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SOLUBILIZATION OF GUINEA PIG KIDNEY ($Na^+ + K^+$)-ATPase WITH LUBROL W AND TRITON X-100

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SUMMARY

1. A comparison has been made of two non-ionic detergents, Lubrol W and Triton X-100 for the solubilization of ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3).

2. The optimum concentrations for maximum solubilization with Triton X-100 and Lubrol W were 0.05 and 0.1 %, respectively.

3. Monovalent cations, such as Na^+ and K^+, and ATP increased the specific activity of the enzyme solubilized by Triton X-100 and decreased that by Lubrol W.

4. Mg^{2+} inhibited solubilization with either of the two detergents.

5. Triton X-100 was found superior to Lubrol W in solubilization of ($Na^+ + K^+$)-ATPase.

6. A procedure for obtaining soluble enzyme preparation with specific activity ranging between 140 and 200 μmoles P_i per mg protein per h is described.

7. The soluble enzyme was more stable in the presence of Na^+, K^+ or ATP, but ATP was inferior to Na^+ or K^+.

INTRODUCTION

($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) which is present in the microsomal fraction of tissue homogenates, participates in the transport of Na^+ and K^+ across cell membrane[1,2]. Since its discovery more than a decade ago[3], this enzyme has not been isolated in a pure form. Failure to purify this enzyme has hampered attempts to define a mechanism for cation transport. Since ($Na^+ + K^+$)-ATPase contains phospholipids as an integral part of the enzyme structure, it is insoluble in water[4–8]. However the enzyme may be solubilized by surface active agents[9–12]. Cationic and anionic detergents have been found to be less effective solubilizing agents than non-ionic detergents[9].

Comparing two non-ionic detergents Triton X-100 and Lubrol WX, MEDZIHRADSKY *et al.*[10] obtained considerably higher recoveries and higher specific activities of ($Na^+ + K^+$)-ATPase with Lubrol. The enzyme solubilized with a detergent in this manner rapidly lost its activity[10]. However monovalent ions such as Na^+ and K^+ exhibited striking stabilizing effects on the soluble enzyme preparation[10]. The specific activity of the soluble enzyme obtained from guinea pig brain was very low and

therefore unsuitable for large scale purification[11]. $(Na^+ + K^+)$-ATPase of higher specific activities could be prepared from NaI-treated beef brain microsomes[11,12]. However, their initial step in the solubilization procedure always resulted in soluble enzyme preparations of either the same or lower specific activity compared to the original enzyme. Thus, the detergent treatment did not purify the enzyme any further and even resulted in inactivation of the enzyme.

In this paper Lubrol W and Triton X-100 are compared for their ability to solubilize $(Na^+ + K^+)$-ATPase. It was observed that Triton with added ligands extracted soluble enzyme of higher specific activity and ouabain sensitivity than Lubrol. The optimum concentration of Triton for maximum solubilization is one tenth that used by previous workers[10]. The specific activity of the soluble enzyme obtained with Triton is 4-fold greater than that reported by UESUGI *et al.*[11] and KAHLENBERG *et al.*[12].

MATERIALS AND METHODS

Materials

Lubrol W Flakes was obtained from Canadian Industries Limited, Montreal. Triton X-100 was purchased from Rohm and Haas, Philadelphia. Na_2ATP, Tris ATP, imidazole and histidine were obtained from Sigma Chemicals.

Methods

Preparation of membrane ATPase. The procedure was a slight modification of the method described by POST AND SEN[13]. Adult guinea pigs were stunned by a blow on the head and kidneys were removed. Approx. 5.0 g of cortex was homogenized in a glass to teflon homogenizer with 35 ml of Solution A containing 0.25 M sucrose 0.02 M NaCl, 5 mM EDTA (disodium salt), 1 mM $MgCl_2$ and 10 mM imidazole (pH, 7.2 ± 0.1) and was centrifuged at 1000 × *g* for 10 min. The supernatant was decanted and saved. The same homogenization procedure was repeated twice on the precipitate followed by centrifugation each time at 1000 × *g* for 10 min. The three supernatants were combined and spun at 9000 × *g* for 10 min. The supernatant was discarded and the sediment was rehomogenized in Solution A and centrifuged at 39100 × *g* for 30 min. The resultant sediment was then homogenized in Solution B containing 0.25 M sucrose, 2 mM EDTA (disodium salt), 0.1 mM $MgCl_2$, 4 mM imidazole and 0.02 % (w/v) sodium heparin (pH 6.9 ± 0.1) and centrifuged at 39100 × *g* for 30 min. The supernatant was removed cautiously by aspiration and discarded. The heparin treatment resulted in a sediment which had two distinct layers, an upper yellowish-white fluffy layer and a lower tightly packed brown layer. The yellow upper layer of the sediment was removed to obtain the microsomal preparation and suspended in a solution containing 10 mM imidazole, 5 mM HCl and 0.1 mM EDTA (pH 6.9 ± 0.05). The specific activity of this fraction was usually between 60 and 75 μmoles P_i per mg protein per h. This isolated fraction was further treated with urea by the method of POST AND SEN[13] to prepare urea-treated enzyme.

Solubilization of $(Na^+ + K^+)$-ATPase. Unless otherwise indicated, solubilization was carried out as follows. The temperature was maintained between 0 and 4°. Detergent of desired concentration was made up in 25 mM imidazole and 12.5 mM histidine–HCl solution (pH 7.1 ± 0.1). When necessary, Na^+, K^+ or ATP were also dissolved in the imidazole–histidine buffer containing the detergent. To 1 ml of the

microsomal suspension 9 ml of this solution were added and homogenized with a teflon pestle. Homogenized material was set aside for 10 min and centrifuged for 1 h at 280000 × *g* in an IEC ultracentrifuge (Model B-60). The resultant supernatant is referred to as the soluble enzyme.

ATPase assay. When Triton was present in the solubilizing solution, the ATPase activity was measured by the method of POST AND SEN[13]. However, Lubrol W interfered with the determination of P_i by forming a white precipitate which prevented extraction of phosphate by butyl acetate. Therefore, the following modifications were made. Following incubation and the addition of 0.5 ml of 1.2 M $HClO_4$, 0.5 ml of 2.5 M H_2SO_4 was added to digest the tissue and facilitate the subsequent extraction. After addition of 0.5 ml of 10 % (w/v) ammonium molybdate, the phosphomolybdate complex was extracted with 4 ml of isobutanol–benzene (1:1, v/v)[9] instead of the butyl acetate. Each tube was agitated vigorously for at least 20 sec at room temperature on a Vortex mixer. The tubes were then centrifuged for 1 min to clear the emulsion formed. 2 ml of the upper layer (organic phase) were transferred to a tube with 4 ml of isopropanol solution containing 0.06 mM $CuCl_2 \cdot 2\ H_2O$ and 0.27 M H_2SO_4. Finally 5 μl of mercaptoethanol were added and the resulting blue color read on a spectrophotometer (Zeiss) at 825 mμ.

The determination of protein was carried out by the method of LOWRY *et al.*[14]. Lubrol (up to 0.4 %) did not interfere in the estimation of protein. The absorbance of a certain quantity of protein nitrogen, however, was greater in presence of Triton than in its absence. Therefore, a standard protein curve containing 0.05 % Triton was plotted. This curve was linear between absorbances of 0.2 and 0.3. Samples of all soluble enzymes containing Triton were so adjusted that the absorbances were within the linear portion of the protein curve. Triton did not interfere in the determination of the total phosphate which was carried out by a modification of the method of BARTLETT[15].

RESULTS

Solubilization of $(Na^+ + K^+)$-ATPase with Lubrol

Results obtained with 0.1 and 0.4 % Lubrol are shown in Table I. Similar amounts of protein were solubilized by these two concentrations of Lubrol. However the specific activity of the soluble enzyme obtained with 0.1 % Lubrol was 3-fold greater than with 0.4 % Lubrol. The sum of the total activity present in soluble and insoluble fractions with 0.1 % Lubrol W was about 100 %. In contrast, total activity found in soluble and insoluble portions after treatment with 0.4 % Lubrol was only 42.6 %. Thus, 0.4 % Lubrol caused an inactivation of the $(Na^+ + K^+)$-ATPase. Furthermore, with 0.1 % Lubrol there was a 14 % increment in ouabain sensitivity. Inactivation by 0.4 % Lubrol was surprising, because, previous workers[9] found maximum solubilization with 1.2 % Lubrol. Therefore, we carried out a concentration curve for Lubrol and results are shown in Fig. 1. There was no significant difference in amount of protein solubilized by different concentrations of Lubrol. The optimum concentration of Lubrol, which gave soluble enzyme with highest specific activity was 0.1 %.

TABLE I

SOLUBILIZATION OF $(Na^+ + K^+)$-ATPase BY 0.1% AND 0.4% LUBROL

The microsomal enzyme was solubilized with 0.1 or 0.4% Lubrol as described under MATERIALS AND METHODS. The enzyme activity was tested with and without 0.25 mM ouabain. The specific activity and the total activity represent the ouabain-sensitive part of the enzyme.

Preparative step	*Protein (mg/ml)*	*Total protein (mg)*	*Protein solubilized or present in pellet (%)*	*Specific activity (μmoles P_i per mg protein per h)*	*Total activity (μmoles P_i per h)*	*Activity solubilized or present in pellet (%)*	*% Sensitive to ouabain*
Original enzyme	4.06	4.06	—	76	309	—	67.51
Soluble enzyme with 0.1% Lubrol	0.25	2.5	61.6	77	196	63.4	81.2
Soluble enzyme with 0.4% Lubrol	0.267	2.69	65.7	25.6	68.8	22.2	—
Pellet obtained with 0.1% Lubrol	0.148	1.48	36.4	79	117	38.0	79.3
Pellet obtained with 0.4% Lubrol	0.148	1.48	36.4	42.5	63	20.4	65.2

TABLE II

SOLUBILIZATION OF UREA-TREATED $(Na^+ + K^+)$-ATPase BY 0.1% LUBROL

Conditions were as described under Table I.

Preparative step	*Protein (mg/ml)*	*Total protein (mg)*	*Protein solubilized or present in pellet (%)*	*Specific activity (μmoles P_i per mg protein per h)*	*Total activity (μmoles P_i per h)*	*Activity solubilized or present in pellet (%)*	*% Sensitive to ouabain*
Original enzyme	0.875	0.875	—	246.6	216	—	76.2
Soluble enzyme with 0.1% Lubrol	0.0272	0.272	31	66.8	18.15	8.4	77.0
Pellet obtained with 0.1% Lubrol	0.04375	0.4375	50	118.5	51.7	24.0	83.2

Solubilization of the urea-treated enzyme

The specific activity and ouabain sensitivity of the insoluble $(Na^+ + K^+)$-ATPase may be increased by the formation of dispersed and activated particles of the microsomal fraction with a detergent, urea or concentrated NaI solution. Since previous workers[11] have shown that soluble enzyme of high specific activity may be prepared from NaI-treated enzyme, it was of interest therefore, to solubilize urea-treated enzyme. Results are shown in Table II. $(Na^+ + K^+)$-ATPase was rapidly inactivated with 0.1 % Lubrol. The total activity present in the soluble enzyme and

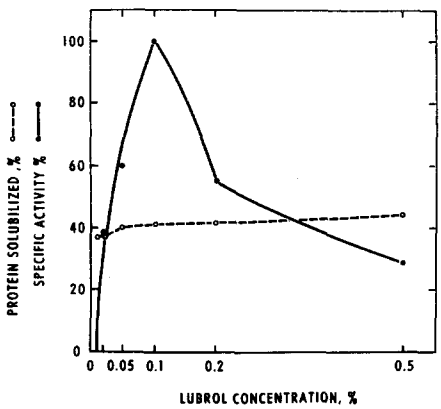

Fig. 1. Solubilization of $(Na^+ + K^+)$-ATPase by different concentrations of Lubrol. The specific activity of the original enzyme was 77.54 μmoles P_i per mg protein per h and contained 3.2 mg of protein per ml of microsomal suspension.

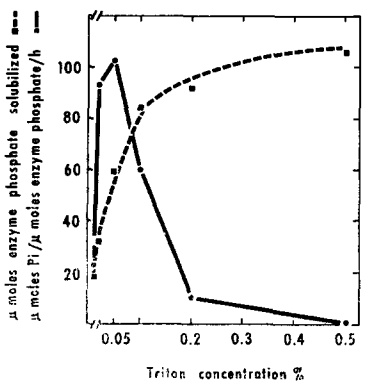

Fig. 2. Solubilization of $(Na^+ + K^+)$-ATPase by different concentrations of Triton. 4 mM of KCl were added to the buffer containing the indicated concentration of Triton. The rest of the procedure was the same as described under the MATERIALS AND METHODS.

the pellet was 32.4 % of the original enzyme (Table II). There was a loss of about 20 % in protein recovery. This may be due to the low concentration of protein used in the experiment. Correction for this amount in the pellet fraction will increase the total activity to 71.0 and the per cent activity in the pellet to 34.0. Even then the total recovery of activity would amount to only 42.4 % of the original enzyme. Thus the urea-treated enzyme was found inferior to the untreated enzyme for solubilization.

Concentration curve for Triton

Before a comparison of the two detergents, Triton and Lubrol, was made, a concentration curve for Triton was obtained. Triton interferes in the protein determination by the method of LOWRY *et al.*[14]. Therefore, instead of protein, total phosphate in the enzyme was estimated. Although the specific activity determined in μmoles P_i per μmole enzyme phosphate per h is different from that expressed as μmoles P_i per mg protein per h for the same enzyme, the method was found suitable to determine the optimum concentration of Triton for maximum solubilization. There was a rapid rise in the specific activity of the soluble enzyme as the concentration of Triton was raised from 0.01 to 0.05 %. Higher concentrations of Triton resulted in an inactivation of the enzyme system. This occurred in spite of steady and exponential increase in the solubilization of the total enzyme phosphate (Fig. 2). Although the specific

TABLE III

A COMPARISON OF THE SOLUBLE ENZYME OBTAINED BY 0.1% LUBROL OR 0.05% TRITON AND THEIR MODIFICATIONS BY DIFFERENT LIGANDS

Preparative step	*Protein (mg/ml)*	*Total protein (mg)*	*Protein solubilized (%)*	*Specific activity (μmoles P_i per mg protein per h)*	*% Sensitive to ouabain*
(*A*) Original enzyme	2.31	—	—	67.14	—
Triton	0.056	0.56	24.22	54.41	96.94
Triton + 3 mM Na_2ATP	0.045	0.45	19.46	157.70	90.53
Triton + 3 mM NaCl	0.075	0.75	32.43	80.86	94.95
Triton + 3 mM Tris ATP	0.049	0.49	21.19	64.18	87.39
Lubrol	0.104	1.04	44.86	70.04	66.48
Lubrol + 3 mM Na_2ATP	0.06	0.60	25.95	18.55	17.58
Lubrol + 3 mM NaCl	0.093	0.93	40.00	41.58	52.58
Lubrol + 3 mM Tris ATP	0.095	0.95	41.08	60.94	59.87
(*B*) Original enzyme	2.94	—	—	74.21	
Triton + 4 mM Mg^{2+} + 1 mM P_i	0.048	0.48	16.34	0.0	—
Triton + 4 mM Mg^{2+}	0.044	0.44	14.89	0.0	—
Lubrol + 4 mM Mg^{2+}	0.061	0.61	20.74	0.0	—
Lubrol + 4 mM Mg^{2+} + 1 mM P_i	0.064	0.64	21.70	7.77	—

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activities of the enzymes solubilized by 0.02 % Triton and 0.05 % Triton were approximately the same, the total phosphate in the former was only 54.3 % of the latter. Solubilization by detergents is influenced by detergent to protein or lipoprotein ratio[9]. The optimum concentration of protein for 0.05 % Triton was, therefore, determined. The optimum Triton to protein ratio was found to be between 0.17 and 0.23 which is close to the Lubrol W to protein optimum ratio of 0.3 (Fig. 1). In all subsequent experiments protein concentration of the microsomal fraction was maintained between 2.5–3.5 mg/ml and optimum concentrations of Lubrol and Triton were used.

Comparison of Triton and Lubrol on solubilization of $(Na^+ + K^+)$-ATPase and effect of different ligands

The results described so far indicate that 0.1 % Lubrol and 0.05 % Triton are the optimum concentrations of detergents for enzyme solubilization. Several recent studies indicate that presence of one or more ligands influences the conformational state of the enzyme[16–19]. Since the conformational state of the enzyme might well influence the solubilization procedure, we tested the effect of different ligands on solubilization by each of the two detergents. The results are shown in Table III. In the absence of added ligands the specific activity of the Lubrol-extracted enzyme was 70.04 μmoles P_i per mg protein per h while that with Triton was 54.41 μmoles P_i per mg protein per h. However, the Lubrol-solubilized enzyme was less sensitive to ouabain in all cases. When 3 mM NaCl or Tris ATP was present in the solubilizing mixture, the two surface active agents exhibited opposite effects. NaCl and/or ATP enhanced the specific activity of Triton-treated enzyme; whereas, the activity of Lubrol-treated enzyme was reduced. Soluble enzyme of highest specific activity (157.7 μmoles P_i per mg protein per h) was obtained with Triton in the presence of 3 mM Na_2ATP. Mg^{2+} with or without P_i inhibited solubilization of the enzyme by the two detergents.

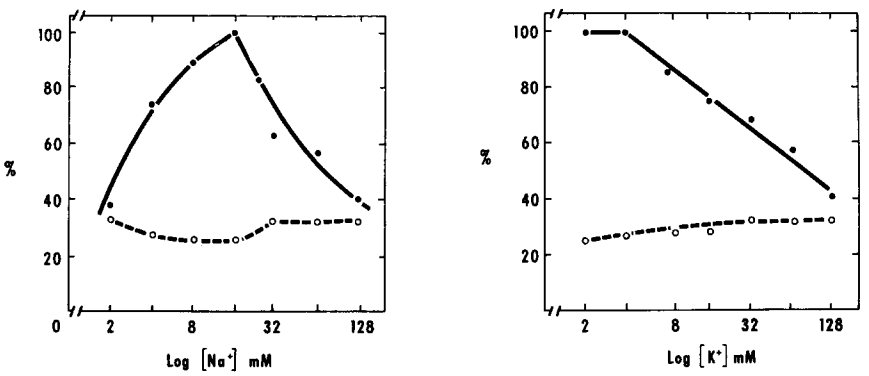

Fig. 3. Effect of different concentrations of Na^+ on the solubilization of $(Na^+ + K^+)$-ATPase by 0.05 % Triton. The per cent of maximum soluble enzyme specific activity is shown by (●—●) and per cent protein solubilized by (○---○). Each point is the average of three experiments. The mean specific activity of the soluble enzyme at 16 mM Na^+ was 125 μmoles P_i per mg protein per h.

Fig. 4. Effect of different concentrations of K^+ on the solubilization of $(Na^+ + K^+)$-ATPase by 0.05 % Triton. The per cent of maximum soluble enzyme specific activity is shown by (●—●) and per cent protein solubilized by (○---○). Each point is average of three experiments and the mean specific activity of the soluble enzyme at 2 mM K^+ was 146 μmoles P_i per mg protein per h.

Effect of Na^+ and K^+ on solubilization of 0.05 % *Triton*

Since NaCl enhanced the specific activity of the enzyme solubilized by Triton, concentration curves for Na^+ and K^+ were plotted. Fig. 3 shows the concentration curve for Na^+. The per cent protein solubilized did not alter with the changes in NaCl concentration in the solubilizing medium. In contrast the specific activity of the soluble enzyme varied with the concentration of Na^+ with a maximum at 16 mM NaCl. Fig. 4 shows the concentration curve for KCl. 2.0 mM of K^+ gave a soluble enzyme of highest specific activity which remained fairly constant till 4 mM KCl. When the concentration of K^+ was raised from 4 to 128 mM there was a linear decrease in the solubilization of the $(Na^+ + K^+)$-ATPase. As with Na^+, changes in K^+ concentration also did not alter the per cent protein solubilized.

Interference of Lubrol and Na_2ATP in protein determination

KAHLENBERG *et al.*[12] and UESUGI *et al.*[11], have employed 4 % or more of Lubrol to solubilize $(Na^+ + K^+)$-ATPase. In the present study Lubrol and Na_2ATP interfered in the protein determination by the method of LOWRY *et al.*[14]. Previous workers[11,12], have employed 4 % or more of Lubrol to obtain soluble $(Na^+ + K^+)$-ATPase of specific activities which were comparable to original insoluble enzyme preparations. Since in the present study even 0.4 % Lubrol inactivated the $(Na^+ + K^+)$-ATPase (Table I), the apparent contradiction could possibly be explained by the interference of higher concentrations of Lubrol in the protein estimations. Fig. 5 shows the effect of 4 % Lubrol on the standard albumin protein curve. Since Na_2ATP was used to stabilize the $(Na^+ + K^+)$-ATPase[11,12], the effect of 4 % Lubrol in presence or absence of 2.5 mM Na_2ATP was examined. Fig. 5 shows that 4 % Lubrol either with or without Na_2ATP interfered in the protein determination.

Stability of $(Na^+ + K^+)$-ATPase solubilized with Triton

Enzyme solubilized with Lubrol may be stabilized by addition of either Na^+ or K^+ (ref. 10). However, no information is available regarding the stability of

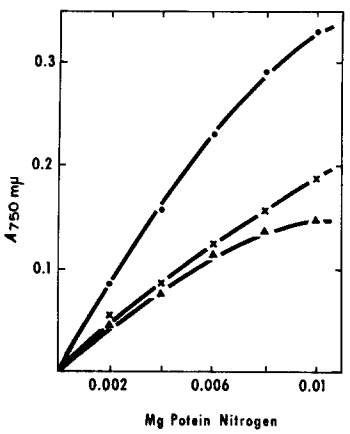

Fig. 5. Interference by 4 % Lubrol and 2.5 mM Na_2ATP in protein determination by the method of LOWRY *et al.*[14]. 0.2 ml of 4 % Lubrol either with or without 2.5 mM Na_2ATP solution was added to the protein reagent. The final volume was 3 ml. Therefore, Lubrol was diluted to 0.27 % and Na_2ATP to 0.17 mM. Absorbance for standard albumin protein is indicated by (●—●), for protein *plus* 0.4 % Lubrol by (×—×) and protein *plus* 2.5 mM Na_2ATP by (▲—▲).

$(Na^+ + K^+)$-ATPase solubilized with Triton. Therefore we examined the effects of different ligands upon the stability of enzyme solubilized in presence of Triton. Results are shown in Fig. 6. In absence of added ligands soluble enzyme rapidly lost its activity. Addition of Tris ATP, Na^+ and K^+ either alone or in combination significantly decreased the rate of inactivation. ATP was found inferior to both Na^+ and K^+. ATP when combined with either Na^+ or K^+ did not enhance the protective effect of the monovalent cations.

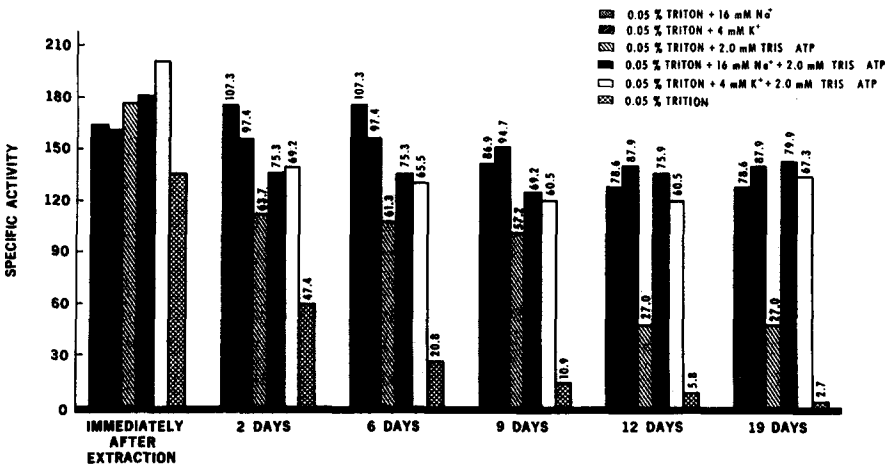

Fig. 6. Effect of different ligands on the stability of the enzyme solubilized with Triton. The number on top of each histogram shows the per cent activity present in the enzyme on the particular day as compared to the specific activity found immediately upon extraction. The specific activity is expressed as μmoles P_i per mg protein per h.

DISCUSSION

In this paper, the clear supernatant fraction of the detergent-treated enzyme obtained following 280000 $\times$ g centrifugation for 60 min has been designated as soluble enzyme. Such a preparation, when centrifuged for 20 h at 114000 $\times$ g in 8.2 % sucrose, gave a sediment which contained most of the $(Na^+ + K^+)$-ATPase activity[11]. This indicates that detergent treatment either resulted in formation of low-density complex with membrane fraction or that the soluble enzyme macromolecules sediment upon prolonged centrifugation. The latter possibility is supported by the finding of UESUGI *et al.*[11], who reported that the sediment obtained after prolonged spinning may be solubilized in detergent-free buffer. However, the possibility of low-density membrane complex formation cannot be completely ruled out. Clarification of this question will be the subject of a future communication.

MEDZIHRADSKY *et al.*[10] have reported that Triton was unsuitable for solubilization of $(Na^+ + K^+)$-ATPase. But ROODYN[20] found maximum solubilization of mitochondrial membrane with Triton. In the present work, the effects of Triton clearly indicate that maximum solubilization of $(Na^+ + K^+)$-ATPase could be achieved with 0.05 % Triton (Fig. 2), whereas 0.5 % Triton completely inactivated the enzyme. The fact that MEDZIBRADSKY *et al.*[10] employed 0.5 % Triton only may explain their inability to obtain good solubilization with Triton.

In agreement with KAHLENBERG *et al.*[12] Na^+ and ATP inhibited solubilization by Lubrol W (Table III). However, it was found that within certain concentration ranges both Na^+ and K^+ facilitated solubilization by Triton (Figs. 3 and 4). Besides enhancing solubilization by Triton, these ions increased the stability of the enzyme activity (Fig. 6).

UESUGI *et al.*[11] concluded from their studies that NaI-treated beef brain microsomes was the most suitable preparation for extraction with 4 % Lubrol. With such a preparation they reported a maximum specific activity of 40.5 μmoles P_i per mg protein per h in the soluble fraction. With guinea pig brain microsomes and 0.2 % Lubrol W SWANSON *et al.*[9] obtained a soluble fraction with a specific activity of 70.0 μmoles P_i per mg protein per h. In the present study, a lower concentration of Lubrol was more effective. With the guinea pig kidney microsomal fraction and 0.1 % Lubrol the specific activity in the soluble fraction was 77.0 μmoles P_i per mg protein per h. With 0.4 % we observed an inactivating effect on the enzyme (Table I). It is possible that there are species and/or tissue differences with respect to the optimal concentration of Lubrol. But similar inactivation effect on guinea pig brain microsomes by 0.4 % Lubrol has been reported by SWANSON *et al.*[9]. Higher concentration of Lubrol not only extracts more of the nonspecific protein but also inactivated the specific protein rendering the soluble fraction unsuitable for further purification as it is extremely difficult to separate the active from the inactive enzyme molecules. Furthermore, high concentrations of Lubrol interfered with the protein determination by the method of LOWRY *et al.*[14] (Fig. 5), this might lead to fortuitous specific activity calculations unless adequate care is taken to correct for this interference.

In conclusion Triton seems to be superior to Lubrol for solubilization of the $(Na^+ + K^+)$-ATPase. In the presence of 2 mM K^+ or 16 mM Na^+, 0.05 % Triton routinely yielded soluble enzymes with specific activities of 140–160 μmoles P_i per mg protein per h.

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