	94
ITP	6	88
IMP	50	0

A variety of physical and chemical procedures was employed in an attempt to modify the ATP-adsorptive activity of sonicated nerve endings (Table VI). Such procedures as boiling, alkali treatment, or exposure to 3 M HCl and formaldehyde had only a slight effect on activity. Occasionally, boiling (at pH 7) enhanced activity, presumably by promoting better dispersion of the sonicated preparation. Complete removal of the lipid by chloroform–methanol markedly reduced activity, while partial

TABLE VI

EFFECT OF VARIOUS PHYSICAL AND CHEMICAL PROCEDURES ON ATP-ADSORPTIVE ACTIVITY OF SONICATED NERVE ENDINGS

Activity is in terms of amount of ATP adsorbed/mg total protein. Each value is an average of 3 determinations performed on 2 separate nerve-ending preparations; agreement is within 7%.

Procedure	*Conditions*	*Activity (nmoles ATP)*	*Decrease (%)*
Control	—	50	—
Boiled	100° for 5 min	55	(−10)
Alkali heated	0.2 M at 100° for 5 min	44	12
3 M HCl	1 h at 27°	48	4
Acetone powder	at 0°; lyophilized	28	44
Lipid free	chloroform–methanol (2:1, v/v)	20	60
Acetic anhydride	1 h at 0°	35	30
5% formaldehyde	4 h at 27°	48	4
Dansyl chloride	5% soln.; 1 h at 27	38	22
Nitrobenzyl bromide	5% soln.; 1 h at 27	45	10
HNO_2	1 h at 27	25	50
3 M HCl hydrolysis	30 min at 100°	5	90
2% sodium lauryl sulfate	5 min at 27	45	10
2% Triton X-100	5 min at 27	40	20
2% Triton X-100–4% ethanol	5 min at 27	10	80

removal, as with acetone, caused less reduction. Acetylation with acetic anhydride and dansylation accounted for some loss in activity; however, exposure to nitrobenzyl bromide was less inhibitory. Exposure to HNO_2 resulted in a 50% reduction, while hydrolysis in 3 M HCl virtually destroyed all activity. Surfactants alone were not inhibitory, but in the presence of 4% ethanol were strongly inhibitory.

ATP adsorption to other proteins and to lipids

A number of crystalline and purified proteins were tested for their ability to adsorb ATP. These included casein, albumin, γ-globulin, protamine, gelatin, collagen, acid phosphatase, phospholipase *c*, lactate dehydrogenase, a purified (Na^+-K^+-Mg^{2+})-ATPase (beef brain; a gift of R. TANAKA), yeast hexokinase, and cytochrome *c*. With the exception of cytochrome *c* and protamine, which exhibited only slight activity, none of the proteins were active at concentrations ranging from 10 to 100 mg/ml. A variety of phospholipids (synthetic and purified from beef brain), cholesterol, neutral fats, and a rat brain lipid mixture were also ineffective, nor did they enhance the adsorptive effect of Pr-2. It should be mentioned, however, that surface films of phospholipids do adsorb ATP after 1 h at room temperature although the amount adsorbed during the first 15 min is negligible[3].

DISCUSSION

The present findings demonstrate that a protein, which is predominantly in the membranous components of the neuron, is capable of adsorbing ATP and other triphosphate nucleotides. Since there is good agreement between the ability of the sonicated subcellular fractions to adsorb ATP and the concentration of endogenous ATP present in the fractions[1], it is likely that the presence of this membranous protein

accounts in part for the subcellular distribution of ATP in rat brain. The results confirm the fact that a large percentage of the ATP in brain is located in membranes, particularly those associated with the nerve endings. Furthermore, the amount of total adenine nucleotides associated with the isolated nerve-ending fraction (about 35 nmoles/mg protein) compares favorably with the amount adsorbed to the sonicated nerve-ending fraction (about 60 nmoles/mg protein). The values may be in closer agreement when one considers the probable loss in bound ATP occurring during the fractionation procedure. Although the greatest activity is found in brain, other tissues contain a significant amount, including other excitatory tissues, such as heart and skeletal muscle. To what extent the bound ATP in such tissues is correlated with the concentration of this protein remains to be investigated.

The type of bonding occurring between the protein and ATP would appear to be primarily electrostatic. Insofar as chelating agents, apart from their non-specific anionic effect, do not inhibit ATP adsorption, such divalent cations as Mg^{2+}, Mn^{2+} and Ca^{2+} do not seem to be involved. Furthermore, addition of a variety of multivalent cations does not enhance the effect, even after prolonged dialysis of the protein against solutions containing citrate, EDTA, or 1 M HCl. It seems likely, therefore, that free amino groups must be involved, and in view of the fact that the activity is unaltered by reagents reacting with N-terminal amino acids the groups are not terminal. Preliminary amino acid analyses of the fraction Pr-2 reveal the presence of relatively large amounts of glutamic and aspartic acid. Since this fraction is not pure, however, such results are no more than suggestive. A detailed chemical and physical analysis of the protein will be presented in a subsequent publication.

It should be emphasized that the measurement of ATP-adsorptive activity was dependent upon the surface activity of the sonicated membranes or beef brain protein. No adsorption could be demonstrated to nerve-ending particles which were either not sonicated, partially sonicated, or not partly solubilized with dilute alkali. Under such conditions the material was not sufficiently surface active. To date, no success has been achieved in demonstrating adsorption of ATP to sonicated microsomes or the purified protein employing techniques of ultracentrifugation, electrophoresis, and ultrafiltration. Both the radiotracer and surface-potential techniques are extremely sensitive requiring only a surface film of monomolecular thickness in order to demonstrate adsorption. Evidently the amount of active protein in the sonicated or purified material is too small to permit measurement of ATP binding by the other techniques employed. Since surface activity was required for the measurement of activity, any alteration in the protein which modified its surface-active properties would influence the measurement of ATP adsorption. Such alterations would include proteolytic cleavage to alter molecular weight, conformation, charge distribution, *etc.* The introduction into the subsolution of detergents and other surfactants greatly reduces ATP adsorption, presumably by forming micelles with the surface protein. Apart from their anionic competitive effect with ATP, electrolytes may diminish ATP adsorption by modifying the charge distribution and solubility which in turn may influence the surface-active property of the protein.

Although the ATP-adsorptive property of the sonicated membrane fragments is considerably greater prior to the removal of lipids, the primary adsorptive sites are on the protein. There are at least three possible explanations for the effect of lipids: (1) they can increase the surface concentration of the protein; (2) they may

increase the number of available sites by altering the tertiary–quaternary structure of the surface protein; or (3) the phospholipids themselves may be interacting with ATP. A detailed study[3] has eliminated the third possibility; for although phospholipids in the presence of Ca^{2+} do interact with ATP, the adsorption does not occur until after 15–30 min and only after partial evaporation of the system. Since the addition of purified lipids or an extract of lipids derived from the sonicated nerve endings will not enhance the adsorptive activity of the protein, the first possibility does not seem likely. Evidently, therefore, the tertiary–quaternary structure of the active protein is influenced by the lipids, and restoration of the favorable structure is not readily possible after the removal of the lipids by organic solvents or detergents. It is expected that the role of the lipids can be investigated more effectively once the protein has been further purified.

The surface potential, ΔV, is a function of the number of molecules, n, per unit area of surface, with a net dipole, μ, *plus* the electrostatic charge, ψ, in the ionic diffuse double layer, given by the expression[14]

$$\Delta V = 4\pi n\mu + \psi$$

In the present case ΔV is a measure of the sum total of dipoles and electrostatic charges of the surface-active protein in the diffuse double layer. Since the adsorption of ATP results in a net increase in ΔV, it must be assumed that the anionic charge contribution of the phosphate groups is either offset by the dipoles of the adenosine and phosphate groups or that the configuration and position of the phosphate groups within the diffuse double layer imparts a net positive charge to the surface film. Another possibility is that the interaction of ATP with the surface protein results in a condensation of the protein film, thus increasing the surface concentration, n. Since the configuration of the protein (or proteins) at the interface is not known, it is not possible to determine the nature of the ATP effect upon the surface potential.

It is now generally recognized that Ca^{2+} may be directly involved in the regulation of Na^+ permeability in the excitatory membrane; while a membranous ATP may, in turn, be regulating the association–dissociation processes of Ca^{2+} (refs. 2, 3). The presence of a large amount of the ATP-adsorptive protein in brain tissue confined largely to the excitatory components—specifically, the membranes of nerve endings—lends further credence to the direct involvement of ATP in controlling membrane permeability.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude for the able technical assistance of Miss EDELGARD GRUNER. This research was supported in part by grants from the National Institutes of Health, NB-05856, and the National Multiple Sclerosis Society.

REFERENCES

1 L. G. ABOOD, K. KURAHASI, E. GRUNER AND M. P. DEL CERRO, *Biochim. Biophys. Acta*, 153 (1968) 531.
2 L. G. ABOOD, *Intern. Rev. Neurobiol.*, 9 (1966) 223.
3 L. G. ABOOD, in S. EHRENPREIS, *Neuroscience Research*, Academic Press, New York, in the press.
4 G. ANIASSON AND O. LAMM, *Nature*, 165 (1950) 357.

5 G. NILSSON, *J. Phys. Chem.*, 61 (1957) 1135.
6 H. G. YAMINS AND W. A. ZISMAN, *J. Chem. Phys.*, 1 (1933) 656.
7 C. D. KINLOCH AND A. I. MCMULLEN, *J. Sci. Instr.*, 36 (1959) 347.
8 R. TANAKA AND L. G. ABOOD, *J. Neurochem.*, 10 (1963) 571.
9 H. FRAENKEL-CONRAT, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. IV, Academic Press, New York, 1957, p. 247.
10 D. E. KOSHLAND, JR., Y. D. KARKHANIS AND H. G. LATHAM, *J. Am. Chem. Soc.*, 86 (1964) 1448.
11 W. R. GRAY AND B. S. HARTLEY, *Biochem. J.*, 89 (1963) 59.
12 G. WEIMANN AND K. RANDERATH, *Experientia*, 19 (1963) 49.
13 W. COHN, *J. Am. Chem. Soc.*, 72 (1950) 1471.
14 J. T. DAVIES AND E. K. RIDEAL, *Interfacial Phenomena*, Academic Press, New York, 1961, p. 72.