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STUDY OF HUMAN RED BLOOD CELL MEMBRANE USING SODIUM DEOXYCHOLATE

II. EFFECTS OF COLD STORAGE, EDTA AND SMALL DEOXYCHOLATE CONCENTRATIONS ON ATPase ACTIVITIES

J. PHILIPPOT and M. H. AUTHIER

Laboratoire de Physico-chimie biologique, Institut de Biologie, 34 Montpellier (France)

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SUMMARY

1. (Na^+-K^+)-ATPase is activated by small deoxycholate concentrations if the membrane preparation has been made in the absence of EDTA, but is not activated if EDTA was present during red cell hemolysis. The activity maximum in the absence of EDTA is never very different from that with EDTA. Storage for more than 24 h at 4 °C or 10 days at −20 °C of membranes prepared with EDTA has an effect comparable to omitting deoxycholate treatment.

2. This maximum occurs at the pH, temperature and concentration of micelle formation in pure deoxycholate solutions. The critical micelle concentration was not changed by the protein concentrations used in our experiments. Micelles appear necessary for enzyme reactivation.

3. This activation by deoxycholate cannot be explained by conversion of ouabain-insensitive ATPase into (Na^+-K^+)-ATPase since increase in one activity is always accompanied by increase in the other. It is tentatively concluded from the detergent action on the membranes that the main effect of deoxycholate is an unmasking of latent ATPase activities.

4. Deleting addition of EDTA during activity determination reveals a high ouabain-insensitive ATPase activity for membranes prepared in the absence of EDTA without changing (Na^+-K^+)-ATPase activity. The simultaneous presence of these two phenomena excludes any activation by free Ca^{2+}. The possible nature of the ouabain-insensitive enzyme is discussed.

INTRODUCTION

Sodium deoxycholate is often used to purify (Na^+-K^+)-ATPase from plasma membrane preparations. The effect of deoxycholate on (Na^+-K^+)-ATPase (EC 3.6.1.3) activity varies with concentration of the bile salt[1–6]. The enzyme is progressively inactivated as deoxycholate concentration rises above 2 mg/ml. This

Abbreviations: cmc, critical micelle concentration.

inactivation corresponds to a solubilizing of the membrane–lipoprotein complex into its two components and an association of phospholipid to deoxycholate in a ratio of 1:13 (refs 1 and 2). On the other hand, the enzyme activity may be markedly enhanced by small deoxycholate concentrations without measurable membrane solubilization[3–6].

The interpretation of this stimulation varies with authors and the tissue studied. According to Ellory and Smith[7] the activation of $(Na^+–K^+)$-ATPase from goldfish intestine is due to an increase in the molecular activity of the enzyme under the combined effect of temperature (37 °C, about 30 °C higher than in the fish habitat) and of deoxycholate. According to Jørgensen and Skou[8] on the outer medulla of rabbit kidney, as for Chan[9] on human red cells, the activation is caused by the dispersing action of the detergent (deoxycholate or dodecyl sulfate), which exposes latent enzymes sites in the membrane fragments.

It was recently shown, however, that the osmolarity and nature of ions present during hemolysis of the erythrocytes, as well as the duration of storage, can change the morphology of membranes. Small osmolarities (< 10 ideal mosM) fragments membranes[10–12] but conversely, small Ca^{2+} or Mg^{2+} concentrations prevent this disaggregation[11–13].

The purpose of the present study was to determine if stimulation of $(Na^+–K^+)$-ATPase activity by small deoxycholate concentrations is related to state of the membranes after different techniques of preparation or after different periods and conditions of storage. We also wanted to settle the question of the existence of a deoxycholate-stimulated ouabain-insensitive ATPase activity in absence of any EDTA treatment. Some hypotheses will be considered on the nature of this activity in the context of the existence of an $(Na^+–K^+)$-ATPase enzyme.

MATERIALS AND METHODS

Incubation of membrane preparations with deoxycholate

Blood was collected on citrate (5.2–6.5 mg/ml) and used the same day or after storage overnight at 4 °C for membrane preparation.

Plasma membrane preparation followed the method of Post *et al.*[14]. In some experiments EDTA was added to a final concentration of 1.0 mM to the hypo-osmotic solution used for red cell hemolysis. These preparations (pH 8.0) were kept either at 4 °C for storage of one week or less or at −20 °C for storage of several weeks. A 241 mM sodium deoxycholate (Fluka, Buchs, Switzerland) stock solution was kept at room temperature, pH 8.1–8.3.

Incubations were done with different concentrations of protein and deoxycholate at varying pH and temperature as specified in the figure legends. In some experiments 2 mM EDTA was present. Samples were preincubated 30 min before enzyme activity measurement.

Measurement of ATPase activity and other chemical determinations

The sample to be analyzed (1.9–5.3 mg/ml protein except for Table III) is incubated 20–60 min at 37 °C in a 2.5-ml solution of 60 mM histidine–imidazole, pH 7.1, 120 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM ATP (as disodium salt) and, in some cases, 0.33 mM EDTA. An identical incubation is made in the presence

of 0.08 mg/ml ouabain (Merck), specific inhibitor of (Na^+–K^+)-ATPase. The reaction is stopped by adding 2.5 ml iced 10% trichloroacetic acid. After centrifugation (Sorvall RC 2B centrifuge, 15 min at 20000 × *g*), 2 or 4 ml supernatant was taken for inorganic phosphate determination according to the method of Beremblum and Chain[15].

(Na^+–K^+)-ATPase activity (ouabain-sensitive activity) in μmoles inorganic phosphate/h per mg protein is equal to the difference in measured activities of the sample in presence (ouabain-insensitive activity) and absence of ouabain (total activity).

Proteins were determined by the Lowry technique[16], with crystallized bovine serum albumin as standard. Measurement of lipid phosphorus followed the method of Bartlett[17] after extraction by chloroform–methanol (2:1,v/v) and perchloric digestion, according to Bradford *et al.*[18]. In two cases the deoxycholate present did not modify the results. Deoxycholate was measured by spectrophotometry at 310 nm, using a technique derived from Singer and Fritschen[19]. Neither proteins nor phospholipids interfered.

Determination of critical micelle concentration

Surface tension and absorption measurements were used to determine critical micelle concentration. Surface tension was measured with a platinum ring and a Du Nouy tensiometer (Simplin model, Jobin-Yvon, France) or with a thin mica plate and a tensiometer made at Montpellier. The first apparatus has a constant temperature jacket which allows measurements at 1, 22 and 36 °C; it was calibrated with weights. Between measurements the platinum ring was thoroughly rinsed and heated to incandescence in a Bunsen flame. The second apparatus, of higher sensitivity, was used only at 22 °C.

The absorption spectrum of certain dyes is changed by their interaction with micelles. This property was also used for determining critical micelle concentration of deoxycholate. Above this critical concentration the absorption peak for methyl orange (4'-dimethylaminoazobenzene-4-sodium sulfonate, R.A.L., Kuhlmann) shifts toward shorter wavelengths giving a maximum absorbance change measured at 484 nm, according to the method of Benzonana[20] with a Beckman DK-2A recording spectrophotometer using thermostated cells of 1 cm optical length. In all cases the solutions to be analyzed were prepared at least 30 min before measurement and the samples remained at the selected temperature throughout the experiment. For each pH and temperature the surface tension or variation in methyl orange absorbance at 484 nm was expressed as a function of log deoxycholate concentration. In two cases the curves showed a sharp break at the onset of micelle formation. The critical micelle concentration corresponded to the sudden slope change on the curves[21].

RESULTS

ATPase activity for membranes prepared with or without EDTA as a function of deoxycholate treatment and storage time

Fig. 1 shows activities obtained on red cell membranes incubated with various deoxycholate concentrations at 22 °C and pH 7.4. Three different samples of blood

were used, each of which provided two membrane preparations, one hemolysed in presence of EDTA, the other without EDTA. Each point in Fig. 1 is thus the average from three identical experiments. 100% activity is defined as the (Na^+-K^+)-ATPase activity of membranes prepared with EDTA the same day as the determination and incubated with deoxycholate.

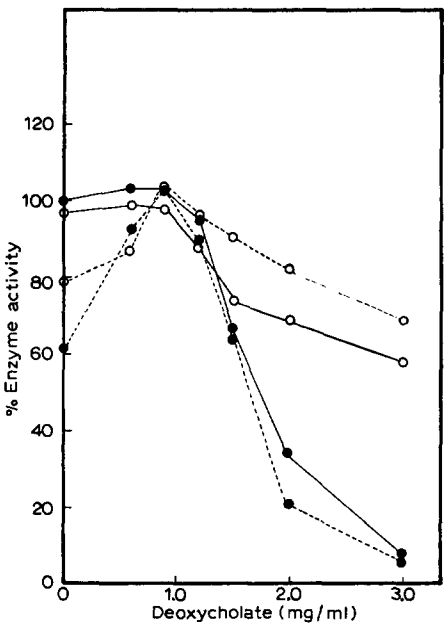

Fig. 1. (Na^+-K^+)-ATPase and ouabain-insensitive ATPase activities on red cell membranes prepared with or without EDTA as a function of deoxycholate concentration. Samples of membranes prepared the same day were preincubated with indicated deoxycholate concentrations, pH 7.4, 22 °C, in a final volume of 0.5 ml. Protein concentration varied from 2.5 to 5.3 mg/ml. After 30 min preincubation ATPase activities were determined as in Materials and Methods on 2 samples of 0.2 ml, one in presence and one in absence of ouabain, with 0.33 mM EDTA present in both cases. Each point is the average of 3 determinations, each determination being on a different preparation. ●, (Na^+-K^+)-ATPase; ○, ouabain-insensitive ATPase; ——, membranes prepared with EDTA; and - - - -, without EDTA.

The following phenomena are revealed by Fig. 1: (i) When deoxycholate is absent from the incubation medium the ouabain-insensitive ATPase and (Na^+-K^+)-ATPase activities of membranes prepared with EDTA are distinctly superior to those of membranes prepared without EDTA. (ii) Small deoxycholate concentrations (0–1.2 mg/ml) do not change the activities of membranes prepared with EDTA but do strongly increase the activities of preparations lacking EDTA. In this last case, however, the maximum activities observed are never very different from those of EDTA-prepared membranes. (iii) In all cases the activities are inhibited at deoxycholate concentrations above 1.2 mg/ml; this is especially so for (Na^+-K^+)-ATPase. The action of the detergent on the enzymes can be described by a curve having a slope sign change which always occurs near the same deoxycholate concentration, 0.9–1.2 mg/ml.

The effect of 4 °C and −20 °C storage on the (Na^+-K^+)-ATPase

activity after incubation is seen from Table I. In the absence of deoxycholate the activity remains at the level found immediately after membrane preparation with or without EDTA for 7–10 days storage at −20 °C but only for 24 h at 4 °C. Afterwards the activity decreases slowly with −20 °C storage (85–90% activity after 2 weeks, 70–75% after 3 weeks, 60–65% after 4 weeks) and rapidly with 4 °C storage. One notes also that weak concentrations of deoxycholate have practically no effect on membranes prepared with EDTA and stored at −20 °C while they do re-establish all or part of the activity lost by the same membranes when stored at 4 °C or of membranes prepared without EDTA.

Three further observations can be made from Fig. 1 and Table I: first, the absence of EDTA during red cell hemolysis seems to have the same effect on (Na^+–K^+)-ATPase as storage at 4 °C for membranes prepared with EDTA. The absence of EDTA[10], as does prolonged storage at 4 °C of fragmented particles[22,25], tends to favor the formation of aggregates. It would seem, therefore that the state of the membranes in the system is one of the factors that must be taken into account in interpreting these results (see Discussion).

TABLE I

EFFECT OF 4 °C AND −20 °C STORAGE ON (Na^+ −K^+)-ATPase ACTIVITY

Each result in this table is the average for three experiments on samples stored at 4 °C and for four experiments on samples stored at −20 °C, using different lots of red cells. For each experiment two membrane preparations were made, one after hemolysis with EDTA, the other after hemolysis in the absence of EDTA. The two preparations were studied simultaneously, after thawing at 4 °C if necessary. Samples were preincubated 30 min at 22 °C, pH 7.4 and their activities then determined in the absence or in various concentrations of deoxycholate (final protein concentration 1.9–5.3 mg/ml). The maximum activity with deoxycholate present (0.9–1.2 mg/ml) appears only in the table, followed by the mean optimum deoxycholate concentration, between parentheses. 100% activity is taken as the (Na^+–K^+)-ATPase activity of membranes prepared with EDTA and incubated the same day in the absence of deoxycholate.

Days storage		*% Activity*			
4 °C	*−20 °C*	*Hemolysis with EDTA*		*Hemolysis without EDTA*	
		No deoxycholate	*With deoxycholate*	*No deoxycholate*	*With deoxycholate*
0		100	104 (0.9)	60	101 (0.9)
1		97	96 (0.9)	47	97 (1.0)
3		51	81 (1.1)	34	85 (1.2)
6		20	59 (1.2)	37	79 (1.1)
9		19	61 (1.2)	36	67 (1.2)
14		19	48 (1.0)	36	46 (0.9)
	0	100	108 (1.1)	38	106 (1.1)
	1	98	107 (1.1)	44	103 (1.1)
	3	97	109 (1.1)	51	109 (1.1)
	7	98	97 (1.2)	52	101 (1.1)
	10	85	93 (1.2)	43	105 (1.1)
	15	86	91 (1.1)	51	90 (1.2)
	20	77	73 (1.1)	31	69 (1.1)
	28	65	59 (1.1)	19	62 (1.2)

Second, it appears clear that the action of deoxycholate is to restore to the membranes the activity they would have had if they had been prepared with EDTA and had not been stored. At −20 °C complete activity restoration is still possible after 2 weeks, but at 4 °C this is only valid for the first few days of storage because of bacterial contamination.

Third, the above results show that the activity is maximal at the same deoxycholate concentration, whatever the mode of membrane preparation or the quantity of membrane incubated (1.9–5.3 mg protein/ml). For this maximum there does not seem to be a constant stoichiometric relation between the quantities of deoxycholate and membrane, as there is for solubilization of these same membranes[2]. Consequently, the increase in activity could be linked to the physical state of the detergent solutions.

Possible relation between (Na^+–K^+)-ATPase activation and critical micelle concentration of deoxycholate

Small[23], Hofmann and Small[24] and Benzonana[20] studied the size and structure of micelles of the different bile acids and showed that the critical concentration for appearance of these micelles depends on temperature and pH. Although they did not have exactly the same experimental conditions as those used here, these authors find critical micelle concentrations (0.8–2.4 mg/ml by Hoffmann, 1.0–1.2 mg/ml by Benzonana) near the deoxycholate concentrations corresponding to the different maxima of Fig. 1 and Table I. A relation between maximum deoxycholate effect on the enzyme and appearance of micelles therefore seemed possible. The existence of this relationship is supported by comparing the deoxycholate concentrations for maximum enzyme activation and the critical micelle concentrations of pure solutions of the detergent, measured at different pH and temperature.

TABLE II

DEPENDENCE OF DEOXYCHOLATE CONCENTRATION AT MAXIMUM (Na^+–K^+)-ATPase ACTIVITY ON pH OF THE PREINCUBATION MEDIUM

For each pH value the samples of red cell membranes prepared without EDTA were incubated (final protein concentration 3–4.8 mg/ml) for 30 min at 22 °C with different concentrations of deoxycholate (0–2 mg/ml) and 24 mM imidazole adjusted to the desired pH with HCl. The activities were determined as in Fig. 1, affording deoxycholate concentrations at the maximum activity. Different membrane preparations were made for each experiment and used after different storage times.

Expt No.	*Days storage*		*Deoxycholate concentration (mg/ml) at maximum activity*			
	4 °C	*−20 °C*	*pH 6.85*	*pH 7.0*	*pH 7.4*	*pH 7.95*
1	1		0.8	1.0	1.2	1.4
2	1		0.6–0.9	0.8–1.1	1.0–1.2	1.3–1.4
3	1		0.9	1.0	1.0–1.2	1.6
4	2	12	0.8	1.0	1.0–1.2	1.4–1.6
5	1	210	0.8	1.0–1.2		
Mean ± S.E.			0.81 ± 0.024	1.01 ± 0.024	1.12 ± 0.025	1.46 ± 0.17
			$P < 0.001$	$P \sim 0.01$	$P < 0.001$	

TABLE III

INFLUENCE OF TEMPERATURE ON THE DEOXYCHOLATE CONCENTRATION AT MAXIMUM (Na^+–K^+)-ATPase ACTIVITY

The activity of red cell membranes prepared without EDTA was determined after 30 min incubation (pH 7.0) with different concentrations of deoxycholate (0–2 mg/ml) at three different temperatures. The protein concentration in preincubation medium varied from 3.1 to 6.5 mg/ml in Expts 1–5, and from 1.0 to 10.0 mg/ml in Expt 6. A membrane preparation was made for each experiment and used after storage (at 4 °C or –20 °C).

Expt No.	*Days storage*		*Protein (mg/ml)*	*Concentration deoxycholate at maximum activity (mg/ml)*		
	4 °C	*−20 °C*		*0 °C*	*22 °C*	*37 °C*
1	1		6.5	1.6	1.2	0.8–1.0
2	2		3.1		0.9–1.0	0.6–0.9
3	2		4.1	1.6	1.0–1.2	0.6
4	16		5.2	1.6–2.0	1.0–1.2	0.6–1.0
5	3	12	3.2	1.6	1.0	0.8–1.0
6	1	120	1.0		1.0–1.2	
			1.0		1.0	
			3.9		1.0–1.2	
			5.0		1.0	
			5.1		1.2	
			6.5		1.0–1.2	
			10.0		1.0–1.2	
Mean ± S.E.	1.65 ± 0.05				1.08 ± 0.03	0.79 ± 0.08
				$P < 0.001$	$P < 0.001$	

Tables II and III show deoxycholate concentration at maximum (Na^+–K^+)-ATPase activity when pH (Table II) or temperature (Table III) varies in the incubation medium. In this investigation membranes prepared without EDTA were stored for different times at 4 °C or −20 °C and in each experiment a curve of enzyme activity variation with deoxycholate concentration was developed for each pH and temperature.

In Table II one sees that this deoxycholate concentration shifted toward smaller values as pH decreased from 7.95 to 6.8. Below pH 6.8 measurements were impossible because of precipitation and gelation. In Table III the deoxycholate concentration shifted toward smaller values as temperature increased from 0 to 37 °C.

For each of the experiments reported in these two tables the maximum enzyme activity varied little with pH or temperature. There were, however, sizeable drops in activity at pH 7.95 and at 0 °C (14% and 22%, respectively, from maximum activity, on the average). As with Table I, one finds that storage time at 4 °C or −20 °C or protein concentration (Expt 6, Table III) did not change the deoxycholate concentration for maximum activation.

Systematic determinations of critical micelle concentration were therefore done at different pH and temperature. Two techniques were used, surface tension

and light absorption, as given in Materials and Methods. As a preliminary step it was verified that the protein concentrations taken in our experiments did not change the deoxycholate critical micelle concentration; this is shown in Fig. 2. Typical curves of surface tension or absorption are shown in Fig. 3 where critical micelle concentration is estimated at 1.8–2.2 mg/ml by surface tension (1 °C, pH 7.0) and 2.0–2.5 mg/ml by absorption measurements (6 °C, pH 7.0). Tables IV and V give the full results.

With both techniques critical micelle concentration increased with pH at constant temperature and decreased when the temperature was raised at constant pH. The concentration value given by absorption was generally slightly higher than that from the surface tension measurements for temperatures of 28 °C and lower; at 36–37 °C the opposite was true.

Thus Tables II, III and IV show good agreement between the deoxycholate concentration for maximum (Na^+–K^+)-ATPase activation and the critical micelle concentration of pure solutions of the bile salt. The role of surface tension in this

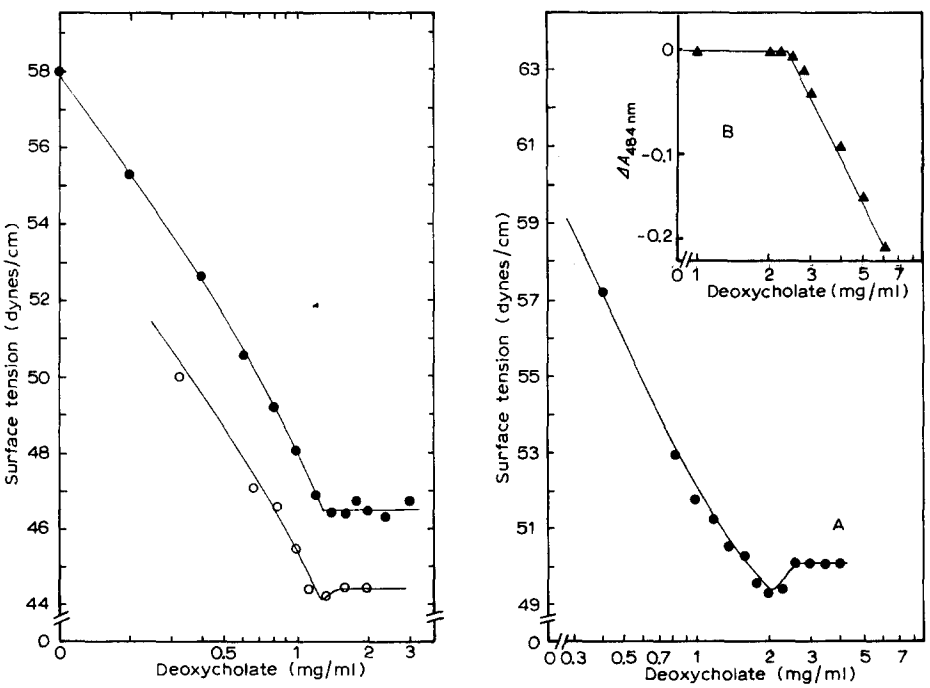

Fig. 2. Surface tension of aqueous solutions containing (○) or not containing (●) 4.2 mg/ml protein as a logarithmic function of deoxycholate concentration. Surface tension was measured as described in Materials and Methods after incubation at 22 °C for at least 30 min. Solutions contained 24 mM imidazole (pH 7.0).

Fig. 3. Graphic determination of critical deoxycholate concentration for micelle formation from surface tension or light absorption measurements. Curve A shows variation of surface tension of pure deoxycholate solutions at 1 °C (24 mM imidazole, pH 7.0) with deoxycholate concentration in log scale. Curve B is from absorbance measurements at 6 °C with 24 mM imidazole (pH 7.0) and 40 μM methyl orange. Blanks (*minus* deoxycholate) had this same composition.

activation does not appear important. In fact we observe (Table V), as have other authors[8], that maximum activation can occur at a variety of values of surface tension. We conclude that maximum stimulation of (Na^+–K^+)-ATPase activity by deoxycholate depends on micelle formation.

TABLE IV

CRITICAL MICELLE CONCENTRATION OF PURE DEOXYCHOLATE SOLUTION AT DIFFERENT pH AND TEMPERATURES FROM SURFACE TENSION OR LIGHT ABSORPTION MEASUREMENTS

For each determination the surface tension was plotted as shown on Fig. 3A. The critical micelle concentration (cmc) lies within the concentration range where surface tension changes from lowest to constant value. The samples, adjusted to selected pH with a 24 mM imidazole–HCl buffer, were kept at constant temperature (±0.1 °C) during the whole experiment. After surface tension determination the pH was never more than 0.05 pH unit from the initial value. Results are averages of three consecutive measurements on each 10-ml sample. To determine cmc by light absorption, the difference in absorbance at 484 nm (ΔA) between a pure solution of methyl orange (40 μM in 20 mM Tris–HCl buffer) and the same solution with different deoxycholate concentrations was plotted against deoxycholate concentration. The cmc in this case is within the range of detergent concentration where ΔA changes from zero to a progressively negative value (Fig. 3B). pH variation was never more than 0.1 pH unit during this measurement. Other conditions as in Materials and Methods.

Temp. (°C)	*Method*	*cmc (mg/ml)*		
		pH 7.0	*pH 7.4*	*pH 7.95*
1	Surface tension	1.8–2.2	2.0–2.3	2.2–2.5
6	Light absorption	2.0–2.5	2.3–2.7	2.8–3.8
22	Surface tension	1.2–1.4	1.3–1.8	1.5–1.8
28	Light absorption	1.5–2.0	1.7–2.2	1.9–2.3
36	Surface tension	0.9–1.2	1.2–1.5	1.7–1.9
37	Light absorption	0.6–1.0	1.0–1.5	1.4–1.9

TABLE V

SURFACE TENSION OF DEOXYCHOLATE SOLUTIONS AT THE CRITICAL MICELLE CONCENTRATION

The data of this table were obtained from the same experiments as in Table IV. For each pH and temperature two values are given. These are the surface tensions at the extremes of the concentration range for micelle formation.

Temp. (°C)	*Surface tension at cmc (dynes/cm)*		
	pH 7.0	*pH 7.4*	*pH 7.95*
1	49.2–50.1	50.9–51.2	50.7–51.4
22	46.7–47.8	48.2–48.7	49.8–50.1
36	46.3–46.6	47.2–47.7	48.8

Simultaneous variation of ouabain-sensitive and ouabain-insensitive activity as a function of deoxycholate concentration

By the action of deoxycholate on preparations from the outer medulla of rabbit kidney Jørgensen and Skou[8] showed that the detergent did not affect ouabain-insensitive ATPase activity while ouabain-sensitive activity could be increased several-fold. Chan[9] obtained different results by treating erythrocyte membranes with sodium dodecyl sulfate, which increases ouabain-insensitive activity without appreciably changing that of (Na^+–K^+)-ATPase.

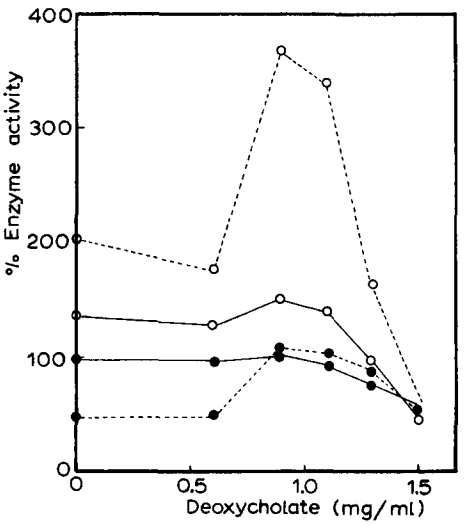

Fig. 4. Same as Fig. 1 except no EDTA addition at activity determination. Average protein concentration of samples 2.7 mg/ml. Each point is the average of four determinations, each determination being on a different preparation. ●, (Na^+–K^+)-ATPase; ○, ouabain-insensitive ATPase; ——, membranes prepared with EDTA; - - - -, without EDTA.

TABLE VI

(Na^+–K^+)-ATPase ACTIVITY AS PERCENTAGE OF TOTAL ATPase ACTIVITY

The percentage of (Na^+–K^+)-ATPase activity was calculated from the results of Figs 1 and 4, in the absence of deoxycholate in the incubation medium and at the maximum activity in presence of detergent (0.9–1.2 mg deoxycholate/ml). The results account for the presence of EDTA during hemolysis and activities determination.

Hemolysis with EDTA	*% (Na^+–K^+)-ATPase activity*			
	Activity measurement with EDTA		*Activity measurement without EDTA*	
	No deoxycholate	*With deoxycholate*	*No deoxcholate*	*With deoxycholate*
+	51	52	41	40
-	44	49	19	22

In fact, variation of (Na^+-K^+)-ATPase and ouabain-insensitive activities of red cell membranes with small deoxycholate concentrations depends on the presence of EDTA during hemolysis as well the activity determination. Figs 1 and 4 and Table VI give results on membranes analysed within 24 h of preparation. The experiments of Fig. 4 were done under the same conditions as those of Fig. 1 except for the addition of EDTA at the time of activity determination. The 100% reference is the same in both figures. Comparison of Figs 1 and 4 shows that EDTA absence at the time of the determination did not affect (Na^+-K^+)-ATPase activity whether or not the preparation contained deoxycholate or has been treated with EDTA. But EDTA absence at the determination considerably increased ouabain-insensitive activity of membranes not previously treated with EDTA; this went from 60% activity to 200% activity in the absence of deoxycholate and from 100% activity to 370% activity with deoxycholate of critical micelle concentration. Only the ouabain-insensitive enzyme, therefore, is affected by EDTA. On the other hand, upon deoxycholate treatment increase in one activity is always accompanied by increase in the other. Table VI shows that, whether or not EDTA is used in the preparation or added at the time of activity determination, the ouabain-sensitive and insensitive activity ratio changes little upon deoxycholate action. Consequently, one concludes that micelles stimulate both (Na^+-K^+)-ATPase and ouabain-insensitive ATPase activities. Therefore the hypothesis, proposed by some authors[5,25] for other types of cells, that one activity can be transformed into the other must be rejected.

DISCUSSION

It is well known that physical and chemical properties, especially enzymatic properties, of membranes, vary with method of preparation of the membranes[26,27]. Recently, Bramley *et al.*[11], Hanahan and Ekholm[12] showed that ATPase activity of red cell membranes depends on the state of fragmentation of the membranes, which in turn depends on preparation techniques. Thus (Na^+-K^+)-ATPase activity increased as membrane fragmentation increased. Bramley *et al.*[11] obtained this result with osmolarities below 20 imosM while Hanahan and Ekholm[12] did so by successive washing of membranes prepared at 20 imosM. In both cases the authors noted protein loss and change in membrane structure. Conversely, with membranes prepared in high osmolarity (80 imosM), therefore less fragmented, it was necessary to sonicate preparations or to treat them with a detergent to obtain elevated (Na^+-K^+)-ATPase activity[11].

A number of authors have described the role of divalent ions in maintaining the lipoprotein structure of membranes. Thus Bramley *et al.*[11] reconstituted acetylcholinesterase activity (measurable only on non-disaggregated ghosts) by small additions of Ca^{2+} to very dilute, hence very fragmented, preparations. This resealing process was also observed by Bodemann and Passow[13] and Duchon and Collier[10] on human red cell ghosts. In general, therefore, divalent ions oppose the effect of dilution.

We observed (Fig. 1) that the absence of EDTA during hemolysis of erythrocytes prepared in low osmolarity gave membranes whose (Na^+-K^+)-ATPase

activity was 40% less than when prepared with EDTA. This difference disappeared, however, upon the addition of small amounts of deoxycholate (which produced little variation in activity of the membranes prepared with EDTA).

By analogy with the above reports, the maximum activity in Fig. 1 should correspond to a state of fragmentation in membranes commensurate with the properties of (Na^+-K^+)-ATPase, deoxycholate and EDTA. (i) The enzyme indeed possesses one of its active sites on the inner membrane, whose substrate accessibility increases with fragmentation of the ghosts. It seems, then, that membrane fragmentation corresponding to maximum activity is obtained in one case by the action of EDTA (membranes prepared with EDTA) and in the other by the action of deoxycholate (membranes prepared without EDTA). (ii) The deoxycholate action in Fig. 1, analyzed below, is to expose latent enzyme sites on (Na^+-K^+)-ATPase by membrane fragmentation and not by activation in the usual enzymology sense. This crypticity (Duchon and Collier[10]) of (Na^+-K^+)-ATPase was not found after EDTA treatment. (iii) The exact mode of action of EDTA was not pursued but it probably fragments ghosts by complexing divalent ions fixed on the membranes, ions which assure the cohesion of the membrane edifice. Marchesi *et al.*[28] have shown that EDTA permits solubilization of part of the proteins of red cell membranes. We have noted that membranes prepared with EDTA were much less colored by hemoglobin, had lower viscosity and contained much less fixed Ca^{2+} (2.54 ± 0.58 *vs* 4.06 ± 0.96 μg Ca^{2+}/mg dry residue) agreeing with the above work of Dodge *et al.*[26], Duchon and Collier[10] and with that of Porzig[29] and Long and Mouat[30] on fixed Ca^{2+}.

Similarly, the results of Table I can be interpreted as were those of Fig. 1 by referring to the state of fragmentation of the membranes, at least for the first 2–3 days storage at 4 °C or the first 1–2 weeks at −20 °C. It seems that during these periods some aggregation of fragmented membranes in suspension occurs, since it is necessary to add deoxycholate to recover maximum activity.

The action of deoxycholate on membranes appears linked to its molecular state in the solution. Comparison of Tables II, III and IV shows that maximum enzyme activity always occurs in the concentration zone for micelle formation, whatever the mode of preparation of the membranes (Figs 1 and 4), storage, or protein concentration (Table III, Fig. 2). Jørgensen and Skou[8] also found agreement between maximum activation by deoxycholate and critical micelle concentration but their concentrations are surprisingly lower than those of Table IV or of those recently measured[20,23,24]. All enzyme activities progressively diminish above the critical micelle concentration.

These apparently conflicting results can be interpreted as being a consequence of the double action of deoxycholate: detergency or disaggregating effect and solubilization or complexing effect. The two effects can be distinguished and their relative importance varies with concentration. Adamson[21] shows that detergency effect rises sharply below the critical micelle concentration and remains practically constant above it, while solubilization of otherwise insoluble organic molecules, such as membrane phospholipids, is seen only above the critical micelle concentration. It appears, therefore, that deoxycholate acts in at least two modes as its concentration increases. Below the critical micelle concentration the bile salt disaggregates fragments; above it there is in addition solubilization of phospholipids

by mixed micelle formation[2]. The detergency property is associated with the monomer ion of anionic detergent[21] while the solubilizing property originates from the hydrophobic part of the deoxycholate molecule[31].

In addition, Figs 1 and 4 and Table VI show that activity variations of the two enzymes produced by deoxycholate are always of the same nature, which ruins the hypothesis that deoxycholate can convert ouabain-insensitive ATPase into (Na^+–K^+)-ATPase and *vice versa.*

However, Fig. 4 is especially interesting because in the presence of Na^+ and K^+ it shows a large ouabain-insensitive activity which is observed only in the total absence of EDTA. This activity is nearly doubled by deoxycholate action, and this is consistent with present knowledge on membranes prepared without EDTA, but the activity is strongly decreases by the addition of EDTA at the time of activity determination (Fig. 1). This activity seems to correspond to that Chan[9] found by using sodium dodecylsulfate on red cell membranes prepared without EDTA. However, comparison between the actions of dodecylsulfate and deoxycholate is difficult since there is no reference activity in Chan's work. In addition his results for the effect of sodium dodecylsulfate on (Na^+–K^+)-ATPase are confusing: 0.2 mM dodecylsulfate caused quite different variations (−15, +51 and −33%) from the activity he measured in absence of this detergent.

Comparison of Figs 1 and 4 indicates that omitting EDTA at the activity determination does not change (Na^+–K^+)-ATPase activity. This could be expected for membranes already treated with EDTA but not for membranes which had had no EDTA contact. This shows, then, that in these experiments (Na^+–K^+)-ATPase activity is completely independent of EDTA action, consequently that EDTA affects only the ouabain-insensitive activity.

It is presently impossible to say if ouabain-insensitive ATPase is in fact Mg^{2+}- or Ca^{2+}-ATPase or another enzyme. Some hypotheses for the action of EDTA on this enzyme can, however, be advanced. First, EDTA can complex Ca^{2+} since identical results are obtained if ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetra-acetic acid (EGTA) replaces EDTA in Fig. 4. But these ions cannot be free, if they were (Na^+–K^+)-ATPase inhibition would be observed[32] but it is not. Thus it would be necessary to conclude that the enzyme is activated by ions fixed on the membrane, which seems highly unlikely. Another possibility is that a large part of the enzyme becomes detached from the membrane through solubilization of proteins by EDTA[28]. This would require that the enzyme lose activity by the solubilization, also that it be very loosely bound to the membrane since the activity of (Na^+–K^+)-ATPase, an enzyme highly sensitive to alteration of the membrane, does not vary. Another hypothesis is that EDTA is competitively fixed to the membrane, playing the role of an inhibitor. Studies in progress will seek to characterize this enzyme and elucidate the action of EDTA on it.

Thus EDTA is needed for studying (Na^+–K^+)-ATPase if deoxycholate use is to be avoided, but EDTA makes ouabain-insensitive ATPase activity study impossible. This summarizes rather well the complexity of the present membrane research. Our results, like those of Bramley *et al.*[11] and Hanahan and Ekholm[12] show the precautions necessary in undertaking determinations of structure or properties of membranes.

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