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PURIFICATION FROM BRAIN OF AN INTRINSIC MEMBRANE PROTEIN FRACTION ENRICHED IN ($Na^+ + K^+$)-ATPase

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Summary

A microsomal fraction from canine brain gray matter has been extracted with the detergent sodium dodecyl sulfate to partially purify the membrane-bound ($Na^+ + K^+$)-stimulated adenosine triphosphatase. Phospholipid, glycolipid, and a family of other glycoproteins are also enriched by the procedure; it is proposed that the product is an intrinsic membrane protein fraction. 6—8-fold purification of ($Na^+ + K^+$)-ATPase is obtained without solubilizing the enzyme and without irreversibly altering its turnover number. Final specific activities are 350—400 μmol of ATP hydrolyzed/h per mg protein. The stimulation and reversible inactivation of the ($Na^+ + K^+$)-ATPase by dodecyl sulfate were examined for information relevant to the mechanism of action of the detergent.

Introduction

A new procedure for the purification of the ($Na^+ + K^+$)-ATPase from brain was sought because the previous procedures have practical disadvantages: complexity, low yield, and instability of the product [1—5]. Several good procedures, however, have been developed for the ($Na^+ + K^+$)-ATPase from renal medulla. If the ($Na^+ + K^+$)-ATPases in the two tissues are identical, one might expect similar techniques to work in both tissues. The simplest of the procedures is that of Jorgensen [6,7], which is based on the extraction of contaminating proteins from membrane-embedded ($Na^+ + K^+$)-ATPase with sodium dodecyl sulfate. Lipid is extracted as well as protein, so that the purified ($Na^+ + K^+$)-ATPase is membrane bound but has approximately the same lipid to

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Abbreviations: ($Na^+ + K^+$)-ATPase, sodium plus potassium-stimulated adenosine triphosphatase (EC 3.6.1.3); Ca^{2+}-ATPase, calcium-stimulated adenosine triphosphatase; Mg^{2+}-ATPase, all adenosine triphosphatase activity not sensitive to ouabain and not stimulated by Ca^{2+}, Na^+, or K^+.

protein ratio as it has before extraction [6]. The method has been successfully applied to the $(Na^+ + K^+)$-ATPase of the nasal salt gland of the duck [8]. Because of the simplicity of the method, its high yield and good purification, its application to the purification of $(Na^+ + K^+)$-ATPase from brain was studied in depth.

Materials and Methods

Materials. Canine brains were the gift of the Cardiovascular Research Laboratory, Massachusetts General Hospital. Sodium dodecyl sulfate from Sigma Chemical Co. (≈95% pure) and from BioRad Laboratories (>99% pure) gave similar purification. Chemicals obtained from Sigma included disodium ATP, *p*-nitrophenyl phosphate, ouabain, strophanthidin, 3-phosphoglycerate, Tris base, bicine, L-α-lecithin, muscle phosphorylase, bovine serum albumin, and ovalbumin. [^{3}H]Ouabain, [^{32}P]phosphate, NaB^3H_4, and sodium dodecyl [^{35}S]-sulfate were from New England Nuclear. Sodium *m*-periodate was from Mallinckrodt. Neuraminidase from *Vibrio cholerae* was from Behring Diagnostics. Galactose oxidase from *Polyporus circinatus* was purified from Worthington galactose oxidase by the method of Hatton and Regoeczi [9], and was the gift of K. Drickamer. Phosphoglycerate kinase, glyceraldehyde-3-*P* dehydrogenase and lysozyme were from Worthington. BioBeads S (BioRad Laboratories) were wetted by equilibration in turn with methanol, methanol/water mixtures, and water [10].

Preparation of microsomes. Cortical gray matter was homogenized at 4°C in nine volumes of 0.32 M sucrose, 1 mM EDTA by a motor-driven teflon-glass homogenizer. It was centrifuged for 20 min at 850 × *g* and 20 min at 8500 × *g*. The supernatant was centrifuged for 1 h at 30 000 rev./min. The pellet was resuspended in sucrose/EDTA and stored at −60°C; this was the microsomal fraction normally used for dodecyl sulfate extraction. When indicated in the text, some microsomes were treated with deoxycholate and NaI by the procedure of Nakao [2] prior to dodecyl sulfate extraction. The procedure of Hart and Titus [11] was used for KI extraction after dodecyl sulfate extraction.

Assay. The $(Na^+ + K^+)$-ATPase reaction mixture contained 140 mM NaCl, 20 mM KCl, 3 mM ATP, 3 mM $MgCl_2$, and 30 mM histidine · HCl, pH 7.25. Incubation was at 37°C for 2–6 min, during which period hydrolysis is essentially linear. Each ml of reaction mixture was quenched with 2.5 ml of 1.25% ammonium molybdate in 0.3125 M H_2SO_4 and was extracted by vortexing for 10 s with 2.5 ml isobutanol/benzene (1 : 1, v/v). The absorbance of the extract containing the yellow phosphomolybdate complex was measured at 380 nm absorbance. $(Na^+ + K^+)$-ATPase activity was defined as the activity sensitive to 100 μM strophanthidin. Ca^{2+}-ATPase activity was that seen in the presence of 100 μM $CaCl_2$, minus that seen in the presence of 1 mM ethyleneglycoltetraacetic acid (EGTA). Mg^{2+}-ATPase activity was defined as that seen in the absence of calcium and insensitive to strophanthidin.

Phospholipid phosphate was analyzed by the method of Ames and Dubin [12]. Protein was measured by the method of Lowry et al. [13]; 1% dodecyl sulfate was included in the reagents.

Incubation with dodecyl sulfate. Unless otherwise stated, dodecyl sulfate

from a stock solution was added with stirring to microsomes in 0.16 M sucrose, 1 mM EDTA, 3 mM ATP, and 30 mM histidine or imidazole · HCl, pH 7.5. Incubation was at room temperature for 30 min.

Sucrose gradients. 10-ml aliquots of microsomes in dodecyl sulfate were layered on 32-ml linear gradients, 30—7% (w/v) sucrose. For routine preparative batches the microsomes were incubated at 4 mg protein/ml with 1.47 mg dodecyl sulfate/ml. Centrifugation was from 4 to 6 h at 27 000 rev./min, 4°C, in a Beckman SW72 swinging bucket rotor. Gradients of 40—15% (w/v) sucrose were used to obtain the data in Fig. 4, but complete separation of dodecyl sulfate from the $(Na^+ + K^+)$-ATPase was not always obtained on the denser gradients. Pelleting the microsomes through a sucrose step gradient is not recommended; at the bottom of the gradient there is always a glassy pellet with an unrelated polypeptide composition, presumably derived from other organelles. Gradient fractions were collected from a puncture made just above this pellet.

Dodecyl sulfate gel electrophoresis was done according to Zeigler et al. [14]. Samples, 10—50 μg of protein, were dissolved in 1 or 2% dodecyl sulfate, 10 mM EDTA, and 0.5% phenylmethylsulfonyl fluoride at room temperature.

Glycoprotein labelling was done essentially by the methods of Steck and Dawson [15]. Pretreatment with neuraminidase was necessary before the galactose oxidase could label any glycoprotein.

Phosphorylation, ouabain binding, and phosphatase activity. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell [16]. The phosphorylation reaction mixture contained 22 μM ATP (1.5 Ci/mmol), 140 mM NaCl, 3 mM $MgCl_2$, 30 mM Tris · HCl pH 7.4, ±20 mM KCl, in 100 μl. $(Na^+ + K^+)$-ATPase specific phosphorylation was defined as the total ^{32}P incorporated in the presence of sodium minus the ^{32}P incorporated in the presence of both sodium and potassium. In the absence of dodecyl sulfate, the amount incorporated in the presence of both sodium and potassium is within 5% of that seen in the presence of potassium alone. The reaction was done at room temperature, and samples were quenched after 10 s with 5% trichloroacetic acid, 1 mM ATP, and 10 mM P_i [6].

For ouabain binding, aliquots of microsomes incubated with dodecyl sulfate were diluted with an equal volume of 10 μM [^{3}H]ouabain (100 Ci/mol), 100 mM NaCl, 3 mM ATP, and 3 mM $MgCl_2$, and incubated for an additional 1 h at room temperature. Controls received 5 mM unlabeled ouabain as well. Samples were centrifuged at 40 000 rev./min for 1 h, and the pellets were dissolved in dodecyl sulfate and counted. The cpm trapped in the control pellets were subtracted from the corresponding experimental points to give the ouabain specifically bound.

K^+-stimulated *p*-nitrophenyl phosphatase activity was assayed as follows. Microsomes incubated with dodecyl sulfate were diluted into 0.5 ml of a reaction mixture containing 30 mM histidine · HCl, pH 7.25, 3 mM *p*-nitrophenyl phosphate, 3 mM $MgCl_2$, with and without 30 mM KCl. Incubation was at 37°C for 2 min, and the reaction was stopped by adding 0.5 ml of 0.5 M NaOH. The absorbance at 410 nm was measured. ϵ was taken to be 18 300 $M^{-1} \cdot cm^{-1}$.

Results

Purification by dodecyl sulfate extraction

Fig. 1 shows the inactivation of brain ($Na^+ + K^+$)-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase by dodecyl sulfate. The difference in response supports the idea that the stability of different proteins to detergent inactivation can vary widely. Similar experiments were performed on brain microsomes at concentrations of protein ranging from 0.14 to 7.0 mg/ml. The concentration of dodecyl sulfate required to produce 50% inactivation of ($Na^+ + K^+$)-ATPase was directly proportional to the concentration of membrane protein. The mean for 12 determinations using seven different microsome preparations was 0.365 (±0.02 S.E.) mg dodecyl sulfate/mg protein at 50% inactivation. Fig. 2 shows the data and contrasts the results with those obtained with renal medulla microsomes, for which the ratio drops from 1.34 to 0.315 as the concentration of membrane is increased. The membranes or ($Na^+ + K^+$)-ATPase from brain and kidney appear to have different affinities for the detergent.

After incubation with dodecyl sulfate, the microsomes are layered onto a continuous sucrose gradient and centrifuged to their equilibrium density. Fig. 3 shows the profiles of brain ($Na^+ + K^+$)-ATPase activity, absorbance, protein, and dodecyl sulfate for a typical gradient. The leading (denser) half of the ATPase activity has a higher specific activity than the trailing half. The purification obtained at a given concentration of protein increases with the concentration of dodecyl sulfate until a plateau is reached (Table I). Maximum specific activities obtained are 350—400 μmol P_i produced/h per mg protein.

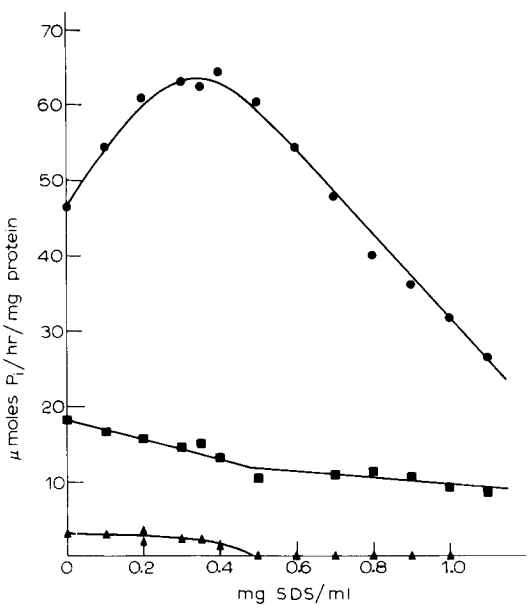

Fig. 1. Inactivation of ($Na^+ + K^+$)-ATPase, Mg^{2+}-ATPase, and Ca^{2+}-ATPase by increasing concentrations of dodecyl sulfate. Brain microsomes at 2.5 mg protein/ml were incubated with different final concentrations of sodium dodecyl sulfate (SDS) for 30 min, and then assayed. Symbols: ●, ($Na^+ + K^+$)-ATPase; ■, Mg^{2+}-ATPase; ▲, Ca^{2+}-ATPase.

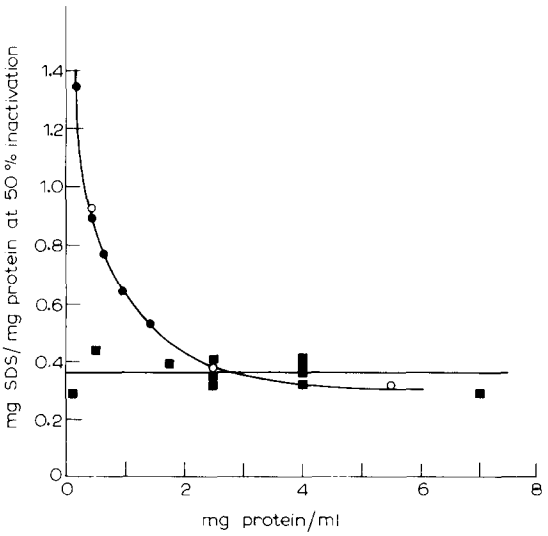

Fig. 2. The ratio of detergent to protein at 50% inactivation of $(Na^+ + K^+)$-ATPase activity, brain vs. kidney. Each point is the mid-point (50% inactivation) of an experiment like that in Fig. 1. Symbols: ■, brain microsomes; ○, canine kidney microsomes; ●, rabbit kidney microsomes calculated from Fig. 6 of Jorgensen [6].

Several other parameters that can affect purification were examined. As reported for the kidney $(Na^+ + K^+)$-ATPase [6], omitting ATP from the incubation mixture makes the enzyme sensitive to dodecyl sulfate at 30—35% lower concentrations, as does incubating at pH 6.5 and below. Any pH from 7.0 to 8.0 is adequate. Lengths of incubation from 30 min to 2.5 h have no effect on the final product. Shorter times were not tried because Mg^{2+}-ATPase activity requires 30 min to inactivate (Fig. 7). After incubation at 37°C, the product has the same density and polypeptide composition as after incubation at room temperature, but it is 30% less active. After incubation at 0°C, the product has both a lower specific activity and a more complex polypeptide composition, as if the detergent extraction is simply less complete. 200 mM NaCl has no effect

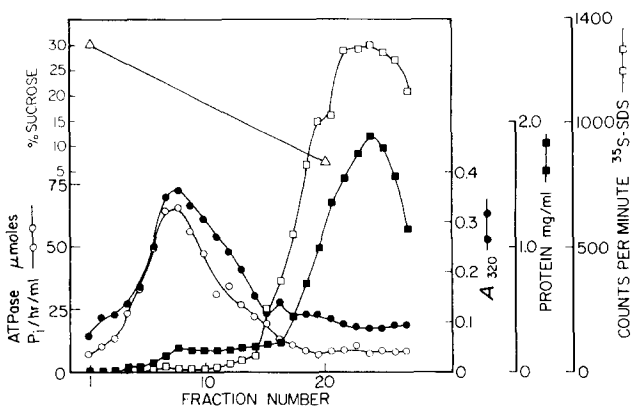

Fig. 3. Sucrose gradient profile of dodecyl sulfate-extracted brain microsomes. Symbols: △, ATPase activity; ●, A_{320nm}; ○, cpm/100 μl; ■, protein (mg/ml). Fractions 21—27 contain the sample region.

TABLE I

PURIFICATION AND RECOVERY AS A FUNCTION OF CONCENTRATION OF DODECYL SULFATE

	Dodecyl sulfate (mg/ml) **				
	0	0.6	0.8	1.0	1.2
$(Na^+ + K^+)$-ATPase activity in detergent: (%)	(100)	75	50	25	0
$(Na^+ + K^+)$-ATPase activity recovered: (%)	(100)	96.5	88.5	67.7	35.0
$(Na^+ + K^+)$-ATPase specific activity *:	44.0	278.0	328.3	323.3	283.5 ***
Mg^{2+}-ATPase specific activity *:	11.7	4.5	3.8	2.4	0.15
$(Na^+ + K^+)$-ATPase specific activity, leading (denser) half of peak:	(44.0)	298.6	366.7	365.1	363.9 ***

* μmol of ATP hydrolyzed/h per mg protein.

** Microsomes were 2.5 mg protein/ml.

*** The specific activities of the trailing half of the peak are always lower, apparently because contaminating proteins also sediment in the gradient. The effect is most pronounced when the density of the microsomes containing the $(Na^+ + K^+)$-ATPase is lowest: i.e. at the highest concentrations of dodecyl sulfate.

on dodecyl sulfate inactivation of $(Na^+ + K^+)$- or Mg^{2+}-ATPase activities, but it dramatically decreases the purification that can be obtained. Omitting the buffer from the incubation mixture to decrease its ionic strength does not improve the purification. Finally, exhaustive pretreatment of the microsomes with β-mercaptoethanol or neuraminidase does not alter either their sensitivity to dodecyl sulfate or their gradient profile.

Unlike kidney microsomes [6], the density of the brain microsomes containing $(Na^+ + K^+)$-ATPase falls as the purification increases, indicating that protein must be extracted in preference to lipid (Fig. 4a). The curve appears to approach a limiting density of 1.07 g/ml, but in reality as more dodecyl sulfate is added both lipid and protein are dissolved, and the yield is reduced (Table I). The density is a function of the ratio of dodecyl sulfate to membrane protein over a wide range of absolute dodecyl sulfate and protein concentrations (Fig. 4b). In other words, the extent of protein extraction, like the extent of inactivation, is a constant function of the ratio of detergent to membrane in the range of conditions tested.

The drop in density of the $(Na^+ + K^+)$-ATPase-containing microsomes is accompanied by an increase in the ratio of lipid to protein. Untreated microsomes had 834 nmol of lipid phosphate/mg protein, while the dodecyl sulfate-purified material had 3670 nmol P_i/mg protein. This was a 4.4-fold enrichment in lipid phosphate in an experiment which gave a 6.2-fold enrichment in $(Na^+ + K^+)$-ATPase.

It appears from the data of Fig. 4 and Table I that a limit product is being reached. Several other lines of evidence support this. (1) Using deoxycholate- and NaI-treated microsomes as starting material, with approximately twice the starting specific activity, has no effect on the final density and polypeptide composition. (2) Reextracting microsomes that have already been treated once

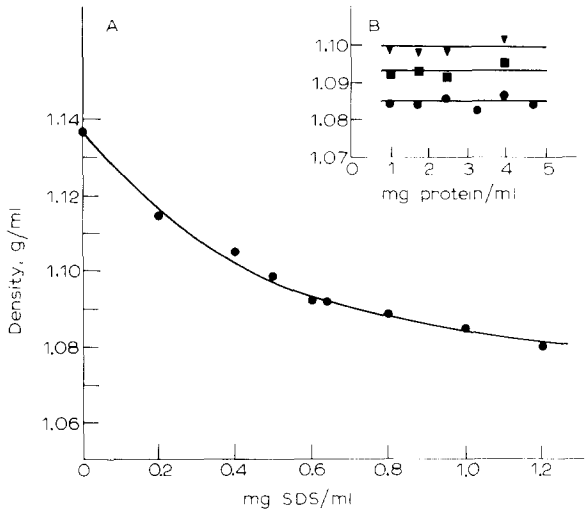

Fig. 4. Density of microsomes containing $(Na^+ + K^+)$-ATPase after extraction with dodecyl sulfate. (A) Microsomes at 2.5 mg protein/ml were treated with dodecyl sulfate at the indicated concentrations, and then sedimented to equilibrium on sucrose gradients. Each point is the density at the peak of ATPase activity. (B) Final density attained when microsomes at the indicated concentrations of protein were treated with dodecyl sulfate sufficient to cause 15% (▲), 30% (■), and 50% (●) inactivation of $(Na^+ + K^+)$-ATPase activity.

with dodecyl sulfate has no effect on the polypeptide composition, although it does cause some irreversible inactivation (approx. 50%). (3) KI treatment after dodecyl sulfate treatment brings about no further purification, except for the removal of a faint residual doublet of protein seen at $M_r \approx 220\,000$ on dodecyl sulfate gel electrophoresis.

Dodecyl sulfate-polyacrylamide gel electrophoresis was used to examine the composition of the starting microsomes and the dodecyl sulfate-extracted material. Fig. 5 shows the pattern of proteins stained by Coomassie Blue and the glycoprotein profiles. The large subunit of the $(Na^+ + K^+)$-ATPase, identified by sodium-stimulated, potassium-sensitive phosphorylation from [γ-^{32}P]-ATP (data not shown) is marked with the arrow. The other polypeptides range in molecular weights from greater than 200 000 to less than 20 000. When gels of different concentrations of acrylamide are used, the apparent molecular weights of these polypeptides change, a phenomenon often seen with glycoproteins [17]. Sialic acid residues were selectively oxidized by periodic acid [18], and galactose and galactosamine residues by a fungal galactose oxidase [19], and then reduced with NaB^3H_4 (267 Ci/mol). It can be seen that there is label in either sialic acid or galactose and galactosamine corresponding to every major band of protein in the dodecyl sulfate-treated material, except for the band corresponding to the catalytic subunit of the $(Na^+ + K^+)$-ATPase. Label in the lipid region (Fig. 5, the large peaks cut off at the far right) is enriched in dodecyl sulfate-extracted microsomes: 3.7-fold in the unoxidized controls, 2.3-fold in periodate-oxidized and in galactose oxidase-treated material, and 3.9-fold in material oxidized with galactose oxidase without prior neuraminidase treatment (not shown). Enrichment of periodate oxidized

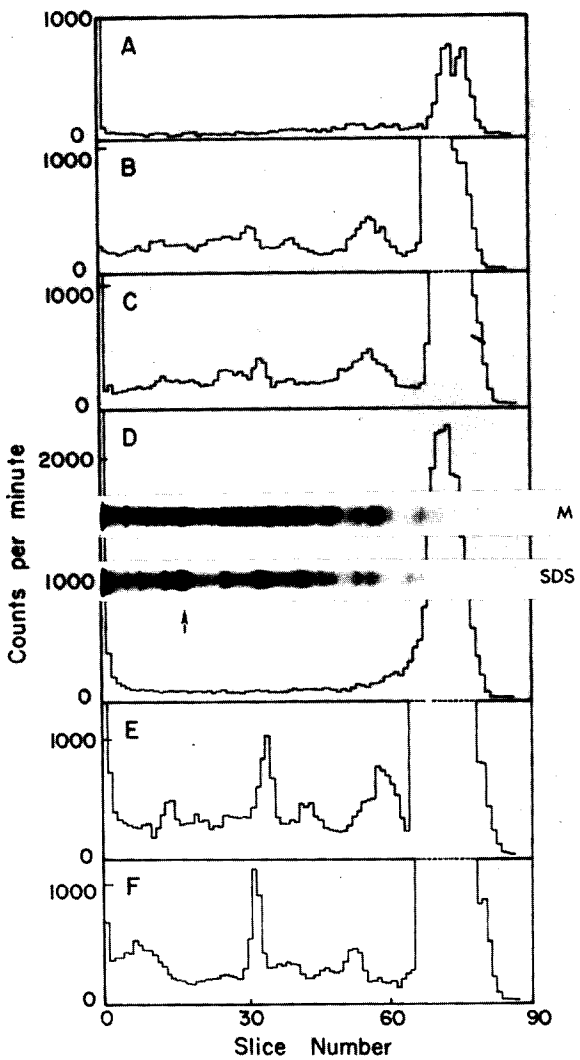

Fig. 5. Protein and glycoprotein composition of brain microsomes before and after dodecyl sulfate extraction. Electrophoresis in dodecyl sulfate on 10% polyacrylamide; the top of the gels is at the left. A,B,C, and M, unpurified microsomes. D,E,F, and SDS, dodecyl sulfate-extracted microsomes. The photographs are of Coomassie Blue-stained gels. A and D, counts per minute from NaB^3H_4 incorporated into unoxidized material (control); B and E, after sialic acid oxidation; C and F, after neuraminidase treatment and galactose oxidase oxidation.

material as high as 4.5-fold was seen in other experiments. The labelling of glycolipid is not quantitative, but the data indicate that the glycolipid of brain microsomes, like the phospholipid, is relatively resistant to dodecyl sulfate extraction.

The effect of dodecyl sulfate on activity and turnover number

Dodecyl sulfate has three different effects on the activity of the $(Na^+ + K^+)$-

ATPase. At low ratios of dodecyl sulfate to protein, it activates it (Fig. 1). At higher ratios it inactivates it, and from Table I it can be seen that some of this inactivation is reversed when the dodecyl sulfate is removed. The rest of the inactivation is irreversible, and since the specific activity remains constant while the total activity recovered decreases (Table I), the irreversibly inactivated enzyme is apparently removed from the membrane. A band of the right molecular weight is detected by dodecyl sulfate gel electrophoresis of protein from the top of the gradient.

To find out if the activation and inactivation changes the turnover number, ATPase activity, ouabain binding, phosphorylation from ATP, and K^+-stimulated *p*-nitrophenyl phosphatase activity were measured (Fig. 6a). Maximum ($Na^+ + K^+$)-ATPase activity was reached at 0.6 mg dodecyl sulfate/ml, and the value for each of the other activities measured at this point is defined as 100%. All of the activities increase in proportion to the increase in ($Na^+ + K^+$)-ATPase

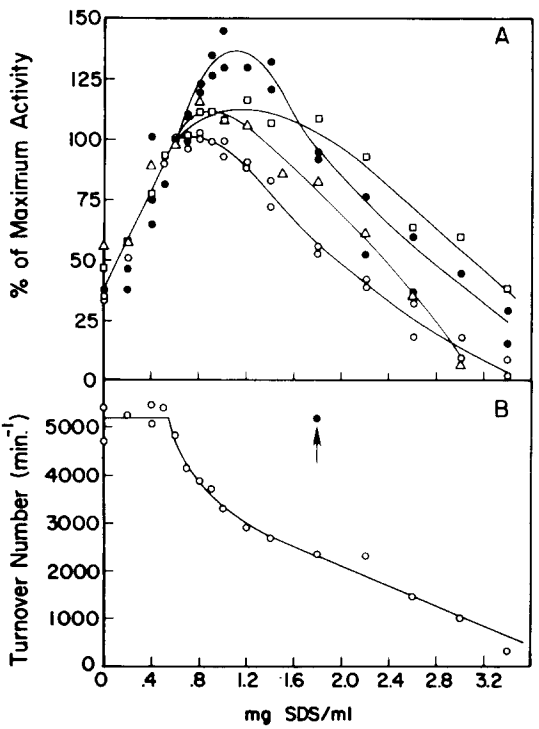

Fig. 6. Effect of dodecyl sulfate activation and inactivation on some partial reactions of the ($Na^+ + K^+$)-ATPase. (A) Brain microsomes at 8.0 mg protein/ml were treated with dodecyl sulfate at the indicated final concentrations. Symbols: ○, ($Na^+ + K^+$)-ATPase activity; △, binding of [^{3}H]ouabain; ●, Na^+-stimulated, K^+-sensitive phosphorylation from [γ-^{32}P]ATP; □, K^+-stimulated hydrolysis of *p*-nitrophenyl phosphate. Absolute values at 0.6 mg dodecyl sulfate/ml were: ($Na^+ + K^+$)-ATPase activity, 50.8 μmol/h per mg protein; ouabain bound, 162.3 pmol/mg protein; ^{32}P incorporated, 165.0 pmol/mg protein; and *p*-nitrophenyl phosphatase, 7.5 μmol/h per mg protein. (B) Turnover number, defined as ATPase activity per phosphorylation site, at the indicated concentrations of dodecyl sulfate. The closed circle is the turnover number of microsomes recovered from a sucrose gradient after extraction with 1.8 mg dodecyl sulfate/ml. The data in this figure were obtained after incubating with dodecyl sulfate in the absence of ATP. Similar results were seen when 3 mM ATP was present with the detergent, except that the curves were all shifted to the right.

activity at low concentrations of dodecyl sulfate. Turnover number, expressed as activity/phosphorylated site, is constant (Fig. 6b). Once reversible inactivation begins, the apparent turnover number drops and each activity displays a different sensitivity to inactivation. Phosphorylation by ATP continues to increase to an apparent value of about 1.25 sites per ouabain binding site, provided that there is no selective loss of extraneous protein during trichloroacetic acid precipitation in the presence of dodecyl sulfate. The turnover number of the dodecyl sulfate-treated ($Na^+ + K^+$)-ATPase, after the dodecyl sulfate is removed on the sucrose gradient, is the same as that of the untreated microsomes (Fig. 6b).

Activation of ($Na^+ + K^+$)-ATPase activity

The constant turnover number during activation suggests that the detergent is unmasking new sites by opening sealed vesicles. Table II presents three indirect lines of evidence. First, if microsomes are prepared from portions of the same brain stored at different temperatures, very different ($Na^+ + K^+$)-ATPase-specific activities are obtained. Dodecyl sulfate, however, activates all of them to the same maximum. Freezing and thawing and ageing appear to act like the detergent in unmasking latent activity. Second, the effect of the detergent can be mimicked, to an extent, by osmotic shock. And third, fragments of

TABLE II

ACTIVATION OF ($Na^+ + K^+$)-ATPase

Assays were performed in parallel, with and without a 30 min preincubation in the presence of 0.3 mg/ml dodecyl sulfate. Specific activity is expressed as μmol of ATP hydrolyzed/h per mg protein.

	Specific activity	
	—SDS	+SDS
(A) Detergent activation removes differences between microsome preparations *		
Microsomes prepared after:		
fresh	11.0	51.0
storage at −15°C	20.6	54.0
storage at −60°C	26.2	52.6
storage at −15°C, with freezing and thawing	30.5	51.3
(B) Osmotic shock mimics the activation by detergent **		
Untreated microsomes:	9.75	40.0
After osmotic shock:	23.7	45.1
Recovery of activity after osmotic shock (%):	147%	64%
(C) Sealed and unsealed microsome fragments can be separated on the basis of density ***		
Banded on top of 15% sucrose:	7.2	46.5
Pelleted through 15% sucrose:	21.0	46.5

* Gray matter from one brain was divided into four aliquots, and microsomes were prepared from each aliquot on different occasions.

** 2.5 ml of microsomes at 8.4 mg protein/ml in 0.32 M sucrose were diluted to 25 ml with deionized water at 0°C, and pelleted by centrifugation at 40 000 rev./min for 15 min. They were resuspended in 0.32 M sucrose.

*** A preparation of microsomes, 14.3 μmol/h per mg protein, in 8% sucrose, was layered on top of 15% sucrose and centrifuged at 30 000 rev./min for 1 h. The band above the 15% sucrose and the pellet were separated and assayed with and without detergent.

microsomes which are sealed and which have trapped within them a less dense sucrose solution can be separated from unsealed or leaky fragments on the basis of density. The denser fraction has a much higher apparent specific activity, but dodecyl sulfate activates both fractions to the same maximum. Neither osmotic shock nor separation on the basis of density was as effective as dodecyl sulfate; this may be due to resealing of vesicles or to some additional activating effect of dodecyl sulfate.

Reversible inactivation of ($Na^+ + K^+$)-ATPase activity

The addition of extra protein (1 mg/ml hemoglobin) or extra phospholipid (1 mg/ml sonicated suspension of egg phosphatidylcholine) has no effect on the inactivation of ($Na^+ + K^+$)-ATPase by dodecyl sulfate, whereas it partially protects the Mg^{2+}-ATPase (data not shown). Dodecyl sulfate thus appears to bind more readily to the site which causes ($Na^+ + K^+$)-ATPase inactivation than to a soluble protein, to phosphatidylcholine vesicles, or to the Mg^{2+}-ATPase. Yet the extent of reversible inactivation of ($Na^+ + K^+$)-ATPase depends on the ratio of detergent to membrane. This is most easily understood if the inactivation is due to the partitioning of the detergent into the lipid bilayer.

The onset of reversible inactivation is very rapid (Fig. 7). Conditions were chosen in which 90% of the ($Na^+ + K^+$)-ATPase activity could be recovered

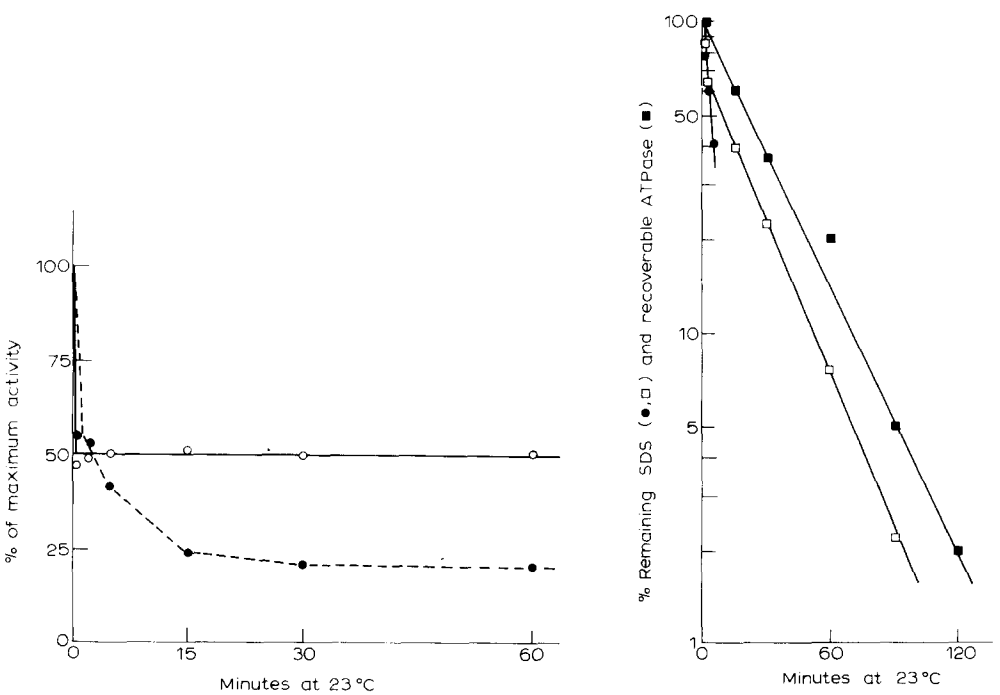

Fig. 7. Rate of inactivation of ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase by dodecyl sulfate. 2.5 mg protein/ml brain microsomes; 0.95 mg dodecyl sulfate/ml. ○, ($Na^+ + K^+$)-ATPase; ●, Mg^{2+}-ATPase.

Fig. 8. Rate of removal of dodecyl sulfate with BioBeads and the recovery of ($Na^+ + K^+$)-ATPase activity. Symbols: ●, removal of dodecyl sulfate from a solution containing only buffer; □, removal of available dodecyl sulfate from brain microsomes; ■, recovery of ATPase activity.

after the dodecyl sulfate is removed. Initial rates of $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase were measured at different times after the addition of dodecyl sulfate. Inactivation of $(Na^+ + K^+)$-ATPase is complete within 30 s, while inactivation of Mg^{2+}-ATPase is much slower.

To determine how quickly the reversible inactivation could be reversed, Biobeads, wettable polystyrene beads with a large hydrophobic surface area, were used to remove the dodecyl sulfate by adsorption. BioBeads took up 83% of dodecyl [^{35}S]sulfate from plain buffer with a half-time of 3.75 min. When microsomes were present, only 72% of the dodecyl [^{35}S]sulfate was taken up. This was arbitrarily designated the "available" fraction, and the rate of its adsorption is shown in Fig. 8. 30% of the available dodecyl [^{35}S]sulfate is taken up with a half-time of 4 min, while the remaining 70% is taken up with a half-time of 19 min. The dodecyl sulfate caused inactivation of 73% of the $(Na^+ + K^+)$-ATPase activity. Of this, 48% was recovered after its removal and 25% was inactivated irreversibly. Less than 10% of the total protein was adsorbed by the BioBeads. The 48% of the activity that was recovered was treated as the only relevant fraction, and the rate of its recovery is compared to the rate of removal of available dodecyl [^{35}S]sulfate in Fig. 8. Activity is recovered with a single half-time of 21 min. The evidence indicates that $(Na^+ + K^+)$-ATPase activity is restored essentially as fast as the slowly relinquished dodecyl sulfate is removed. The rapid removal of 30% of the available dodecyl sulfate is probably the adsorption of dodecyl sulfate not bound to protein or lipid.

Discussion

The dodecyl sulfate-resistant fraction

The dodecyl sulfate extraction procedure developed by Jorgensen [6] for the purification of $(Na^+ + K^+)$-ATPase from kidney works differently when applied to $(Na^+ + K^+)$-ATPase from brain. That dodecyl sulfate causes a substantial extraction of lipid from renal medulla membranes [6], while it does not from brain membranes, supports the hypothesis that the composition or structure of the lipid bilayer is different in the two tissues. It is proposed that the dodecyl sulfate resistance of the $(Na^+ + K^+)$-ATPase of brain, along with lipids and other glycoproteins, reflects the relative resistance of the lipid bilayer and its deeply embedded constituents. The dodecyl sulfate is acting primarily as a protein perturbant, and is selectively removing peripheral proteins [20—22], leaving a residue enriched in phospholipid, glycolipid, and the glycosylated intrinsic membrane proteins, including the $(Na^+ + K^+)$-ATPase. Although the detergent may partition into the membrane (see below), concentrations may be used at which it interacts with the membrane only enough to cause activation and reversible inactivation of ATPase activity, but not enough to solubilize the membrane. This is in contrast to its action of on other sources of $(Na^+ + K^+)$-ATPase [6,8].

Dodecyl sulfate effects on $(Na^+ + K^+)$-ATPase activity

The activation of $(Na^+ + K^+)$-ATPase activity by low concentrations of detergent has been suggested to be due to enzyme activation or uncoupling, to the removal of inhibitors, or to the unmasking of latent sites [1,23—25]. The

action of dodecyl sulfate reported here is consistent with the unmasking of sites by opening sealed vesicles. The turnover number of the enzyme does not change during activation.

The reversible inactivation is a function of the ratio of detergent to membrane over the range of concentrations studied. This leads to the hypothesis that some dodecyl sulfate is partitioning into the membrane and is perturbing the lipid environment of the $(Na^+ + K^+)$-ATPase in addition to removing peripheral proteins. The partitioning appears to occur rapidly, and the membranes relinquish the dodecyl sulfate slowly. Ouabain binding has been observed before to be more resistant than ATPase activity to a variety of protein perturbants, including dodecyl sulfate [26,27]. Rodnight [28] saw much the same kind of stimulation of phosphorylation during ATPase inactivation as is reported here. The data presented here indicate that the stoichiometry of $(Na^+ + K^+)$-ATPase phosphorylation rises to more than one site per ouabain binding site. If the enzyme has half-of-the-sites reactivity (reviewed in ref. 29), this would indicate uncoupling of the two interacting halves of the enzyme complex. Phlorizin and dimethyl sulfoxide may have a similar action to dodecyl sulfate in that they stimulate *p*-nitrophenyl phosphatase activity at the expense of $(Na^+ + K^+)$-ATPase activity [29]. It is plausible that the reversible effects of all three reagents are due to a disruption of inter-subunit interactions which are essential for $(Na^+ + K^+)$-ATPase activity but not for the phosphatase activity, ouabain binding, or phosphorylation, and that the disruption is accomplished by reversible alteration of the lipid environment of the enzyme complex.

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