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Preparation of a ouabain-binding membrane fraction from brain

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SUMMARY

A procedure employing 2 M $NaClO_4$ is presented that allows the mild disruption of brain microsomal membranes and yields three distinct fractions. One fraction has a relatively high ouabain-binding capacity but low (Na^+, K^+)-ATPase activity. This fraction was shown by polyacrylamide gel electrophoresis to be very similar in protein composition to that of the initial microsomes but the phospholipid content was lower. It is suggested that the enzyme in this fraction lacks either necessary phospholipids or a membrane matrix needed for complete activity. The presence of enzyme as indicated by the binding of this specific inhibitor may be useful in further purification of the (Na^+, K^+)-ATPase enzyme system.

The importance of the (Na^+, K^+)-ATPase enzyme system in the maintenance of active cation transport in biological membranes has been stressed by a number of investigators [1-3], but the success in its purification has thus far been limited. The enzyme system is known to be membrane bound and under certain conditions has a requirement for phospholipid [4,5]. Several investigators [3,7,9,11] have demonstrated that subcellular fractions which contain (Na^+, K^+)-ATPase activity will specifically bind radioactively labelled ouabain, a cardiac glycoside that is a specific inhibitor of enzyme activity. The procedure presented here, which may be accomplished without appreciable loss of enzymatic activity, uses 2 M $NaClO_4$ at pH 7.4 to disrupt the membrane suspension followed by high-speed centrifugation. In addition to determining the enzymatic specific activity in fractions obtained during purification procedures, it seemed reasonable to also monitor the distribution of ouabain binding as an indication of the presence of inactivated enzyme.

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Beef brain microsomes were prepared by differential centrifugation in 0.32 M sucrose at pH 7.4 (ref. 12). Either freshly prepared or lyophilized microsomes were used. The brain microsomes were dispersed with a glass homogenizer in water at pH 7.4 and an equal volume of 4 M $NaClO_4$ was added. The protein concentration of this dispersion ranged between 2-10 mg/ml. The dispersion was either sonicated in 10-sec bursts for a total of 1 min or maintained at room temperature for 1 h. The dispersion was then centrifuged at 104 000 × g (40 rotor) for 1 h at 0° in a Spinco Model L-2 ultracentrifuge. At the end of the centrifugation there were three distinct fractions in the centrifuge tube: a dense white floating pellicle (Fraction 1), a clear to slightly opalescent supernatant (Fraction 2), and a pellet (Fraction 3). Where indicated these fractions were dialyzed against 5 mM imidazole, pH 7.4 at 4° for 12-16 h. ATPase, K^+-stimulated *p*-nitrophenyl phosphatase, protein and phospholipid phosphorus were assayed by standard methods [8,12,1]

Table I shows the distribution of protein, phospholipid phosphorus and enzyme activities in the ClO_4^- fractions prepared by the two methods. It is evident that with either method some disruption and redistribution of membrane components took place. (Na^+, K^+)-ATPase and K^+-stimulated *p*-nitrophenyl phosphatase activities had similar distributions. The enzyme determinations were run on dialyzed material. During dialysis,

TABLE I

TREATMENT OF BRAIN MICROSOMES WITH $NaClO_4$

Fractionation was carried out as described in text. Values reported are means ± S.E. with the number of experiments in parentheses. The relative specific concentrations and relative specific activities indicate the distributions relative to starting material for the number of experiments in parentheses.

Fraction	*Protein distribution (%)*	*Phospholipid phosphorus*		*(Na^+, K^+)-ATPase*		*K^+-stimulated p-nitrophenyl phosphatase*	
		μmoles/mg protein	*Relative specific concentration*	*Specific activity**	*Relative specific activity*	*Specific activity**	*Relative specific activity*
			Method 1 (Sonicated)				
Initial microsomes	100	0.76 ± 0.20	1.00	12.3 ± 2.3	1.00	0.92 ± 0.38	1.0
1	11 ± 4	2.65 ± 0.13	3.50	40.8 ± 14.0	3.26	3.38 ± 3.10	3.70
2	55 ± 8	0.54 ± 0.14	0.71	7.4 ± 1.3	0.56	0.51 ± 0.50	0.55
3	34 ± 7	0.32 ± 0.05	0.42	9.7 ± 6.2	0.77	0.79 ± 0.20	0.85
No. of experiments	(9)	(4)	(4)	(3)	(3)	(3)	(3)
			Method 2 (Room temperature, 1 h)				
Initial microsomes	100	0.88 ± 0.32	1.00	15.3 ± 2.3	1.00	2.54 ± 0.68	1.00
1	17 ± 8	2.64 ± 1.81	2.06	26.2 ± 1.4	1.71	2.19 ± 1.14	0.80
2	28 ± 9	0.59 ± 0.11	0.66	4.3 ± 2.0	0.31	0.32 ± 0.24	0.12
3	53 ± 12	0.81 ± 0.46	0.92	14.2 ± 4.6	0.93	2.70 ± 1.40	1.04
No. of experiments	(8)	(3)	(3)	(5)	(5)	(3)	(3)

*(μmoles/mg protein per h).

Fraction 2, which had previously been clear, became opalescent or slightly turbid indicating reaggregation of components. Sonication appeared to be the preferred method of preparation because of greater preservation of enzyme activities. However, this procedure was difficult to reproduce and control. If disruption by sonication continued too long all enzymatic activity was lost. Treatment for 1 h at room temperature without sonication increased the (Na^+, K^+)-ATPase activity of Fraction 1 and the procedure was very reproducible.

To determine if there was a unique protein composition in each fraction, polyacrylamide gel electrophoresis of the initial microsomes and the ClO_4^- fractions was run. The procedure was that described by Lim and Tadayyon [6]. The dialyzed ClO_4^- fractions were lyophilized to dryness and approximately 5 mg of protein was dissolved in 1 ml of a solubilization solution consisting of 10% mercaptoethanol, 5% Triton X-100, 8 M urea and 50 mM K_2CO_3. The gels also contained Triton and urea. A 100-μl aliquot was applied to each gel. The gels were stained with 0.1% Amido Schwartz dye and destained by diffusion in 7.5% acetic acid. Fig. 1 shows the protein patterns obtained by electrophoresis of the initial microsomes and the ClO_4^- fractions. It is evident that each fraction contained many protein bands and the band patterns appear to be similar. The concentrations of the individual components appear to vary, however. Since the protein contents of the fractions were apparently similar the differences in enzymatic properties must lay elsewhere.

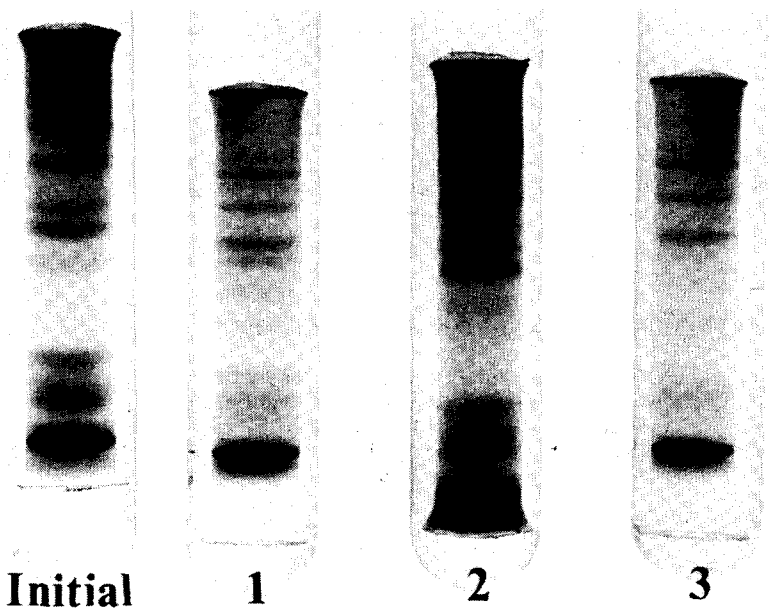

Fig. 1. Polyacrylamide gel electrophoresis of initial brain microsomes and ClO_4^- fractions. The complete method is described in the text. Lyophilized protein was dissolved in sample solution at a concentration of 10 mg/ml; 100 μg of this solution was applied to each gel and a constant current of 0.75 mA/tube was applied for 11 h. Gels were stained with Amido Schwartz dye and destained by diffusion in 7.5% acetic acid.

Binding of [^{3}H]ouabain was carried out by the method described by Tobin and Sen [7] except the levels of ouabain were designed to be nonsaturating. Brain microsomes (15-20 mg) were dispersed in a 6-ml solution of either 4 mM $MgCl_2$, 1 mM H_3PO_4, 10 mM imidazole (pH 7.4) or 200 mM NaCl, 2 mM ATP and 10 mM imidazole (pH 7.4). 100 μl of [^{3}H]ouabain (11.7 C/mM, 8.4 pmoles) was added. Approximately 350-400 pmoles/mg protein would be necessary to saturate all available binding sites. This solution was incubated either for 20 min without ATP or for 3 min with ATP at 37°. Following incubation the sample was centrifuged at 48 000 × g for 20 min; the pellet was resuspended in 12 ml of the labelling solution and was then recentrifuged. The pellet was washed three or more times until the radioactivity of the supernatant had reached background. The final pellet was resuspended in the medium prior to ClO_4^- treatment. Binding of ouabain was approximately equal in both systems so the system without ATP was used routinely. All fluids used for dialysis and other treatments contained 4 mM $MgCl_2$ and 1 mM PO_4^{3-} *plus* buffer. Between 75-80% of the ouabain applied was bound and retained through the washing procedure.

Table II indicates the [^{3}H]ouabain binding to the initial microsomes and the ClO_4^- fractions. The absolute quantity of ouabain bound to the ClO_4^- fractions could not be established prior to dialysis since any unbound or free ouabain would have appeared in the supernatant fraction. Also, a portion of the protein (5-10%) was always lost during dialysis so that all the [^{3}H]ouabain lost in this step would not necessarily have been removed during the ClO_4^- treatment. The pattern of (Na^+, K^+)-ATPase specific activity and amount of ouabain bound in Fractions 1 and 3 roughly corresponded. However, Fraction 2 bound much more ouabain than would have been predicted from its enzymatic activity. In contrast, ouabain binding was reduced by 96-98% in microsomes that were incubated at 55° for 45-60 min.

It has been reported that lecithin or phosphatidyl serine is necessary for (Na^+, K^+)-ATPase enzymatic activity [4,5]. Since Fraction 2 had a lower phospholipid content than the initial material, reactivation with a variety of concentrations of these compounds was attempted but was unsuccessful.

TABLE II

DISTRIBUTION OF [^{3}H]OUABAIN BOUND TO BRAIN MICROSOMES

Ouabain binding was carried out as described in the text using Method 2. Subsequent ClO_4^- fractionation was carried out in the presence of 4 mM $MgCl_2$ and 1 mM H_3PO_4. Data shown are after 14 h dialysis against the labeling solution without ouabain. Relative specific activity is the specific activity in the fraction relative to the specific activity in the initial microsomes. Values are means ± S.E. for the number of experiments in parentheses.

Fraction	*Specific activity (counts/min per mg protein × 10^{-3})*	*Relative specific activity ratio*
Initial microsomes	134 ± 5.6	1.00
1	81 ± 7.7	0.60
2	180 ± 40	1.34
3	68 ± 10	0.51
No. of experiments	(5)	(5)

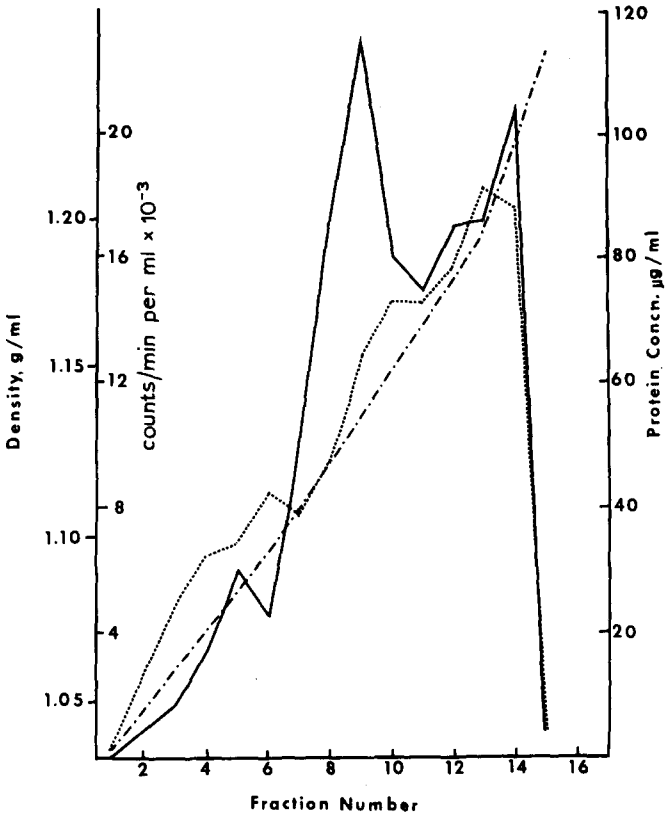

Fig. 2. Density gradient centrifugation of Fraction 2. The gradient is from 2 M $NaClO_4$ - 20% sucrose to 0.5 M $NaClO_4$ and was run in a Beckman SW-27 rotor for 14 h at 25 000 rev./min. 2-ml fractions were collected with the use of a Beckman fraction recovery system. Density (•—•—•—) is in g/ml; protein concentration (•••••••) is in µg/ml; and radioactivity (———) is expressed as counts/min per ml X 10^{-3}. Radioactivity and protein data were obtained after 12 h dialysis in labeling solution.

Since the protein composition of the fractions had been shown to be heterogeneous, a density gradient centrifugation was carried out to determine the particle (density) size distribution. Since reaggregation occurred when salt was removed, a linear gradient of 20% sucrose, 2 M $NaClO_4$ to 0.5 M $NaClO_4$ was run. Fig. 2 shows a density gradient centrifugation of Fraction 2 after 12 h at 20 000 rev./min in a Spinco SW-27 rotor. Banding was found over a rather broad density range of 1.07-1.22 g/ml. There appeared to be two bands. The small peak in Fraction 5 probably represents contamination from Fraction 1 since all of the material from Fraction 1 banded at that density. At this stage the preparation was clearly heterogeneous.

Non-ionic detergents have been used as an effective means for the disruption of biological membranes when studying membraneous enzymes such as the (Na^+, K^+)-ATPase Unfortunate[1]y, there are several difficulties inherent in their use. Non-ionic detergents are quite difficult to remove completely from an enzyme solution. In one of the most successful purification methods of the (Na^+, K^+)-ATPase to date [3], the final product contained 16.5% Lubrol by weight. Another disadvantage to the use of non-ionic

detergents is their stimulation of enzyme activity at certain concentrations [8,10]. This makes it difficult to distinguish between the degree of purification and detergent stimulation of enzyme activity. By comparison, $NaClO_4$ may be very easily removed from any enzyme preparation by dialysis and it does not stimulate (Na^+, K^+)-ATPase activity.

Presently there is some controversy concerning the reversibility of the binding of ouabain to microsomal preparations [7,11]. Under the conditions specified in this communication, Mg^{2+} and P_i were present throughout the procedure, and the majority of the [^{3}H]ouabain did remain bound. Thus, the radioactive label which is specifically bound to the (Na^+, K^+)-ATPase may allow the enzyme to be followed through attempted purification procedures. It must be stressed that there is a difference between the inactive enzyme which may lack some critical component and a denatured enzyme. The evidence presented suggests that Fraction 2 of the ClO_4^- fractionation contains an inactivated form of the enzyme which, since it binds [^{3}H]ouabain, is not denatured. Present studies are designed to try to reactivate this protein and also to purify the ouabain-binding component.

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