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PURIFICATION AND CHARACTERIZATION OF $(Na^+ + K^+)$-ATPASE

I. THE INFLUENCE OF DETERGENTS ON THE ACTIVITY OF $(Na^+ + K^+)$-ATPASE IN PREPARATIONS FROM THE OUTER MEDULLA OF RABBIT KIDNEY

PETER LETH JØRGENSEN AND JENS CHR. SKOU
Institute of Physiology, University of Aarhus, 8000 Aarhus C (Denmark)
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SUMMARY

1. Incubation of a microsomal fraction from the outer medulla of rabbit kidney with deoxycholate rapidly increases the specific activity of $(Na^+ + K^+)$-ATPase (ouabain sensitive; ATP phosphohydrolase, EC 3.6.1.3) from 45 to 270 μmoles P_i per mg protein per h if the conditions for incubation are optimal with respect to temperature, pH and concentrations of protein and detergent. A procedure for evaluation of the conditions for maximum activation by deoxycholate is described.

2. Measurements of the surface tension show that the marked influence of changes in the pH on the activation by deoxycholate is due to changes in the capillary activity of deoxycholate. The optimum concentrations of deoxycholate, sodium dodecyl sulfate, and Lubrol-14 for activation of $(Na^+ + K^+)$-ATPase are different, but it is common for the three detergents that maximal activation is obtained when the critical micelle concentration is reached.

3. Fractionation by zonal centrifugation shows that the $(Na^+ + K^+)$-ATPase remains associated with membranes after activation by deoxycholate, whereas inactive protein is removed and solubilized by the detergent. The treatment with deoxycholate reduces the content of Mg^{2+}-ATPase (ouabain insensitive; ATP phosphohydrolase, EC 3.6.1.3) in the fractions which contain the $(Na^+ + K^+)$-ATPase.

4. Tracer studies show that the activation of $(Na^+ + K^+)$-ATPase is not associated with binding of significant amounts of deoxycholate to the membranes. The activation does not change the molecular activity of $(Na^+ + K^+)$ATPase.

5. The data suggest that the activation of $(Na^+ + K^+)$-ATPase is due to exposure of latent enzyme sites in the preparation. The removal of protein may lead to opening of vesicular structures resulting in free access of substrate and activators to their respective sites on the membrane.

INTRODUCTION

In freshly prepared subcellular fractions of mammalian tissues the specific activity of $(Na^+ + K^+)$-ATPase (ouabain sensitive; ATP phosphohydrolase, EC

Abbreviation: c.m.c., critical micelle concentration.

3.6.1.3) is low, and only a small fraction of the total ATPase activity is ($Na^+ + K^+$)-dependent and inhibited by ouabain[1]. The enzyme system is firmly associated with subcellular particles and the activity levels obtained in preparations of ($Na^+ + K^+$)-ATPase vary greatly with the pretreatment of the fractions assayed.

Under proper conditions, incubation of subcellular fractions from brain and kidney with detergents such as deoxycholate[2–4], sodium dodecyl sulfate[5], and Lubrol[6], gives rise to a marked increase in the specific activity of ($Na^+ + K^+$)-ATPase, whereas the activity of Mg^{2+}-ATPase (ouabain insensitive; ATP phosphohydrolase, EC 3.6.1.3) remains unchanged or decreases. This activation of ($Na^+ + K^+$)-ATPase occurs within a certain range of concentrations of detergent, whereas higher concentrations inactivate the enzyme[4,6]. A careful control of the conditions for treatment with detergent is therefore essential in attempts to purify the enzyme system[7] and for determinations of the total activity of ($Na^+ + K^+$)-ATPase in a given preparation[4,8].

The purpose of the present study was to analyse in detail the effect of detergents on preparations from the outer medulla of rabbit kidney, which are used as starting material in an attempt to purify ($Na^+ + K^+$)-ATPase[7,9]. The dependence of the activation of ($Na^+ + K^+$)-ATPase on the composition of the incubation medium and the influence of deoxycholate on the preparative separation of ($Na^+ + K^+$)-ATPase were assessed. In order to learn more about the mechanism by which detergents influence the enzyme activity, the changes in enzyme activity were related to the surface tension of the incubation media, and the extent of binding of detergent to the enzyme preparation was determined. Further, the binding of [^{3}H]ouabain was measured to see if the activation is due to an increase in the number of enzyme sites in the preparation or to an increase in the molecular activity of ($Na^+ + K^+$)-ATPase.

EXPERIMENTAL

Tissue preparation

Rabbits (2–3 kg) were killed by a blow on the head and exsanguination from the neck. The kidneys were rapidly removed and placed in ice-cold 0.03 M histidine, 0.25 M sucrose, pH 7.2 (20°). Tissue from the outer medulla was obtained by dissection of transverse sections with a scalpel on a block of frozen histidine–sucrose, covered with filter paper and moistened with histidine–sucrose. The kidney was cut in transverse sections of 3-mm thickness and the light grey inner medulla or papilla was removed. Tissue from the dark red outer medulla was obtained by incisions along the inner side of the boundary to the inner cortex. On an average, 0.6 g of tissue was obtained from the outer medulla of one kidney. The tissue was homogenized in 10 vol. of histidine–sucrose with 5 stokes in a Teflon glass homogenizer (Braun, Melsungen). To obtain the preparation which in the following is called the microsomal fraction, the homogenate was centrifuged at 6000 × *g* for 15 min in a Sorval RC-2B centrifuge. The sediment was resuspended by homogenization with 4 strokes and centrifuged again at 6000 × *g* for 15 min. The combined supernatants from the two centrifugations were centrifuged at 48000 × *g* for 30 min (20000 rev./min in Rotor SS 34 of the Sorvall RC-2B centrifuge). The resulting pellet was resuspended by gentle homogenization in histidine–sucrose to a concentration of 2.5 mg protein per ml. The prepara-

tion was stored in 1–3 ml aliquots at —25° and was used for a period of 1 week. Once thawed the aliquots were discarded.

Incubation of the microsomal fraction with detergent and enzyme analysis

The composition of the media for incubation with deoxycholate was varied with respect to pH, temperature, concentration of deoxycholate and EDTA and of microsomal protein. Details are given in the figure legends. For enzyme analysis, 25 μl of the incubation media was transferred to test tubes of 1 ml. In this way the concentration of deoxycholate in the test tubes was kept below 0.025 mg/ml, *i.e.* 2–3-fold lower than the concentration which inhibits the enzyme reaction[2]. Assays for ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase were done as before[4,7]. Cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) activity was determined by the method of WHARTON AND TZAGOLOFF[10]. Protein was determined by the micro-Kjeldahl method or by the method of LOWRY *et al.*[11] after precipitation and wash with 5% trichloroacetic acid at 0–4°. The method of LOWRY *et al.* was standardized with micro-Kjeldahl determinations, and bovine serum albumin (Armour) was used as the working standard.

For fractionation of the microsomal fraction by zonal centrifugation the Beckmann Ti-XIV or Ti-XV rotors were used, and the sucrose gradients were formed by a Beckmann Model 131 gradient pump. The details of the procedure are given in the figure legends.

Surface tension was measured by means of a platinum ring and a du Noüy tensiometer (SEELICH's[12] model). The instrument was thermostated at 20° and calibrated with redistilled water and pure ethanol. The results, expressed in dyn·cm^{-1}, are averages of three consecutive measurements on each sample of 3 ml at 20°.

The light scattering of detergent solutions was measured as the ratio between the intensity of the light scattered at 90° and the intensity of the transmitted light (520 nm) in a Brice Phoenix light scattering photometer using a 20-mm circular cell[13].

[^{14}C]Deoxycholate with a specific activity of 7.62 mC/mmole was obtained from Mallinckrodt Nuclear, St. Louis, Mo., U.S.A. Counting was done in a Packard Tri Carb scintillation counter in vials with 15 ml of scintillator[14]. Correction for quenching was made by the aid of an internal standard.

For determination of [^{3}H]ouabain binding[15,16], an amount of the enzyme preparation corresponding to 0.2–0.3 mg protein was incubated in 1 cm × 6 cm centrifuge tubes for 30 min at 37° with 2 mM EDTA, 10 mM NaCl, 5 mM $MgCl_2$, 3 mM ATP, 10^{-5} M [^{3}H]ouabain, 30 mM Tris, pH 7.5, (37°) in a total volume of 1 ml. For the estimation of unspecific binding of [^{3}H]ouabain and of radioactivity trapped in the pellets, the enzyme preparation was incubated in parallel in media devoid of NaCl, $MgCl_2$, and ATP. After the incubation at 37° the tubes were coooled on an ice-bath and centrifuged for 30 min at 260000 × *g* (0°). The supernatants were removed, and remaining droplets wiped off with filter paper. The pellets were dissolved in 0.2 ml 1 M NaOH in a water bath at 60° for 30 min. The solution was transferred to counting vials with 15 ml of scintillator[14] and counted in a Packard TriCarb scintillation counter. Correction for quenching was made by the aid of an internal standard. [^{3}H]ouabain with a specific activity of 3.7 C/mole was obtained from New England Nuclear.

Phospholipid and cholesterol were determined on extracts of the fractions by

the method of FOLCH *et al.*[17]. Phospholipid was determined as inorganic phosphate by the method of BARTLETT[18], and cholesterol was estimated using the Tschugaeff reaction in the modification of HANEL AND DAM[19].

RESULTS

Evaluation of the conditions for maximum activation by deoxycholate of ($K^+ + Na^+$)-ATPase in the microsomal fraction

In Fig. 1 is shown the time-course of the increase in specific activity of ($Na^+ + K^+$)-ATPase when the microsomal fraction is incubated with varying concentrations of deoxycholate at 20° and at pH 7.0. It is seen that the activity increases with time until a stable level is reached after about 30 min. The specific activity at the stable level increases with the deoxycholate concentration up to 0.6 mg/ml.

The effect of deoxycholate on the ouabain-insensitive Mg^{2+}-ATPase is not shown in Figs. 1–4, but it remains unchanged or decreases slightly during the incubation with deoxycholate (*cf.* Fig. 5).

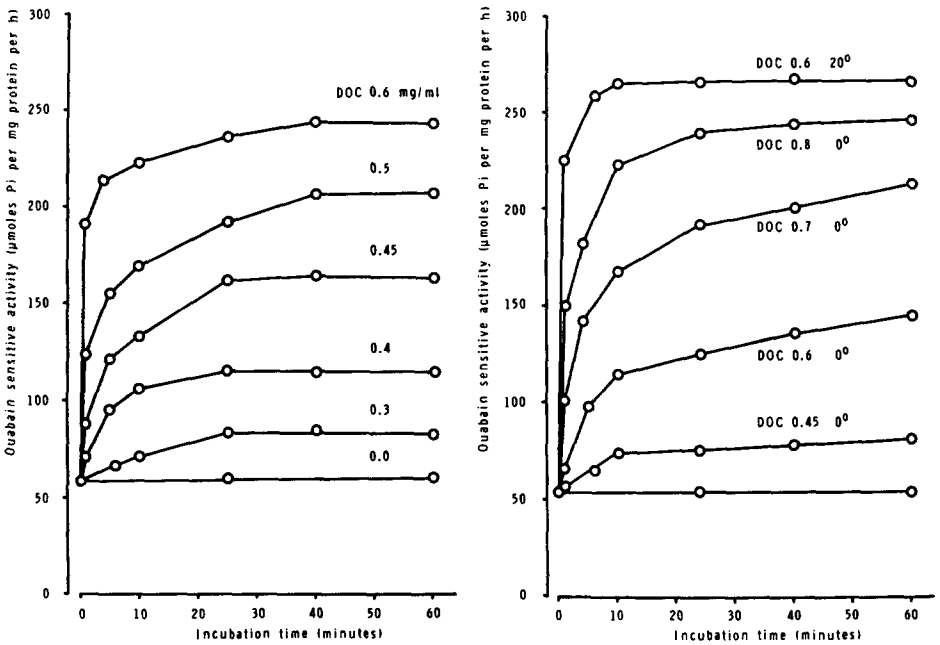

Fig. 1. The time-course of the changes in activity of ($Na^+ + K^+$)-ATPase during incubation with increasing concentrations of deoxycholate (DOC) at 20°. An amount of the microsomal fraction corresponding to 0.25 mg protein was incubated in 1 ml with the indicated concentrations of deoxycholate and 25 mM imidazole, pH 7.0, at 20°. At the indicated intervals 25 μl was transferred to test tubes (1 ml) containing 3 mM Mg^{2+}, 130 mM Na^+, 20 mM K^+, 3 mM ATP (Tris salt), 30 mM histidine, pH 7.5 (37°). After 10 min at 37° the reaction was stopped with 100 μl 50% trichloroacetic acid and cooling on an icebath. The ouabain-sensitive activity was calculated as the difference in activity with an without 1 mM ouabain present in the test tubes.

Fig. 2. The time-course of the changes in activity of ($Na^+ + K^+$)-ATPase during incubation at 0° with increasing concentrations of deoxycholate (DOC). For comparison the time-course of the changes in activity during incubation at 20° with 0.6 mg deoxycholate per ml is shown. The pH of the incubation media was 7.0 (20°). Experimental conditions as in Fig. 1.

At a lower temperature, 0°, the rise in specific activity of ($Na^+ + K^+$-)ATPase during incubation with deoxycholate is slower, and the concentration necessary to give the same stable level of activity as at 20° is increased (Fig. 2, *cf.* Fig. 1).

The activating effect of deoxycholate is highly dependent on the pH of the incubation medium. This is shown for 0.45 mg/ml deoxycholate and at 20° in Fig. 3. It is seen that both the rate of activation and the stable level obtained increases when the pH is decreased. The activity of ($Na^+ + K^+$)-ATPase in the controls, incubated in buffers devoid of deoxycholate, remained unchanged.

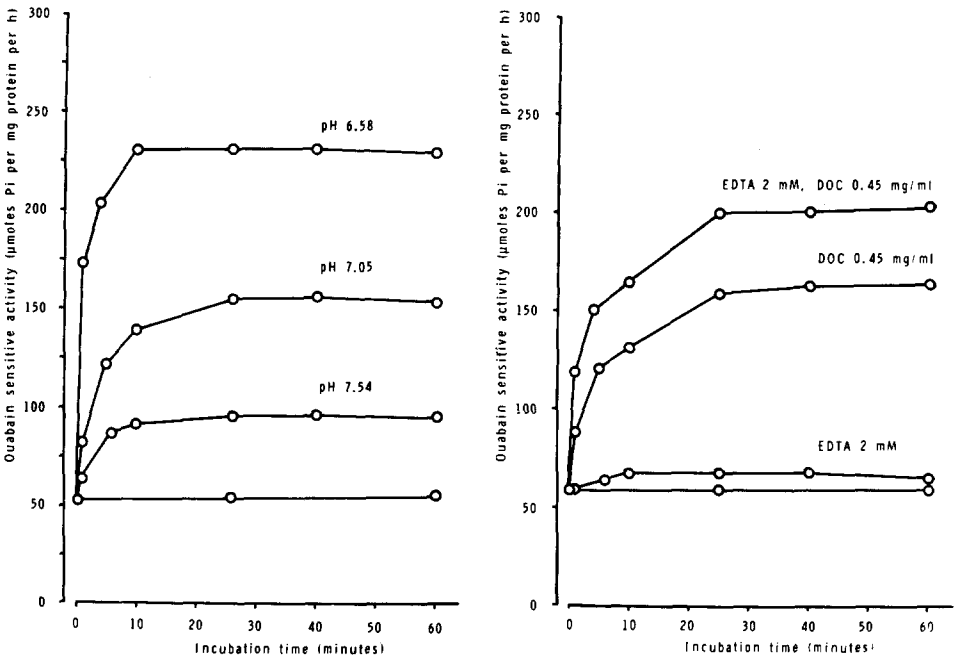

Fig. 3. The influence of pH on the time-course of the changes in activity of ($Na^+ + K^+$)-ATPase during incubation with 0.45 mg deoxycholate per ml. Experimental conditions as in Fig. 1.

Fig. 4. The influence of EDTA on the time-course of the changes in activity of ($Na^+ + K^+$)-ATPase during incubation with 0.45 mg deoxycholate (DOC) per ml at 20°, pH 7.0. Experimental conditions as in Fig. 1.

Incubation of the microsomal fraction with 2 mM EDTA without deoxycholate gives a slight increase in the activity of ($Na^+ + K^+$)-ATPase (Fig. 4). With 0.45 mg/ml deoxycholate addition of 2 mM EDTA moderately increases the level of activity above that obtained without EDTA at pH 7.0 and 20°.

The results presented in Figs. 1, 3 and 4 show that the activity of ($Na^+ + K^+$)-ATPase measured after incubation with deoxycholate for 30 min or more at 20° depends on the pH and on the concentration of deoxycholate in the medium but is independent of the time of incubation.

This means that the most direct approach in evaluating the optimum conditions for activation of ($Na^+ + K^+$)-ATPase by deoxycholate at 20° is to determine the concentration dependence of the activation by deoxycholate at different pH values

and after 30 min of incubation. Such an experiment is shown in Fig. 5. At each pH value, the specific activity of ($Na^+ + K^+$)-ATPase measured after incubation for 30 min with increasing concentrations of deoxycholate follows a biphasic curve with a narrow optimum. The optimum concentration of deoxycholate at different pH values decreases from 1.0 to 0.3 mg/ml as the pH of the incubation medium is lowered from 7.6 to 6.3. The highest degree of activation is obtained at pH 7.1 and 6.6 with a deoxycholate concentration of 0.6 mg/ml and 0.45 mg/ml, respectively.

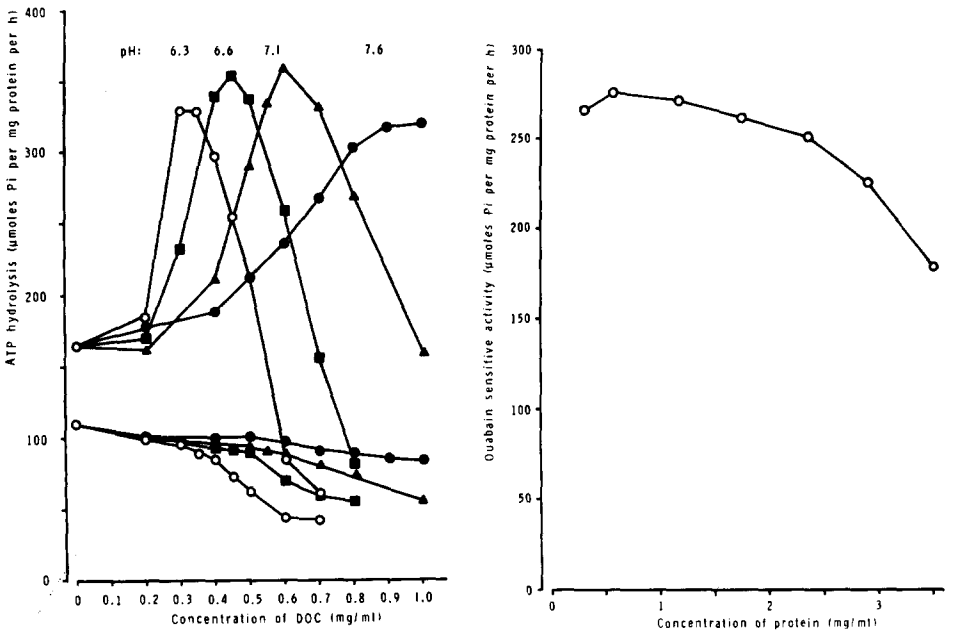

Fig. 5. The influence of pH on the concentration dependence of the activation by deoxycholate (DOC) of ($Na^+ + K^+$)-ATPase in the microsomal fraction. The pH of the incubation media was ○—○, 6.3; ■—■, 6.6; ▲—▲, 7.1; and ●—●, 7.6; at 20°. The microsomal fraction (0.25 mg protein per ml) was incubated for 30 min with the indicated concentrations of deoxycholate and 25 mM imidazole adjusted to the desired pH with HCl. Other experimental conditions as in Fig. 1. The upper set of lines represent the total ATPase activity and the lower set of lines, the activity with 1 mM ouabain added to the test tubes.

Fig. 6. The influence of the concentration of microsomal protein on the level of ($Na^+ + K^+$)-ATPase activity reached after incubation with 0.6 mg deoxycholate per ml, 1 mM EDTA, 25 mM imidazole, pH 7.0 at 20° for 30 min. Other experimental conditions as in Fig. 1.

In the experiments shown in Figs. 1–5, the protein concentration was 0.25 mg/ml. As seen from Fig. 6 the activating effect of deoxycholate with a concentration of 0.6 mg/ml is independent of the protein concentration when this concentration is less than about 1.5 mg/ml, while at higher concentrations the activating effect of deoxycholate decreases.

The relationship between the activation of ($Na^+ + K^+$)-ATPase and the surface tension of the incubation media

A possible explanation for the effect of changes in pH on the activating effect of deoxycholate is that the capillary activity of this anionic detergent changes with

the pH. To examine this the surface tension of the incubation media which gave the ascending part of the biphasic curves in Fig. 5 was measured, and the activating effect of deoxycholate at the different pH values was plotted as a function of the surface tension (Fig. 7). It is seen that deoxycholate starts to activate when the surface tension has decreased to a value around 50 dyn·cm^{-1} and that this value is nearly independent of the pH. Furthermore, that an increase in the deoxycholate concentration at a given pH value only gives a slight further decrease in surface tension. This suggests that the concentration of deoxycholate which activates is close to the critical micelle concentration (c.m.c.).

In order to examine whether the increase in activity of ($Na^+ + K^+$)-ATPase is due to a reduction in the surface tension *per se* to a certain level, or whether the detergent must be present in concentrations high enough to form micelles, the effect of deoxycholate was compared with the effect of sodium dodecyl sulfate and Lubrol-14.

Fig. 8 shows the concentrations of the three detergents which gave maximum activation at pH 7.0, 20° after an incubation time of 30 min. The maximum activity seen after incubation with deoxycholate and sodium dodecyl sulfate is the same (Fig. 8), whereas the optimal concentration of sodium dodecyl sulfate was 6-fold lower on weight basis and 4-fold lower on molar basis than the optimum concentration of deoxycholate. The maximum activity reached after incubation with Lubrol-14 was consistently lower than with deoxycholate and sodium dodecyl sulfate.

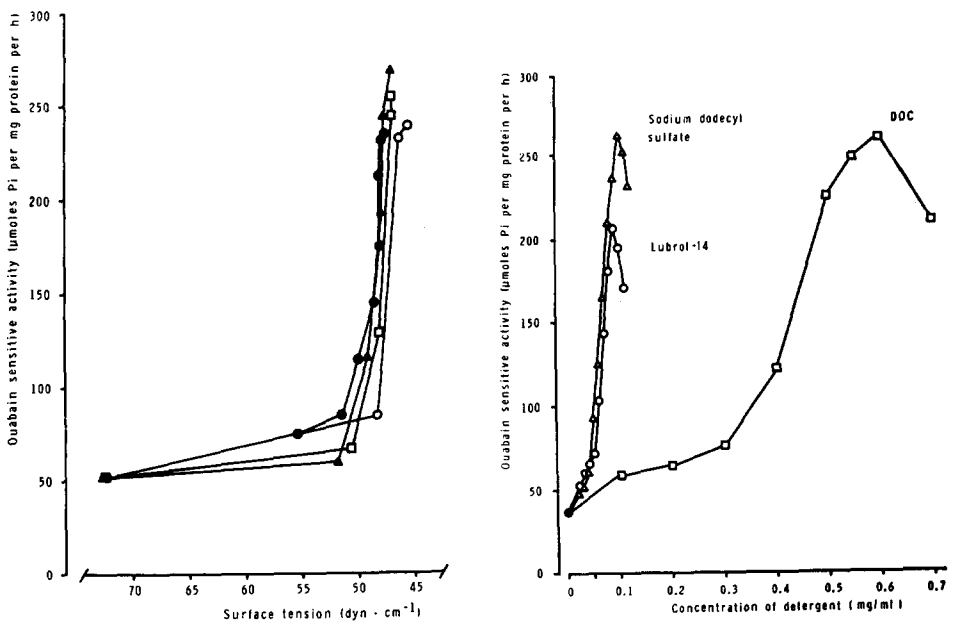

Fig. 7. The relationship between the activity of ($Na^+ + K^+$)-ATPase and the surface tension of the incubation media used for the experiment shown in Fig. 5. The surface tension was measured as described under EXPERIMENTAL after incubation for 30–60 min at 20°. Only the results for media with concentrations of deoxycholate at or below the maxima of the biphasic curves in Fig. 5 are shown. ○—○, pH 6.6; □—□, 6.6 pH; ▲—▲, pH 7.1; ●—●, pH 7.6; at 20°.

Fig. 8. The concentration dependence of the activation of ($Na^+ + K^+$)-ATPase by sodium dodecyl sulfate (△), Lubrol-14 (○) and deoxycholate (DOC, □). Incubation for 30 min at 20°, pH 7.0. Other experimental conditions as in Fig. 1.

Fig. 9 shows that the surface tension at which optimum activation is reached is lower with sodium dodecyl sulfate and Lubrol-14 than with deoxycholate.

In Table I are shown the c.m.c. of the three detergents in buffer solutions and in buffer solutions with microsomes in a concentration of 0.25 mg protein per ml. Table I also shows the surface tension of the buffer solutions at the c.m.c. The addition of microsomal protein increases the c.m.c. for sodium dodecyl sulfate and Lubrol-14, but not for deoxycholate. The c.m.c. in solutions of buffer with microsomes is slightly lower than the concentration of the detergents which are necessary for maxi-

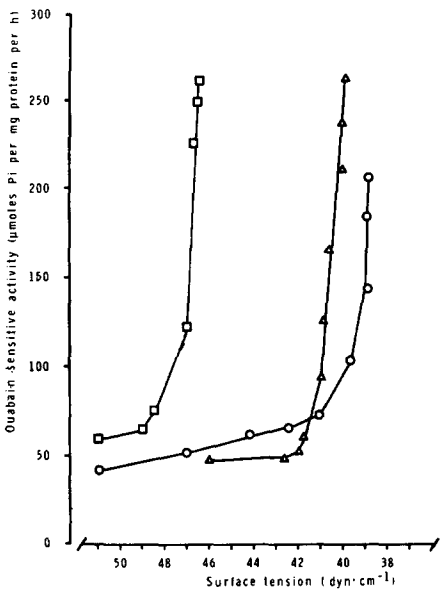

Fig. 9. The relationship between the activity of ($Na^+ + K^+$)-ATPase and the surface tension of the incubation media used in the experiment shown in Fig. 8. Experimental conditions as in Fig. 7. □, deoxycholate; △, sodium dodecyl sulfate; ○, Lubrol-14.

TABLE I

THE CRITICAL MICELLE CONCENTRATION (c.m.c.) AND THE SURFACE TENSION AT THE c.m.c. FOR SOLUTIONS OF DEOXYCHOLATE, SODIUM DODECYL SULFATE, AND LUBROL-14 IN 25 mM IMIDAZOLE, pH 7.0 (20°), WITH AND WITHOUT THE ADDITION OF MICROSOMES (0.25 mg/ml PROTEIN)

Curves for the variations with concentrations of the surface tension and the light scattering were plotted for solutions of the three detergents in buffer. The c.m.c. was determined as the range of detergent concentrations within which the surface tension changed to a low and constant value[20]. For solutions in buffer alone, a sudden increase in light scattering occurred within the same range of concentrations[20]. For solutions in buffer with microsomes, only the surface tension was measured.

Detergent	*c.m.c. (mg/ml)*		*Surface tension at the c.m.c. (dyn·cm⁻¹)*
	Buffer	*Buffer with microsomes*	*Buffer*
Deoxycholate	0.50 –0.55	0.50–0.60	46–47
Sodium dodecyl sulfate	0.047–0.050	0.07–0.08	39–40
Lubrol-14	0.022–0.025	0.06–0.07	38–39

mum activation (*cf.* Fig. 8). For all three detergents the surface tensions measured at optimum activation (Fig. 9) are within the range of surface tensions measured at the c.m.c.

The data in Figs. 8 and 9 and in Table I thus show that maximum activation of $(Na^+ + K^+)$-ATPase is obtained only when the concentration of the detergents has reached the c.m.c., *i.e.* when aggregates or micelles have been formed in the detergent solutions[20].

The influence of detergents on the preparative separation of $(Na^+ + K^+)$-ATPase by centrifugation

In a previous report[7] it was shown that incubation of the microsomal fraction with detergents not only leads to an increase in the specific activity of $(Na^+ + K^+)$-ATPase but also to a release of protein from the microsomal particles. After high-speed centrifugation of the deoxycholate-treated microsomal fraction, 44% of the total protein remained in the supernatant, whereas all of the $(Na^+ + K^+)$-ATPase activity was recovered in the sediment. A further separation of active and inactive protein could be obtained by differential centrifugation and sucrose gradient centrifugation.

To explore this in more detail, we studied the influence of deoxycholate on the distribution of protein, $(Na^+ + K^+)$-ATPase, and Mg^{2+} in a density gradient after zonal gradient centrifugation under conditions which favour isopycnic separation of $(Na^+ + K^+)$-ATPase[9]. The results are given in Figs. 10–12.

Fig. 10 shows the distribution of the three components after centrifugation of a microsomal fraction which has not been activated by deoxycholate. It is seen that

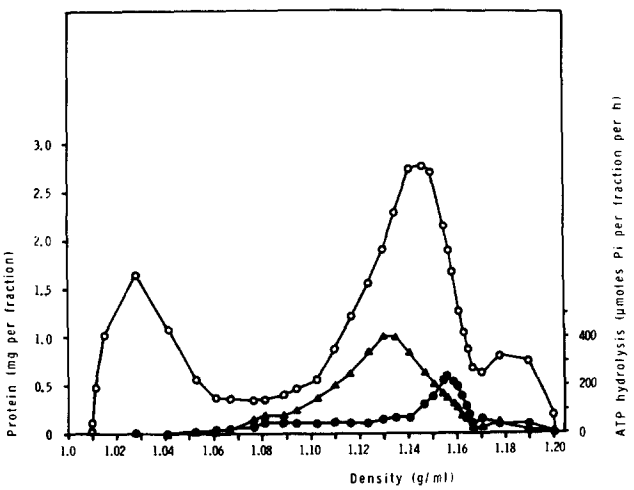

Fig. 10. The distribution of protein (○), $(Na^+ + K^+)$-ATPase (●) and Mg^{2+}-ATPase (▲) after zonal gradient centrifugation of a fresh microsomal fraction in the Ti-XIV rotor at 40000 rev./min for 90 min ($\omega^2 t = 10.4 \cdot 10^{10}$). The sample contained 36.2 mg of microsomal protein in a total volume of 50 ml, 25 mM imidazole, 3% (w/v) sucrose, pH 7.0 (20°). A sucrose gradient of 500 ml was formed from 15% (w/v) sucrose and 45% (w/v) sucrose in 25 mM imidazole, 1 mM EDTA, pH 7.0 (20°), and pumped into the rotor at a rate of 28 ml/min. The gradient was followed by a cushion of 55% (w/v) sucrose. Sample and overlay, 60 ml 25% (w/v) sucrose, were injected with a syringe. After centrifugation, the gradient was displaced from the rotor with 55% (w/v) sucrose at a rate of 16 ml/min. 42 fractions of 16 ml were collected and analysed for protein and enzyme activity as in Fig. 1. Density measurements were made with a refractometer at 20°.

a major part of the protein is found in a band at densities between 1.10 and 1.17 g/ml, while a small part was found in a band near the sample zone (1.1–1.15 g/ml) (Fig. 10). There is a small peak of ($Na^+ + K^+$)-ATPase at 1.15–1.16 g/ml, while the activity is low in fractions between 1.12 and 1.15 g/ml where a broad band of Mg^{2+}-ATPase is located.

The fractions collected after the centrifugation were treated with deoxycholate under optimum conditions. The results are given in Fig. 11. It is seen that the ($Na^+ + K^+$)-ATPase activity in the small peak which coincides with the peak of cytochrome oxidase is not changed by treatment with deoxycholate. At lower densities, however,

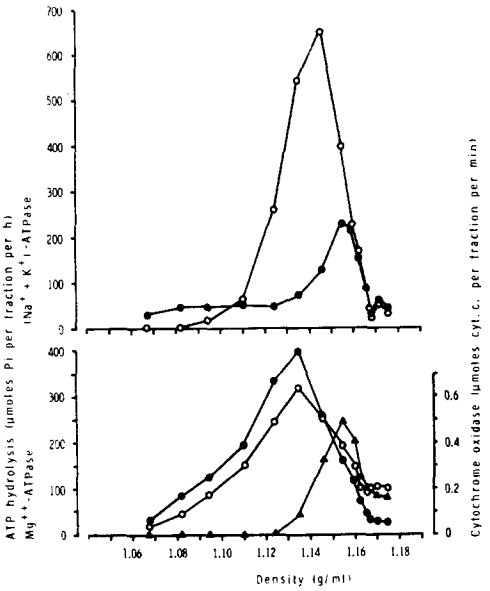

Fig. 11. The effect of deoxycholate on the activity of ($Na^+ + K^+$)- ATPase and Mg^{2+}-ATPase in fractions obtained by zonal gradient centrifugation of the fresh microsomal fraction. Aliquots of the fractions obtained in the experiment shown in Fig. 10 were incubated for 30 min at 20° with (○) and without (●) 0.6 mg deoxycholate per ml, 2 mM EDTA at pH 7.0 (20°). Enzyme analysis as in Fig. 1. ▲ denotes the activity of cytochrome oxidase in the untreated fractions.

treatment with deoxycholate gives a large increment in ($Na^+ + K^+$)-ATPase activity, and a peak appears at a density of 1.145 g/ml. The specific activity of ($Na^+ + K^+$)-ATPase at this peak is 260 μmoles P_i per mg protein per h. The treatment with deoxycholate gives only small changes in the activity of Mg^{2+}-ATPase.

In the experiment shown in Fig. 12 the microsomal fraction was treated with deoxycholate before the centrifugation in order to give optimum activation of the ($Na^+ + K^+$)-ATPase. After the centrifugation, a major part of this ($Na^+ + K^+$)-ATPase activity is found in a peak at densities between 1.12 and 1.15 g/ml. The specific activity of ($Na^+ + K^+$)-ATPase in the three fractions at the peak is 983, 1176, and 947 μmoles P_i per mg protein per h.

A comparison between Figs. 10 and 12 shows that the treatment with deoxycholate before the centrifugation grossly alters the distribution of the protein. The band between 1.10 and 1.17 g/ml is reduced and that near the sample zone is increased. Further, the broad band of Mg^{2+}-ATPase with the peak around 1.13 g/ml

seen in Fig. 10 has disappeared, and the Mg^{2+}-ATPase activity is more widely distributed.

The experiments with the zonal centrifugal system thus show that the particles with which the $(Na^+ + K^+)$-ATPase is associated have the same localization in the density gradient whether or not the microsomal fraction is treated with deoxycholate before the centrifugation. The high specific activity obtained by centrifugation of the material treated with deoxycholate before the centrifugation is mainly explained by removal of inactive protein and of Mg^{2+}-ATPase from the fractions which contain the $(Na^+ + K^+)$-ATPase.

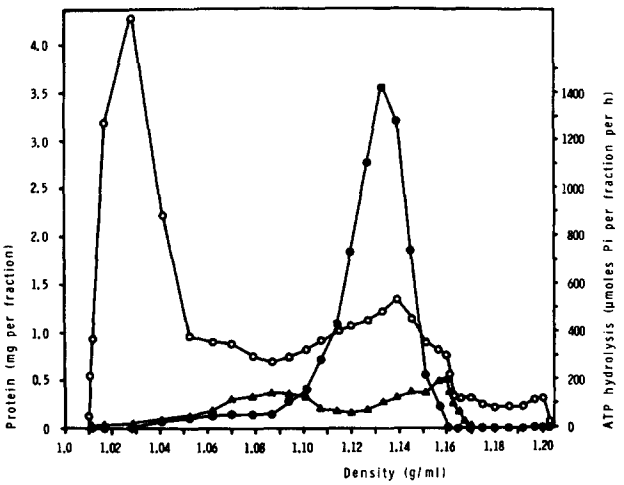

Fig. 12. The distribution of protein (○), $(Na^+ + K^+)$-ATPase (●) and Mg^{2+}-ATPase (▲) after zonal gradient centrifugation of a microsomal fraction treated with deoxycholate and EDTA. 29.9 mg of microsomal protein was incubated for 45 min at 20° with 0.6 mg deoxycholate per ml, 2 mM EDTA, 25 mM imidazole, 3% (w/v) sucrose, pH 7.0 (20°) in a total volume of 50 ml and introduced as a sample into the zonal rotor. Other experimental conditions as in Fig. 10.

The binding of deoxycholate to the microsomal particles

[^{14}C]Deoxycholate was used to determine whether the activation by deoxycholate of $(Na^+ + K^+)$-ATPase is accompanied by binding of the detergent to the particles with which the enzyme activity is associated.

In the experiment shown in Fig. 13, the microsomal fraction was incubated with [^{14}C]deoxycholate and subjected to zonal gradient centrifugation as in Fig. 12. It is seen that only a small fraction of deoxycholate in the sample enters the gradient along with the microsomal particles. Only 0.21% of the total amount of deoxycholate in the incubation medium is found in the 10 fractions (No. 17–27) containing the $(Na^+ + K^+)$-ATPase.

In the fractions at the peak of $(Na^+ + K^+)$-ATPase (No. 23–25) the amount of deoxycholate is 3.7 ± 0.2 (3) μg or 9.4 ± 0.5 (3) nmoles deoxycholate per mg protein. After dilution and centrifugation of these fractions in an angle rotor the $(Na^+ + K^+)$-ATPase activity is recovered in the sediments. The amount of deoxycholate bound to the sediments is 3.9 ± 0.3 (3) nmoles deoxycholate per mg protein.

For comparison it may be mentioned that the total content of phospholipid

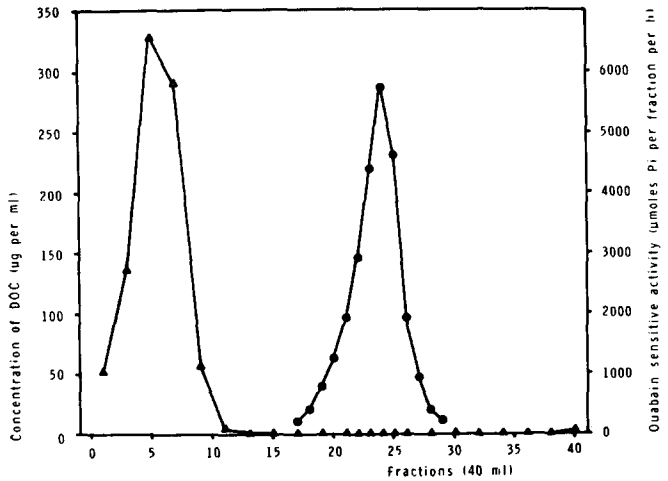

Fig. 13. The distribution of [^{14}C]deoxycholate (DOC; △) and ($Na^+ + K^+$)-ATPase (●) after zonal gradient centrifugation of a microsomal fraction incubated with [^{14}C]deoxycholate and EDTA. 134.1 mg of microsomal protein was incubated for 45 min at 20° in 200 ml 0.6 mg [^{14}C]-deoxycholate per ml, 2 mM EDTA, 25 mM imidazole, 3% (w/v) sucrose, pH 7.0 (20°) and introduced as a sample into the Ti-XV zonal rotor. The volume of the sucrose gradient was 1200 ml and the overlay, 150 ml. Centrifugation at 35000 rev./min for 120 min ($\omega^2 t = 10.7 \cdot 10^{10}$). 40 fractions of 40 ml were collected. Other experimental conditions as in Fig. 10.

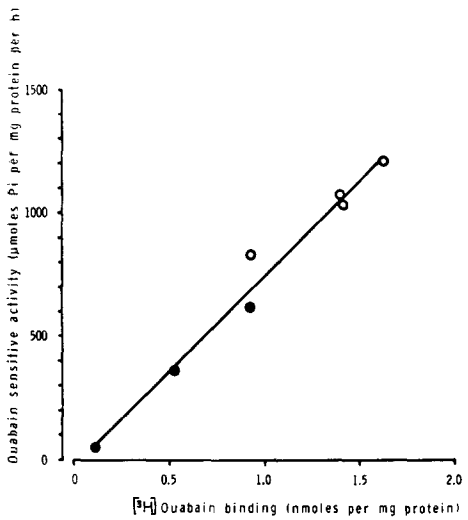

Fig. 14. The relationship between the specific activity of ($Na^+ + K^+$)-ATPase and the binding of [^{3}H]ouabain for preparations obtained by subfractionation of microsomal fractions treated with increasing concentrations of deoxycholate. ● denotes preparations obtained by differential centrifugation of microsomal fractions incubated for 30 min at 20° with 2 mM EDTA, 25 mM imidazole, pH 7.0, and increasing concentrations of deoxycholate: 0, 0.4, and 0.6 mg deoxycholate per ml, from the left to the right, respectively. The incubation media were subjected to differential centrifugation and the sediment after 25000 × g^7 was used. ○ denotes preparations obtained by sedimentation in an angle rotor of fractions at the peak of ($Na^+ + K^+$)-ATPase after zonal gradient centrifugation of a microsmal fraction incubated with 0.6 mg deoxycholate per ml and centrifuged as in Fig. 12. Enzyme analysis as in Fig. 1, and measurement of [^{3}H]ouabain binding as described under EXPERIMENTAL.

in the same fractions is 882 ± 29 (3) nmoles/mg protein and that the content of cholesterol is 523 ± 37 (3) nmoles/mg protein.

These observations show that the activation by deoxycholate of $(Na^+ + K^+)$-ATPase is not associated with binding of significant amounts of detergent to the microsomal particles.

The molecular activity of $(Na^+ + K^+)$-ATPase before and after treatment with deoxycholate

The increase in the specific activity of $(Na^+ + K^+)$-ATPase induced by incubation with deoxycholate might be due to an increase in the molecular activity of the enzyme or to an increase in the number of enzyme sites in the preparation.

The binding of [^{3}H]ouabain has been used to estimate the number of enzyme sites and the molecular activity of the enzyme in preparations of $(Na^+ + K^+)$-ATPase[21,22]. The same quantitative relationship between [^{3}H]ouabain binding and $(Na^+ + K^+)$-ATPase activity has been found for a number of preparations with widely different specific activities[16,21,22].

Fig. 14 shows the relationship between the [^{3}H]ouabain binding and the specific activity of $(Na^+ + K^+)$-ATPase for the untreated microsomal fraction and for preparations obtained by differential centrifugation and zonal gradient centrifugation of microsomal fractions treated with deoxycholate. The specific activity of $(Na^+ + K^+)$-ATPase varies between 44 and 1212 μmoles P_i per mg protein per h. It is seen that there is a linear relationship between the two parameters for these preparations. The molecular activity of $(Na^+ + K^+)$-ATPase as calculated from the slope of this line is 12850 ± 810 P_i per min, *i.e.* within the range previously found for this enzyme[21,23].

DISCUSSION

Maximum and reproducible activation of $(Na^+ + K^+)$-ATPase can be obtained within 30 min of incubation with low concentrations of detergent if the conditions for incubation are carefully controlled. The results suggest that the existence of aggregates or micelles in the detergent solution is necessary for this activation. The $(Na^+ + K^+)$-ATPase activity remains associated with membrane structures after differential centrifugation[7] or zonal centrifugation[9], whereas inactive protein or lipoprotein is removed and rendered soluble by the detergent. The membrane structure seems to have changed irreversibly, since the enzyme activity is retained after resuspension of the membranes in media devoid of detergent[9].

The content of deoxycholate is so low in the preparation of membranes with a high specific activity of $(Na^+ + K^+)$-ATPase obtained by zonal centrifugation that it is unlikely that the activation is due to binding of deoxycholate to the membranes. This is in agreement with the observation of CHAN[5] that the change in alkali metal activation of erythrocyte ATPase after incubation with sodium dodecyl sulfate is not due to binding of the detergent molecules to the membranes. It seems therefore that the important event in the action of the detergents is the removal of protein and lipid from the membrane and not penetration of detergent molecules into the membrane structure.

It is known that the plasma membrane of the cells can be transformed into

vesicular structures during the homogenization procedure[24]. The ($Na^+ + K^+$)-ATPase requires an effect of K^+ from one side of the membrane, in the intact cell the outside, and an effect of Na^+ and of Mg^{2+} and ATP from the other side, the inside of the membrane[1]. In view of this it seems a likely possibility that the activation of ($Na^+ + K^+$)-ATPase by detergents can be due to an increase in the permeability of the membranes or to opening of the vesicles resulting in free access of substrate and activators to their respective sites on the membrane.

The hypothesis that the effect of deoxycholate is to expose latent enzyme sites in the preparation is supported by the observation that the molecular activity of ($Na^+ + K^+$)-ATPase is not changed by treatment with detergent. Similar results have been obtained with preparations from ox brain treated with increasing concentrations of deoxycholate[16]. In contrast to this, ELLORY AND SMITH[25] suggested that the effect of deoxycholate is to increase the molecular activity of the enzyme. They found that incubation of preparations from goldfish intestine with high concentrations of deoxycholate increased the molecular activity of ($Na^+ + K^+$)-ATPase from 2000 to 8000 P_i per min. This discrepancy may be explained by differences in the nature of the preparations. The increase in the specific activity of ($Na^+ + K^+$)-ATPase in the preparation from goldfish intestine is small, namely 1.3-fold and the treatment with detergent causes a decrease in the total ATPase activity. In the present experiments the total ATPase activity increases in parallel with the 5–6-fold increase in ($Na^+ + K^+$)-ATPase activity obtained by treatment with deoxycholate under optimum conditions.

It has been suggested that the effect of deoxycholate can be explained by a conversion of the Mg^{2+}-ATPase to the ($Na^+ + K^+$)-activated enzyme[26,27]. This is not in agreement with the present observations because the detergents can increase the activity of ($Na^+ + K^+$)-ATPase several-fold without noticeable changes in the activity of Mg^{2+}-ATPase. The distribution of the Mg^{2+}-ATPase in the density gradient is widened by treatment with deoxycholate, whereas the distribution of ($Na^+ + K^+$)-ATPase is the same whether or not the microsomal fraction is treated with deoxycholate before the zonal centrifugation. This suggests that at least part of the extramitochondrial Mg^{2+}-ATPase is bound to particles other than the membranes which possess the ($Na^+ + K^+$)-ATPase activity.

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