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PARTIAL PURIFICATION AND OUABAIN SENSITIVITY OF LUBROL-EXTRACTED SODIUM-POTASSIUM TRANSPORT ADENOSINE TRIPHOSPHATASES FROM BRAIN AND CARDIAC TISSUES*

D. Y. SHIRACHI,† A. A. ALLARD and A. J. TREVOR

Department of Pharmacology, University of California, San Francisco Medical Center, San Francisco, Calif. 94122, U.S.A.

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Abstract—Lubrol extracts containing (Na+K)-activated adenosine triphosphatase of specific activity 70–100 μmoles P_i/mg of protein/hr were prepared from rat brain microsomal fractions. The enzyme was stable in 0·32 M sucrose containing 1 mM EDTA at pH 6·9 and had properties, regarding optimal concentrations of ATP, Na and K required for maximal activation, similar to those of particulate preparations of the rat brain enzyme. The apparent K_m for the enzyme was $5{\cdot}0 \times 10^{-4}$ M and the optimal pH was 6·8. Sucrose density gradient separation further purified the (Na+K)-activated adenosine triphosphatase, but the resulting preparation was not homogeneous, despite high specific activity (400 μmoles P_i/mg of protein/hr), and contained at least five protein staining bands on disc gel electrophoresis. Lubrol treatment was effective in extracting the enzyme from cardiac tissue of both rat and guinea pig and appeared to increase the sensitivity of the enzyme to ouabain. Enzymes of cerebral origin from the two species were of equal sensitivity to ouabain. The enzyme extracted by Lubrol treatment from guinea pig heart microsomes was 23 times more sensitive to ouabain than a similar preparation from rat heart.

SINCE Skou[1] first presented evidence for an association of an (Na+K)-activated ATPase derived from nerve membranes with active cation transport, there have been numerous attempts to purify the enzyme system.[2–9] The extraction of the enzyme from particulate preparations of brain tissue with the non-ionic detergent, Lubrol, has been accomplished with varying degrees of success.[3,6] More recently, further purification of the enzyme has been reported after Lubrol extraction of brain microsomes,[8,9] but such studies have been directed primarily toward isolation rather than characterization of the resulting preparations. The present report describes a simple procedure for extracting (Na+K)-activated ATPases from particulate preparations of brain and heart using low concentrations of Lubrol. Data are presented concerning the properties of the extracted enzyme and its further purification by density gradient fractionation to obtain a highly active Na-K ATPase. In addition, the results of a comparative investigation on the effects of ouabain on the enzyme activity of various preparations from brain and heart of rat and guinea pig are reported.

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MATERIALS AND METHODS

Preparation of microsomal fractions

The microsomal fractions were prepared by a procedure modified from that reported by Schwartz *et al.*[10] Male Sprague–Dawley rats (200–250 g) or male guinea pigs (approximately 700 g), sacrificed by decapitation or by a blow on the neck, respectively, were exsanguinated. The brains were removed and cleared of white matter and capillaries. They were homogenized in 9 vol. of 0·32 M sucrose, containing 1 mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 6·9 with Tris(hydroxymethyl)aminomethane, using a Potter–Elvehjem homogenizer and a Teflon pestle (clearance 0·004–0·006 in.) mechanically driven at 2000 rev./min. Ten strokes were used with intermittent cooling in ice. The hearts were removed and the large vessels and excess connective tissue were dissected from them. The tissue was minced with a scissor in approximately 5 vol. of sucrose and homogenized using a series of three pestles: smooth Teflon using 10 strokes, scored Teflon using 5 strokes, ground glass using 5 strokes. In all cases a mechanical rotor driven at 2000 rev./min was used. During homogenization, 4 vol. of sucrose was added so that a total of 9 vol. was used. Subcellular fractionation of this homogenate was carried out in a Lourdes model A2 Betafuge using the 9 RA rotor according to the fractionation scheme shown in Fig. 1. The final microsomal pellets were resuspended in 0·32 M sucrose containing 1 mM

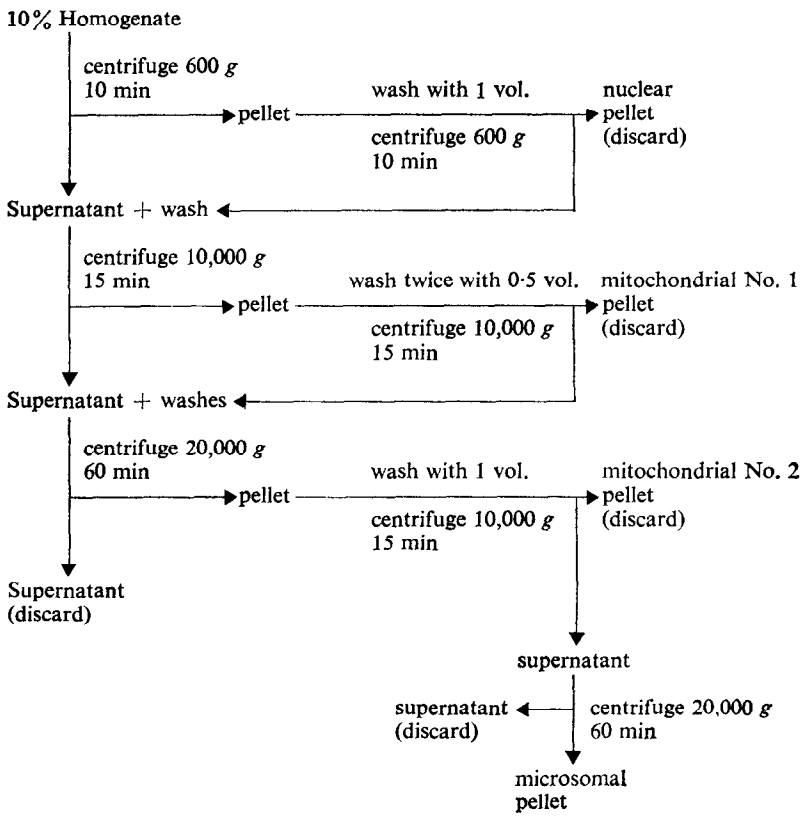

FIG. 1. Fractionation scheme for microsomal fraction.

EDTA with a Dounce homogenizer. The final protein concentration was 4–10 mg/ml for the cardiac preparations and 8–16 mg/ml for brain preparations. They were stored at −10° until use.

Lubrol extraction of microsomal fractions

A 0·4 per cent Lubrol WX solution (w/v) in 0·32 M sucrose, 1 mM EDTA and pH adjusted to 6·9 with Tris buffer was added to an equal volume of the microsomal fraction, resulting in a final Lubrol concentration of 0·2 per cent, which was found to be optimal for the extraction of the Na-K ATPase (see Results). This suspension was homogenized in a Dounce homogenizer (B pestle) using 10 strokes. It was kept on ice for 15 min and mixed occasionally. The suspension was then centrifuged in the FA40 rotor of a Spinco model L centrifuge at 100,000 *g* (av.) for 1 hr. The nonsedimentable supernatant was removed and stored in the refrigerator for routine use or frozen at −10° for longer storage periods (up to 2 months).

Sodium iodide treatment of microsomal fractions

The microsomal fractions were treated with 2 M NaI, following the procedure of Nakao *et al.*[4] as reported by Matsui and Schwartz,[5] with the following minor modifications. A total of four washes with 1 mM EDTA were used. The pellet was resuspended in a small Tenbroeck homogenizer and centrifuged at 20,000 *g* for 20 min after each wash. The final pellet was resuspended in 0·32 M sucrose, 1 mM EDTA, pH 6·9. The iodide-treated fractions were stored frozen and used within 1 week.

Density gradient centrifugation

Discontinuous sucrose gradients were prepared using equal volumes (5 ml) of 15·0, 17·5, 20·0, 22·5 and 25·0 per cent (w/v) sucrose solutions containing 0·5 mM EDTA with the pH adjusted to 6·9 with Tris buffer.

These solutions had densities (g/ml) of 1·056, 1·065, 1·075, 1·084 and 1·094 respectively. Five ml of the Lubrol extract from rat brain microsomal fraction (2–3 mg protein/ml) was layered on the gradient and centrifuged in the SW 25·1 rotor of a Spinco model L centrifuge at 90,000 *g* for 40 hr. The fractions corresponding to the different sucrose concentrations were separated and stored in the refrigerator.

Polyacrylamide gel electrophoresis

Polyacrylamide gels were prepared in glass tubes, 100 × 5 mm (i.d.). The separating gels contained 7% acrylamide, 0·184% *N,N'*-methylenebisacrylamide (Bis), 0·029% *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0·07% ammonium persulfate in a Tris-HCl buffer, pH 8·9. Gels were 50 or 75 mm in length. They were polymerized for 1 hr at room temperature under an incandescent light. The stacking gels contained 2·5% acrylamide, 0·625% Bis, 0·058% TEMED, 0·5% riboflavin and 20% sucrose in a Tris-HCl buffer, pH 6·7. Approximately 5 mm gel was placed on the separating gel and they were polymerized for 15 min at room temperature under a fluorescent light. A 0·2-ml layer of sample gel containing 4% acrylamide, 1% Bis, 0·092% TEMED and 32% sucrose in Tris-HCl buffer, pH 6·7, was layered on the stacking gel. The protein sample (0·1–0·4 ml) was added and mixed with the gel, and the gel was polymerized at 4° under a fluorescent light for 15 min. A 2·5 mM Tris buffer (pH 9·5) containing the trailing ion, alanine (19·2 mM), was placed in the upper and lower

chambers, and electrophoresis was carried out at 4° for 4 hr. A current of 1·33 mA/tube was applied, attaching the cathode to the top electrode. Upon completion of electrophoresis, the gels were removed from glass tubing, and the protein bands stained with 1% Amido-Schwartz in 7% acetic acid for 30 min. The gels were destained with 7% acetic acid for 12–18 hr.

Assays

ATPase. The conventional system used for the assay of the Na-K-Mg ATPase activity was: ATP (Tris salt), 5 mM; KCl, 10 mM; NaCl, 110 mM; $MgSO_4$, 5 mM; imidazole buffer, 10 mM; and the appropriate enzyme preparation in a total volume of 0·5 ml. The pH of the incubation mixture was 6·8 at 37°. The protein concentration in the final mixture was 50–300 μg/ml depending upon the experiment. Mg-stimulated activity was determined in a similar system containing choline chloride rather than NaCl and KCl. This activity was subtracted from the total activity to give values reported as Na-K-stimulated ATPase activity. The medium, containing everything except ATP, was incubated at 37° for 1 min, and the reaction was allowed to run for 10 min after addition of substrate. It was terminated by the addition of 0·05 ml of 10 per cent perchloric acid. A 0·25-ml aliquot was taken for phosphate determination by the procedure of Martin and Doty.[11] To insure proper buffering, the assay for the pH curve was run in incubation media containing 100 mM imidazole buffer. The pH was determined after the addition of all ingredients at 37°. Appropriate sucrose or sucrose-Lubrol blanks were run for all experiments.

Acetylcholinesterase. Acetylcholinesterase was measured using the thiocholine colorimetric procedure[12] at 25°.

Adenyl cyclase. The radioisotopic assay method of Krishna *et al.*[13] was used to measure adenyl cyclase.

Lubrol. Lubrol was determined using a modification of method II reported by Stevenson.[14] The method involved the precipitation of a detergent–molybdate complex. The precipitate was washed with deionized water, dissolved in H_2SO_4, reacted with ammonium thiocyanate, and reduced with 0·5 ml of 10 per cent stannous chloride. The method is suitable for the estimation of 10–50 μg Lubrol.

Protein. Protein was determined by the method of Lowry *et al.*[15] Bovine serum albumin standards were used and appropriate sucrose or sucrose-Lubrol blanks were included in the assay.

Materials. Tris ATP (Sigma) solution was prepared in de-ionized distilled water and the pH was adjusted to 7·15. A stock ouabain (Sigma) solution, 1×10^{-2} M, in 0·32 M sucrose with 1 mM EDTA (pH 7·0) was prepared and serial dilutions with sucrose-EDTA were used to obtain the desired concentrations for assays. Lubrol WX, batch No. SCD-SR-59, was a gift from ICI America Inc. All reagents for the polyacrylamide gels were obtained from Eastman Organic Chemicals. Other reagents or chemicals used were reagent grade.

RESULTS

Lubrol extraction of rat brain microsomal fractions

Microsomes prepared from rat cerebral cortex tissue were treated with Lubrol at concentrations ranging from 0·05 to 0·4 per cent (w/v). Protein concentrations and

TABLE 1. LUBROL EXTRACTION OF Na-K ATPASE ACTIVITY FROM RAT BRAIN MICROSOMES*

Lubrol concn (%)	Protein extracted (%)	Sp. act. of Lubrol extract (μmoles P_i/mg protein/hr)		Total recovery of Na-K ATPase (%)
		Na-K	Mg	
0·05	11	4	0·7	149
0·10	22	14	3·6	193
0·20	34	71	5·2	219
0·30	39	70	6·3	212
0·40	46	68	6·1	195
Sp. act. of original microsomal preparation		19	8·3	

* Microsomes were suspended in 0·32 M sucrose containing 1 mM EDTA at pH 6·9 at a concentration of approximately 10 mg protein/ml. The suspension was mixed with an equal volume of Lubrol solution to give the percentages indicated above. After Lubrol treatment and centrifugation, ATPase activity and protein were determined in supernatant and pellet. Recoveries of ATPase activity are the sum of that in the supernatant plus the pellet. Protein recoveries were approximately 80 per cent. Recovery of the Mg ATPase activity averaged 70 per cent.

ATPase activities of the nonsedimentable supernatant in the presence of Mg only, and Na, K and Mg are shown in Table 1. Extraction of microsomal protein into the supernatant increased with increasing Lubrol concentration. Highest specific activity of the Na-K ATPase occurred in the extract following 0·2 per cent Lubrol treatment and was 71 compared to 19 μmoles P_i/mg of protein/hr for the original microsomal preparation. Total recovery of Na-K ATPase (pellet and supernatant combined) was above 200 per cent, indicating activation of the enzyme by Lubrol treatment. Mg ATPase activity of the 0·2 per cent Lubrol extract was decreased and equalled only 6·5 per cent of the total Na-K-Mg ATPase activity as compared to 17·5 per cent in the case of the microsomal preparation. This suggests an inhibition of the activity of the Mg-dependent component by Lubrol treatment. Treatment of microsomal fractions with higher concentrations of Lubrol (0·4 per cent) did not increase specific activity of the Na-K ATPase. Although higher concentrations of the detergent have been used in other investigations, such concentrations have caused inhibition of the Na-K ATPase enzyme.[3,6] In the present studies, higher concentrations of Lubrol also inhibited the enzyme. If microsomal preparations were treated with sodium iodide prior to exposure to 0·2 per cent Lubrol, no ATPase activity or protein could be detected in the nonsedimentable supernatant. The extract obtained by 0·2 per cent Lubrol treatment was very stable with respect to Na-K ATPase activity and did not require addition of substrate or monovalent cations for protection. No appreciable loss of activity was observed after 7 days' storage at 4°, and only a 10 per cent decrease occurred after 14 days at this temperature. When stored at −10°, the preparation retained 85–90 per cent of the Na-K ATPase activity after 60 days. However, repetitive freezing and thawing caused a rapid decline in activity, and only 39 per cent remained after three such treatments.

Characterization of ATPase activities of Lubrol extracts of rat brain

Hydrolysis of ATP by the 0·2 per cent Lubrol extracts of the microsomal fraction was examined with respect to variations in concentration of enzyme, Na, K and

substrate. Total Na-K-Mg ATPase activity was directly proportional to enzyme concentration over the range 20 to 100 μg protein in 0·5 ml of reaction mixture. With protein concentrations in this range, release of P_i was linear during a 10-min incubation period at 37°. Half-maximal activation of ATPase in the presence of 10 mM K occurred at a Na concentration of 12·5 mM, and maximal stimulation was observed at 100 mM. K caused half-maximal activation in the presence of 100 mM Na at a concentration of 3 mM, and maximal activity occurred at 10 mM. Optimal substrate concentration for the Na-K ATPase activity was 5 mM ATP, and concentrations of 10 mM and above caused substrate inhibition. The relationship between substrate concentration and velocity is shown in Fig. 2, a Hanes plot[17] of S/V against S. The straight line was obtained by linear regression and extrapolated to determine

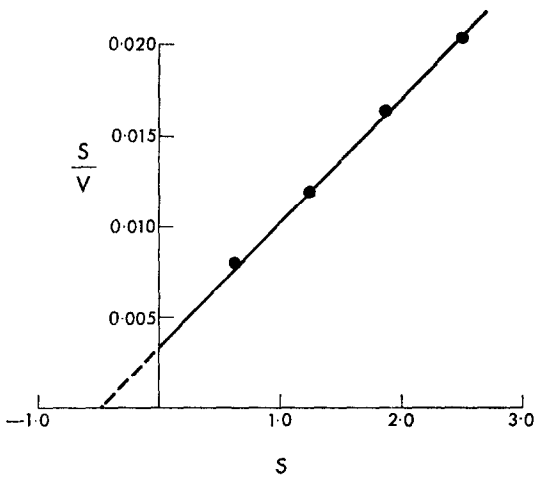

FIG. 2. Hanes plot (S/V against S) of the effect of substrate concentration on Na-K ATPase activity. Lubrol extracts of rat brain microsomal fractions were assayed for Na-K ATPase activity as in Methods. Substrate concentrations ranged from 0·625 to 10 mM ATP, enzyme protein 85 μg in a final volume of 0·5 ml. The straight line was determined by linear regression and extrapolated to estimate K_m.

apparent K_m, which was 5×10^{-4} M. The slope of the line equals $1/V_{max}$, and the derived maximal velocity of the Na-K ATPase was 147 μmoles P_i/mg of protein/hr. Maximum activity of the Mg ATPase occurred at a substrate concentration of 2·5 mM.

The pH sensitivity of the ATPase activities was examined in 100 mM imidazole buffer (Fig. 3). This concentration of imidazole caused approximately 20 per cent inhibition of the Na-K ATPase, but was used to ensure adequate buffering capacity over the pH range studied. Determinations of pH were made immediately after starting the reaction and were monitored during the 10-min incubation at 37°. The pH of the reaction mixture changed less than 0·1 unit during this period. Optimum pH of the Na-K ATPase was 6·8, while Mg ATPase activity did not change significantly over the pH range studied. A pH optimum of 6·8 for the Na-K ATPase was also observed in Tris buffer, which caused greater inhibition of the activity than did imidazole.

The Na-K-dependent component of the ATPase activity of Lubrol extracts of microsomal fractions was sensitive to ouabain, 50 per cent inhibition occurring at a

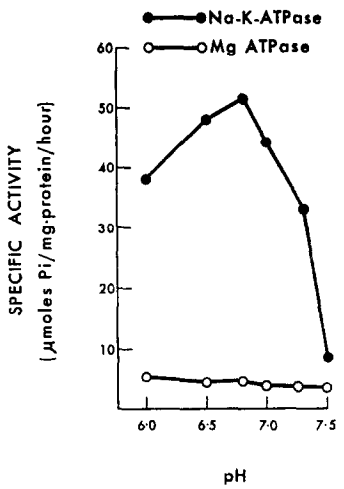

FIG. 3. Effect of pH on ATPase activities of Lubrol extract. Rat brain microsomal fractions were treated with Lubrol as detailed in Methods. The ATPase activities of extracts were determined in a reaction medium containing 100 mM imidazole. The pH values shown are those determined after initiation of the reaction and did not change by more than 0·1 unit during the 10-min incubation.

concentration of 1×10^{-6} M. Concentrations of ouabain between 10^{-7} and 10^{-3} M had no effect on Mg ATPase. The effect of K concentration on ouabain inhibition of enzyme activity is shown in Table 2. The concentration of K used did not completely abolish the inhibitory action of ouabain, but the effect of the glycoside was decreased markedly by increase in K, particularly at the lower concentration of the drug.

TABLE 2. EFFECT OF POTASSIUM ON OUABAIN INHIBITION OF LUBROL-EXTRACTED NA-K ATPASE FROM RAT BRAIN*

Potassium concn (mM)	Per cent inhibition by ouabain concentrations of	
	1×10^{-7}M	1×10^{-6}M
5	37·3	51·8
10	22·9	48·0
20	16·2	42·0
40	14·5	38·1
80	11·8	32·6

* Lubrol extracts of rat brain microsomal fractions were prepared as described in Methods. Na-K ATPase activity was measured in an incubating medium containing 100 mM NaCl, the indicated concentrations of KCl, and appropriate concentrations of choline chloride to maintain constant ionic strength. Control activity of Lubrol-extracted Na-K ATPase was 64 μmoles P_i/mg protein/hr.

Comparison of ATPase activities of various preparations obtained from rat and guinea pig brain and heart tissue

The relatively high sensitivity of the Na-K ATPase activity of Lubrol extracts of rat brain microsomes to ouabain was of interest, in view of the reported insensitivity of this species to cardiac glycosides.[16] Ouabain sensitivity of similar preparations from

cardiac tissue of the rat was therefore examined to determine if treatment with the detergent effected any changes as compared to more conventional particulate preparations of the enzyme. For comparative purposes, preparations were also examined from the brain and heart of guinea pig, a species of established sensitivity to the glycosides. The ATPase activities of microsomal fractions of sodium iodide-treated microsomes and of 0·2 per cent Lubrol extracts derived from cerebral cortex tissue of the

TABLE 3. ATPASE ACTIVITIES OF DIFFERENT PREPARATIONS FROM RAT AND GUINEA PIG BRAIN*

Microsomal preparations	Rat			Guinea pig		
	Na-K-Mg	Mg	$\frac{\text{Na-K-Mg}}{\text{Mg}}$	Na-K-Mg	Mg	$\frac{\text{Na-K-Mg}}{\text{Mg}}$
Microsomal fraction	44·1	13·6	3·2	40·5	14·5	2·8
Sodium iodide treated	78·6	10·0	7·9	70·5	12·1	5·8
Lubrol extract	100·0	8·7	11·5	53·0	7·0	7·6

* Microsomal fractions were prepared from rat and guinea pig cerebral cortex tissue by differential centrifugation and treated with sodium iodide or Lubrol as indicated in Methods. ATPase activities were determined using 50–100 μg protein in 0·5 ml of reaction medium and are expressed as μmoles P_i released/mg protein/hr. Results given are mean values derived from four distinct experiments.

rat and guinea pig are given in Table 3. Lubrol extracts of rat brain microsomes exhibited higher Na-K ATPase activity and a more favourable ratio of Na-K-Mg/Mg activities than did sodium iodide treatment. In contrast, sodium iodide preparations of guinea pig brain microsomes contained higher Na-K ATPase activity than did Lubrol extracts of the fraction, although the detergent procedure was more effective in reducing the Mg-dependent component of the enzyme system. The ATPase activities of microsomal fractions, sodium iodide-treated microsomes and Lubrol extracts of microsomes derived from cardiac tissue of the rat and guinea pig are presented in Table 4. Although the experimental conditions were identical to those used for brain preparations, neither Lubrol nor sodium iodide treatment enhanced the total Na-K-Mg ATPase activities. Lubrol treatment of guinea pig cardiac microsomes improved

TABLE 4. ATPASE ACTIVITIES OF DIFFERENT PREPARATIONS FROM RAT AND GUINEA PIG HEART*

Microsomal preparations	Rat			Guinea pig		
	Na-K-Mg	Mg	$\frac{\text{Na-K-Mg}}{\text{Mg}}$	Na-K-Mg	Mg	$\frac{\text{Na-K-Mg}}{\text{Mg}}$
Microsomal fraction	94·3	31·9	3·0	37·0	16·5	2·2
Sodium iodide treated	85·0	32·3	2·6	26·1	10·8	2·4
Lubrol extract	27·0	7·7	3·5	28·8	7·2	4·0

* Microsomal fractions were prepared from rat and guinea pig cardiac tissue by differential centrifugation and treated with sodium iodide or Lubrol as indicated in Methods. ATPase activities were measured in incubation media containing protein concentrations of 40–90 μg (microsomal fraction), 25–75 μg (sodium iodide treated), and 25–150 μg (Lubrol extract) in a final volume of 0·5 ml. Results are expressed as μmoles P_i released/mg protein/hr and represent mean values derived from four distinct experiments.

the ratio of Na-K-Mg/Mg activities compared to the original microsomal fraction. Treatment of heart microsomes with 0·2 per cent Lubrol resulted in extraction of 18–20 per cent of the total protein of this fraction, as contrasted with 28–38 per cent for cerebral microsomes. Despite the limited efficiency of Lubrol treatment of cardiac microsomal fractions, the procedure did result in extraction of Na-K ATPase activity in the nonsedimentable supernatant. Such preparations were of adequate specific activity for investigation of ouabain sensitivity.

TABLE 5. OUABAIN CONCENTRATIONS ($\times 10^{-6}$ M) PRODUCING 50 PER CENT INHIBITION OF NA-K ATPASE PREPARATIONS*

	Rat		Guinea pig	
Microsomal preparations	Brain	Heart	Brain	Heart
Microsomal fraction	3·9		1·4	
Sodium iodide treated	5·4		1·0	
Lubrol extract	1·0	60	0·7	2·6

* Microsomal fractions were prepared from rat and guinea pig cerebral cortex and cardiac tissue by differential centrifugation and treated with sodium iodide or Lubrol as indicated in Methods. Control values of Na-K ATPase activity of the different preparations are given in Tables 3 and 4. ATPase activities were measured in the presence of various concentrations of ouabain (10^{-7}–10^{-3} M). The values above represent ouabain concentrations ($\times 10^{-6}$ M) producing 50 per cent inhibition of Na-K ATPase activity and were determined by linear regression using data from four experiments.

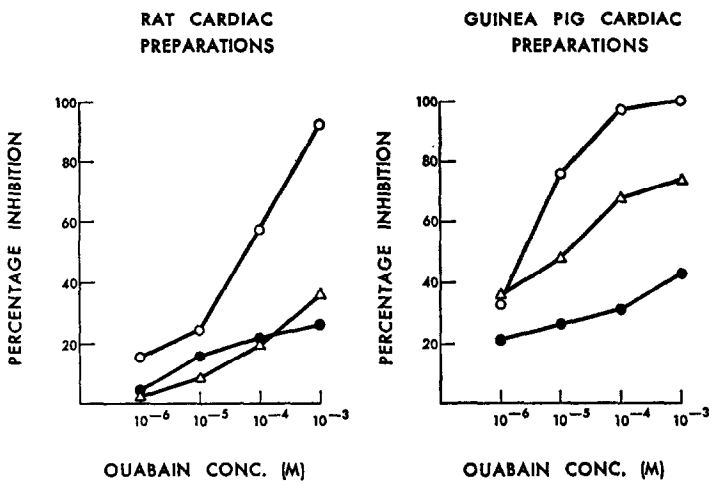

FIG. 4. Effect of ouabain on Na-K ATPase activities of rat and guinea pig cardiac preparations. Microsomal fractions were prepared from rat and guinea pig cardiac tissue by differential centrifugation and treated with sodium iodide or Lubrol as indicated in Methods. Control values of Na-K ATPase activity of the different preparations are given in Table 4. Na-K ATPase activities were measured in the presence of ouabain concentrations shown. Each point represents a mean value derived from four experiments. Lubrol extract, ○—○; sodium iodide-treated, △—△; microsomal fraction, ●—●.

The concentrations of ouabain ($\times 10^{-6}$) producing 50 per cent inhibition of the Na-K ATPase of the different preparations are shown in Table 5. Both sodium iodide- and Lubrol-extracted Na-K ATPase activities from rat brain were slightly less sensitive to ouabain than comparable preparations from guinea pig brain. Lubrol extracts of cardiac tissue microsomes, whether from rat or guinea pig, required much greater concentrations of ouabain to effect 50 per cent inhibition than similar preparations from brain tissue. Indeed, the sensitivity of the Lubrol-extracted Na-K ATPase of rat brain was 60 times that of the cardiac Lubrol extract. With concentrations of ouabain up to 10^{-3} M, it was not possible to obtain accurate estimates of 50 per cent inhibitory concentrations on microsomal fractions or on sodium iodide preparations of cardiac origin. The effect of ouabain on the Na-K ATPase activities of cardiac preparations is shown in Fig. 4. Ouabain sensitivity of the enzyme present in Lubrol extracts of heart microsomes was greater than that of the original microsomal fraction or sodium iodide-treated preparations. Note that the Na-K ATPase activity of Lubrol extracts of guinea pig cardiac microsomes was approximately 23 times more sensitive to ouabain than that derived from rat heart tissue.

Density gradient fractionation of Lubrol extracts of rat brain microsomes

Lubrol extracts of rat brain microsomal fractions were fractionated on a discontinuous density gradient of sucrose ranging in density from 1·037 to 1·094. The six fractions were separated and analysed for protein, ATPase activity (Fig. 5) and Lubrol concentration. Over 80 per cent of the total protein of the original extract was found in the lower density sucrose layers (1·037–1·065), and the major protein peak occurred at a density of 1·056. Approximately 55 per cent of the total Na-K ATPase was associated with these fractions. The highest specific activity of the Na-K ATPase occurred in the 22·5 per cent sucrose fraction (density 1·084). This fraction was capable of hydrolyzing ATP at a rate of 400 as compared to 70–80 μmoles P_i/mg of protein/hr for the original Lubrol extract. The distribution of Mg ATPase did not parallel that of the Na-K-activated enzyme and was of highest specific activity in the most dense sucrose layer (density 1·094). The distribution of Lubrol was similar to that of protein. Concentrations in the six fractions in order of increasing density were 0·65, 0·66, 0·38, 0·20, 0·13 and 0·15 mg/ml respectively. The fraction containing Na-K ATPase of highest specific activity contained approximately 7 per cent of the original Lubrol concentration. After subjection of the detergent (0·2 per cent) to density gradient fractionation in the absence of subcellular components of tissue, 80 per cent of the Lubrol was recovered in the least dense sucrose layer and the remainder in the fraction of density 1·056.

Despite the relatively high specific activity of the Na-K ATPase present in the 22·5 per cent sucrose fraction from the gradient, the preparation was not homogeneous. The original Lubrol extract of rat brain microsomes contained acetylcholinesterase activity (10·3 μmoles/mg of protein/hr). This was increased by a factor of 2·5 by density gradient centrifugation in the fraction containing the highest Na-K ATPase activity. Preliminary experiments also demonstrated the presence of adenyl cyclase in this extract. The protein staining bands present in the original Lubrol extract, the fractions separating at densities of 1·056 and 1·075, and that containing the highest specific activity of the Na-K ATPase (density 1·084) separated by disc gel electrophoresis are demonstrated in Fig. 6. Between 20 and 25 protein bands were separated

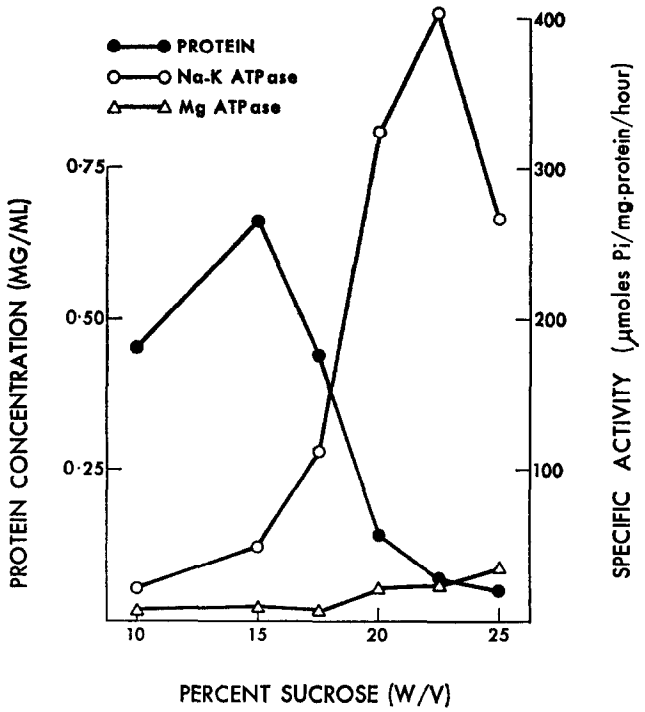

FIG. 5. ATPase activities and protein concentration of fractions obtained by density gradient centrifugation of Lubrol extract from rat brain microsomes. Microsomal fractions were prepared from rat cerebral cortex tissue and treated with Lubrol as indicated in Methods. The Lubrol extract was layered on a discontinuous gradient of sucrose (containing 0·5 mM EDTA, pH 6·9) and centrifuged at 90,000 *g* for 40 hr. The values for ATPase activity and protein represent the mean of three experiments.

from the 0·2 per cent Lubrol extract. The density gradient fraction containing the highest protein concentration also separated into numerous protein staining bands, a large number of which exhibited fast migration rates. Note the absence of a dense staining band at the origin of this sample. The fraction containing the highest Na-K ATPase specific activity separated into at least five lightly staining protein bands of slower migration rate with a heavily staining band at the origin. Since the total protein of this fraction applied to the gel was only $\frac{1}{10}$ of that of the other fractions, the observed electrophoretic pattern does not necessarily indicate that a purification has been achieved by the density gradient separation. Attempts to estimate the Na-K ATPase activities of the individual bands separated by gel electrophoresis have been unsuccessful to date.

DISCUSSION

Detailed studies on the characterization of the Na-K transport ATPase system are dependent on purification of the enzyme. The application of procedures involving detergents in attempts to purify the enzyme are numerous, and there have been a number of reports concerning the use of the non-ionic detergent, Lubrol, in this respect. Schwartz *et al.*[10] were unsuccessful in extracting Na-K ATPase activity from a brain

microsomal preparation using Lubrol W. Swanson *et al.*[3] demonstrated an activation of approximately 60 per cent of the Na-K ATPase of cerebral microsomes by treatment with low concentrations of the detergent and inhibition of activity at concentrations above 0·4 per cent. Hokin *et al.*[6,8,9] have reported the solubilization and further purification of the enzyme from various brain preparations. These studies involved the use of high concentrations of Lubrol and resulted initially in Na-K ATPase preparations of relatively low specific activity and marginal stability unless protected by the addition of substrate and Na. In the present investigation, concentrations of Lubrol (0·2 per cent) which have no inhibitory action on the enzyme were effective in extracting Na-K ATPase activity from brain and heart tissue of rat and guinea pig. Much of the data reported above concerns the enzyme preparation obtained by Lubrol treatment of a microsomal fraction from rat cerebral cortex. Such preparations contained Na-K ATPase of high specific activity and a low percentage (6·5 per cent) of Mg ATPase activity (Table 1). They were stable in 0·32 M sucrose containing EDTA and did not require ATP or sodium for protection, losing only 10 per cent of their Na-K ATPase activity after 14 days at 4°. When microsomal fractions were pretreated with sodium iodide, subsequent treatment with 0·2 per cent Lubrol failed to extract Na-K ATPase into the nonsedimentable supernatant. This is in accord with Uesugi *et al.*,[8] who found that high concentrations of the detergent were necessary to extract the enzyme after sodium iodide treatment.

The Na-K ATPase activity of the Lubrol extracts exhibited many properties in common with various particulate preparations of cerebral origin. Concentrations of Na and K required for both half-maximal and maximal activation were close to previously reported values.[3,7] The optimum substrate concentration (5 mM ATP) found in this study was slightly higher than that used by other investigators,[4,17] but the apparent K_m of 5×10^{-4} M was similar to previously reported values, which range from 3 to 7×10^{-4} M.[3,18] Maximum Na-K ATPase activity was observed at a pH of 6·8, which is lower than that reported by others,[4,7] although Swanson[19] observed a biphasic relationship of activity to pH with one peak occurring at pH 6·6. The Na-K ATPase activity of the Lubrol extract was sensitive to ouabain, half-maximal inhibition occurring at a concentration of 1×10^{-6} M. High concentrations of K protected the enzyme from ouabain inhibition (Table 2), demonstrating that Lubrol treatment did not alter this rather characteristic property of Na-K-activated ATPase systems.

Inhibition of the Na-K ATPase of Lubrol extracts of rat brain microsomes by low concentrations of ouabain is interesting, since this species is reported to be relatively insensitive to the action of cardiac glycosides.[16] Repke[20] has suggested that species variations in response to cardiac glycosides may be related in part to differences in sensitivity of Na-K ATPases to such agents. There are a few reports in the literature concerning the sensitivity of rat brain Na-K ATPase to ouabain.[18,21–23] With the exception of the lipoprotein enzyme of Ahmed and Judah,[21] most rat brain enzyme preparations were considerably less sensitive to ouabain than the Lubrol-extracted Na-K ATPase examined in the present study. No great differences existed in the ouabain concentrations required to produce 50 per cent inhibition of the enzyme in Lubrol extracts of rat and guinea pig brain microsomes (Table 5). Lubrol treatment of microsomal fractions of cardiac tissue of both rat and guinea pig resulted in extraction of Na-K ATPase activity of greater sensitivity to ouabain than the microsomal or sodium iodide preparations (Fig. 4). Ouabain concentration required to effect 50 per cent

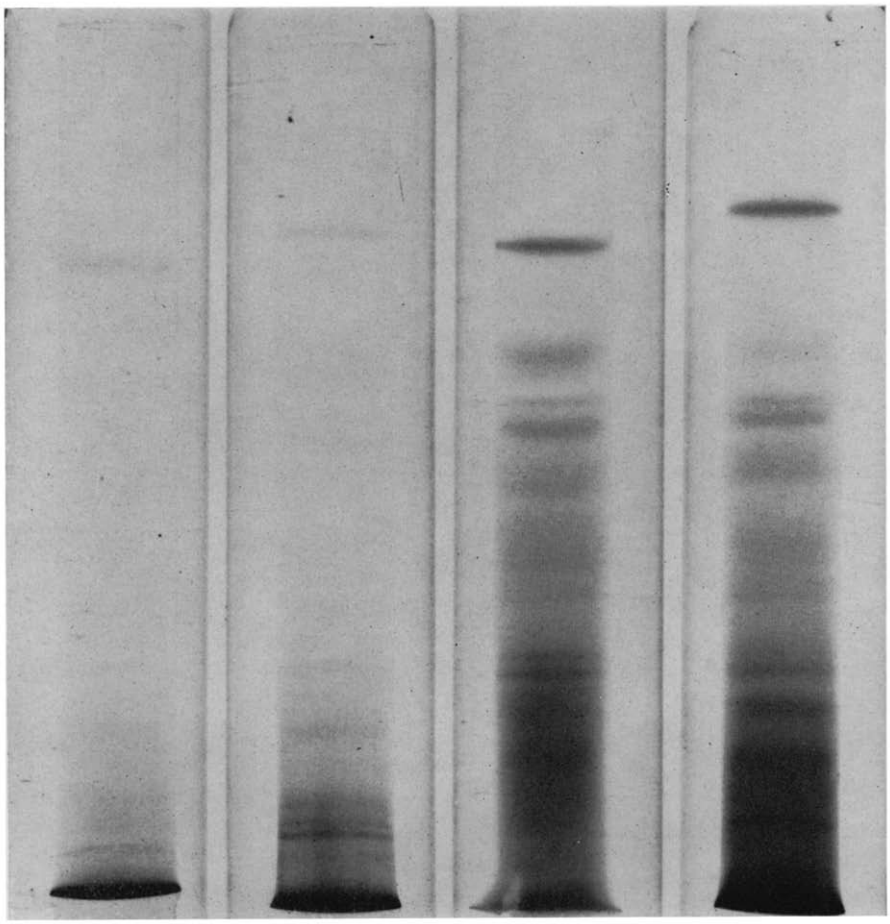

FIG. 6. Disc gel electrophoretic separation of Lubrol extract and density gradient fractions. Lubrol extracts of rat brain microsomes were subjected to density gradient centrifugation and the separated fractions were compared to the original sample by electrophoretic separation on 7% polyacrylamide gels using the procedure detailed in Methods. From left to right, the samples are: original 0·2% Lubrol extract; high protein sample in 15% sucrose; 20% sucrose sample; and the fraction containing high specific activity Na-K ATPase (22·5% sucrose). Total protein applied to gels was 110, 92, 46 and 10 μg respectively.

inhibition of the Na-K ATPase activities of Lubrol extracts of rat and guinea pig heart microsomes were $6{\cdot}0 \times 10^{-5}$ and $2{\cdot}6 \times 10^{-6}$ M respectively. Thus, in the case of heart tissue, a species difference in ouabain sensitivity of the enzyme may well exist. Schwartz *et al.*[24,25] have examined species differences in the sensitivity of cardiac Na-K ATPase to ouabain in some detail. They report a greater degree of non-specific binding and more labile specific binding of ouabain to the rat heart enzyme than to enzymes derived from beef and dog heart. Our data on the heart enzyme preparations support suggestions[20,25] that species differences in cardiac glycosides may have a correlate in Na-K ATPase susceptibility to such drugs. Such correlations appear to be tissue specific, since no great differences in ouabain sensitivity exist in enzymes of cerebral origin.

Subjection of the Lubrol extract of rat brain microsomes to sucrose density gradient fractionation resulted in further purification of the Na-K ATPase. Peak specific activity (400 μmoles P_i/mg of protein/hr) occurred at a density of 1·084, the fraction containing 5 per cent of the protein content of the original extract. Uesugi *et al.*[8] have reported the further purification of Na-K ATPase solubilized by higher concentrations of Lubrol by separation on continuous sucrose gradients. In their hands, the peak specific activity occurred at a density of 1·05 and the ouabain-insensitive ATPase "equilibrated" with the major protein peak at an even lower density. By contrast, the Mg ATPase activity of the 0·2 per cent Lubrol preparation in this study reached peak activity at a density of 1·094. Lubrol distribution appeared to follow that of protein and was found in highest concentrations in sucrose fractions of density 1·037 to 1·065. The fraction containing the highest specific activity of the Na-K ATPase contained Lubrol at a concentration of 130 μg/ml and protein of 70 μg/ml. The data suggest that a considerable proportion of the detergent exists in complex with protein, and further suggests the possibility that such complex formation is essential to maintain the Na-K ATPase system in a nonsedimentable form.

The results from disc gel electrophoretic studies indicated that the original Lubrol extract and the separated density gradient fractions are quite heterogeneous. Despite the high specific activity of Na-K ATPase in the fraction separated in sucrose of density 1·084, it contains at least five protein staining components, and the densely stained origin suggests that certain components did not enter the 7 per cent polyacrylamide gel. This may be the case for the Na-K ATPase, since other investigators[9] failed to demonstrate its entry into gels containing greater than 3 per cent polyacrylamide. The heterogeneity of the separated fraction is also shown by the presence of other enzyme species of membrane origin, namely acetylcholinesterase and adenyl cyclase. Nonetheless, the preparation, by virtue of high and stable Na-K ATPase activity, appears suitable for further purification and characterization studies of this enzyme system.

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