BBA 75326

DEOXYCHOLATE STIMULATION OF GOLDFISH INTESTINAL ($Na^+ + K^+$)-ATPase AND ITS RELATION TO DIGOXIN BINDING

J. C. ELLORY AND M. W. SMITH

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge (Great Britain)

(Received April 24th, 1969)

SUMMARY

1. Low concentrations of deoxycholate inhibit Mg^{2+}-activated ATPase, enhance the ($Na^+ + K^+$)-activated ATPase and leave unchanged the total ATPase activity of goldfish intestinal membrane preparations.

2. Tritiated digoxin has been shown to bind to the membrane preparation. A large part of the total digoxin bound (approx. 65 %) was dependent on the presence of $Mg^{2+} + Na^+ + ATP$, and this fraction has been taken to represent digoxin specifically bound to the ($Na^+ + K^+$)-activated ATPase.

3. The deoxycholate-induced changes in ($Na^+ + K^+$)-ATPase activity are not accompanied by parallel changes in digoxin binding.

4. There is a marked increase in the catalytic centre activity of the ($Na^+ + K^+$)-ATPase at high deoxycholate concentrations (1.25–2.5 mM).

5. It is concluded that deoxycholate inhibition of Mg^{2+}-ATPase is not associated with its transformation to the ($Na^+ + K^+$)-activated enzyme. The deoxycholate activation of ($Na^+ + K^+$)-ATPase probably represents an increase in the effective turnover rate of a fixed number of enzyme sites.

INTRODUCTION

A membrane ATPase system isolated from the goldfish intestinal mucosa has been shown previously to vary its properties with the environmental temperature of the fish[1]. As the environmental temperature is raised the Mg^{2+}-ATPase activity increases while that of the ($Na^+ + K^+$)-ATPase decreases[2]. It may be that the conversion of one form of enzyme to another provides the means by which Na^+ transport is regulated in this tissue. Superficial evidence that such an interconversion might occur has been obtained recently by treating isolated membranes with deoxycholate. This detergent can, under certain conditions, increase the activity of the ($Na^+ + K^+$)-ATPase and decrease that of the Mg^{2+}-ATPase, without changing the total ATPase activity of the system[3]. The present work tests whether this increase in ($Na^+ + K^+$)-ATPase activity was associated with an absolute increase in the amount of enzyme present.

Cardiac glycosides inhibit ($Na^+ + K^+$)-ATPase by virtue of their high affinity for a K^+-activated site on the enzyme[4]. The use of radioactively labelled glycosides has recently shown that this binding is quantitatively related to the activity of the ($Na^+ + K^+$)-ATPase present[5–8]. Provided that experiments are rigorously controlled

and that it can be assumed that each molecule of glycoside binds only to one K^+-site, then the number of $(Na^+ + K^+)$-ATPase sites can be calculated from a knowledge of the amount of glycoside bound. Tritiated digoxin was used in this way in the present work to test whether deoxycholate could convert Mg^{2+}-ATPase to its $(Na^+ + K^+)$-activated form. In fact the results obtained ruled out this possibility and suggested an alternative explanation for the increase in $(Na^+ + K^+)$-ATPase activity.

MATERIALS AND METHODS

Storage of fish and preparation of membranes

Goldfish measuring 15–20 cm were obtained locally and were kept in aerated water for 3–4 weeks before use. They were fed once or, in the case of warm-adapted fish, twice daily with Duffields Anglers ground bait (Buxton Distributors, Norwich). The temperature of the water was maintained constant at values ranging from 3 to 32°, but most of the experiments were carried out using membranes prepared from fish acclimatised to 8°.

The procedure for killing fish and for collecting mucosal scrapings from the anterior intestine has already been described in detail[2]. Homogenisation was in 10 parts (v/w) of 0.25 M sucrose containing 5 mM EDTA, adjusted to pH 7.1 by the addition of Tris buffer. Each homogenate, containing the pooled mucosal scrapings from at least 4 fish, was centrifuged first at 10000 × *g* for 15 min to remove nuclei and most of the mitochondria. A second pellet was sedimented from the first supernatant by a further centrifugation at 20000 × *g* for 60 min. This pellet was suspended in fresh homogenisation medium and was again sedimented by centrifugation at 20000 × *g* for 60 min. All centrifugations were carried out in the cold using a Spinco model L preparative ultracentrifuge (rotor AH50). The final pellet, found previously to consist almost exclusively of membrane-like material[2], was resuspended in 6.5 ml of 10 mM histidine (pH 7.1) for immediate use.

Treatment of membranes with deoxycholate and subsequent incubation with tritiated digoxin

Aliquots of the membrane suspension, each containing about 1 mg of protein, were incubated with equal volumes of histidine or histidine–deoxycholate mixtures (pH 7.1) for 15 min at 37°. These mixtures were then diluted 10 times with ice-cold 10 mM histidine before centrifugation at 100000 × *g* for 15 min. Pellets were resuspended in 1.6 ml of 10 mM histidine, and aliquots were taken for protein and ATPase estimations. The remainder was incubated with tritiated digoxin.

Each membrane preparation was divided into two, half being incubated in 10 mM histidine, the rest in 3 mM Tris–ATP, 3 mM $MgCl_2$, 40 mM NaCl and 10 mM histidine (pH 7.1). About 40 μg of membrane protein were present in each case. Tritiated digoxin (specific activity 40 mC/mmole) was added to a final concentration of 3 μM, and the tubes were then incubated for 15 min at 37°. The optimal concentration of digoxin and the time for incubation to be used were determined separately in preliminary experiments. The reaction was stopped by the addition of 1 mM ouabain in 10 mM histidine, and the membranes were sedimented in the cold by centrifugation at 100000 × *g* for 15 min. The resulting pellets were twice resuspended in ouabain–histidine solution and recovered by centrifugation at 100000 × *g* for 15 min before being finally dispersed in

0.3 ml water. Part of this suspension was assayed for protein and the rest transferred quantitatively to counting vials. Nuclear-Chicago Solubilizer was added to each suspension followed by a toluene-based scintillator. The tritium label was counted after a 24-h storage in a Packard Tri-Carb scintillation spectrometer.

Analytical procedures

The ATPase activity of each microsomal fraction was determined by adding 0.1 ml, 30 mM Tris–ATP to 0.9 ml of solution containing 6 mM $MgCl_2$, 10 mM histidine (pH 7.1), 60–100 μg of membrane protein, with or without the addition of 40 mM NaCl *plus* 20 mM KCl. Incubation was stopped after 15 min at 37° by the addition of 0.1 ml of 50 % (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation at 1400 × *g* for 5 min. P_i was estimated[9] in 0.5-ml samples of the clear supernatants, and a correction was applied for the small amount of P_i present in samples incubated without enzyme. All estimations were carried out in duplicate.

Protein was estimated colorimetrically[10] using crystallised bovine serum albumin as standard.

Reagents

Tris–ATP was prepared from the disodium salt (Sigma Chemical Co., St. Louis) by passing 90 mM ATP (disodium salt) through a cation exchange column (Dowex 50) in the H^+ form. The effluent was adjusted to pH 7.1 with Tris, and the final solution was diluted to give 30 mM Tris–ATP which was stored at —18°. Deoxycholic acid was obtained from British Drug Houses, Ltd. (Poole, England). Digoxin, specifically labelled in the 12α position, was kindly supplied by Dr. R. Rutschmann of Sandoz A.G., Basle. All other reagents were of A.R. grade.

RESULTS

Deoxycholate-induced changes in membrane ATPase activity measured at different temperatures

The effect of deoxycholate on Mg^{2+}- and $(Na^+ + K^+)$-ATPase depends on the previous environmental temperature of the fish[3]. In initial experiments this relation was investigated in greater detail, and the temperature dependence of the deoxycholate effect was measured on different ATPase preparations. Table I shows the results obtained using membranes prepared from fish acclimatised to 3, 14, 23 and 32° incubated both at 32° and at their previous environmental temperatures. Deoxycholate inhibited the Mg^{2+}-ATPase. This inhibition was most noticeable when a high incubation temperature was combined with a high acclimatisation temperature. Deoxycholate had little effect on the $(Na^+ + K^+)$-ATPase incubated at temperatures equal to the previous environmental temperatures of the fish. However, deoxycholate did activate the $(Na^+ + K^+)$-ATPase when the incubation temperature exceeded the previous environmental temperature, conditions for maximal activation being a big difference between the environmental and incubation temperatures. It was decided on the basis of these results to confine future experiments to membranes prepared from cold-adapted fish used at a high incubation temperature.

TABLE I

ATPase activities of membrane fractions measured before and after treatment with 1.25 mM deoxycholate

Suspensions of membranes were incubated in 10 mM histidine or 10 mM histidine *plus* 1.25 mM deoxycholate. T_{acc}, temperature of acclimatisation; T_{inc}, incubation temperature. ATPase activities were determined afterwards in the presence of Mg^{2+}, with or without $Na^+ - K^+$, at temperatures equal to those used previously with deoxycholate. Each value is the mean of a duplicate estimate on a membrane fraction obtained from the pooled mucosal scrapings of five fish.

Temperature		*ATPase activity (μmoles P_i per h per mg protein)*			
T_{acc}	T_{inc}	*Mg^{2+}-ATPase*		*$(Na^+ + K^+)$-ATPase*	
		Untreated	*Deoxycholate treated*	*Untreated*	*Deoxycholate treated*
3	3	8.3	8.3	0.5	0.4
14	14	22.8	18.9	2.3	2.7
23	23	42.4	30.0	4.5	6.4
32	32	67.9	45.5	6.4	5.4
3	32	31.9	24.5	14.5	27.0
14	32	32.7	22.1	12.2	22.8
23	32	45.8	29.6	13.1	19.5
32	32	67.9	45.5	6.4	5.4

Digoxin inhibition of membrane ATPase and the time-course for digoxin binding

Membrane fractions prepared from fish adapted to 8° were incubated for 15 min at 37° in the presence of various concentrations of digoxin. Fig. 1 shows the inhibition curve obtained, the concentration of digoxin needed to cause half-maximal inhibition lying between 1 and 0.1 mM (*cf.* 0.5 mM for similar preparations incubated with ouabain[1]). High concentrations of digoxin (100 and 10 μM) caused slightly more inhibition

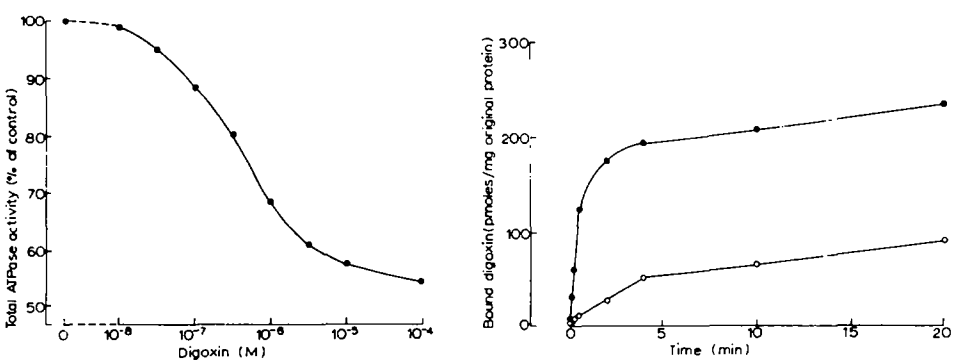

Fig. 1. Digoxin inhibition of total membrane ATPase. Membrane protein (approx. 75 μg) was incubated at 37° for 15 min in media containing 3 mM Tris–ATP, 20 mM KCl, 40 mM NaCl, 6 mM $MgCl_2$ and various concentrations of digoxin. ATPase activity is expressed as a percentage of that measured in the absence of digoxin. Each gives the mean of duplicate estimates carried out on two different membrane preparations. Fish acclimatised previously to 8°.

Fig. 2. Time-course of digoxin binding. Enzyme suspensions (approx. 400 μg protein) were incubated in 1 ml of 10 mM histidine (pH 7.1) (○——○) or 1 ml of 3 mM Tris–ATP, 6 mM $MgCl_2$, 40 mM NaCl, 10 mM histidine (pH 7.1) (●——●) at 37°. The reaction was stopped with ice-cold 1 mM ouabain in 10 mM histidine (10 ml), and the samples were washed 3 times by centrifugation (100000 × *g* for 15 min).

than the lower concentration of 3 μM. Digoxin was dissolved in a 50 % (v/v) ethanol solution to give a 1 mM solution and the final concentrations of ethanol in incubation media (5 and 0.5% respectively) were high enough to inhibit the ATPase. It was decided to use 3 μM tritiated digoxin in the labelling studies, since this fully inhibited the $(Na^- + K^+)$-ATPase at an ethanol concentration (0.15 %, v/v) which was too low to affect the enzyme.

Fig. 2 shows the amount of digoxin bound to membranes prepared from 8°-adapted fish incubated at 37° for different periods of time. The amount bound in the presence of $Na^+ + Mg^{2+} + ATP$ increased rapidly during the first 5 min and then more slowly over the next 15 min. Binding in the presence of 10 mM histidine was much less rapid over the first 5 min, but later the rate of increase corresponded to that found under test conditions. 15 min was chosen as incubation time for future experiments. This was the same length of time as that used to estimate ATPase activity and the binding of digoxin under both control and test conditions had by this time reached a steady rate.

When Mg^{2+} was present with histidine, the binding of digoxin, measured after 15 min incubation, was about two thirds that found with $Mg^{2+} + Na^+ + ATP$ present. Similar findings have been reported by ALBERS *et al.*[5] who concluded that Mg^{2+} could eventually transform the ATPase system to a state where it bound ouabain. For this reason Mg^{2+} has been omitted from media used to measure the nonspecific binding of digoxin (Fig. 2, lower curve).

Digoxin binding to deoxycholate-treated membranes and its relation to ATPase activity

Fig. 3 shows changes recorded in digoxin binding following the incubation of membranes with deoxycholate. The amount bound remained unaffected by pretreatment with deoxycholate up to and including 1.25 mM. This concentration of deoxycholate increased the activity of the $(Na^+ + K^+)$-ATPase and inhibited the Mg^{2+}-ATPase, leaving the total ATPase activity unchanged. The increase in $(Na^+ + K^+)$-ATPase activity was significant using 1.25 mM deoxycholate ($t = 3.45$, $P < 0.05$), but the

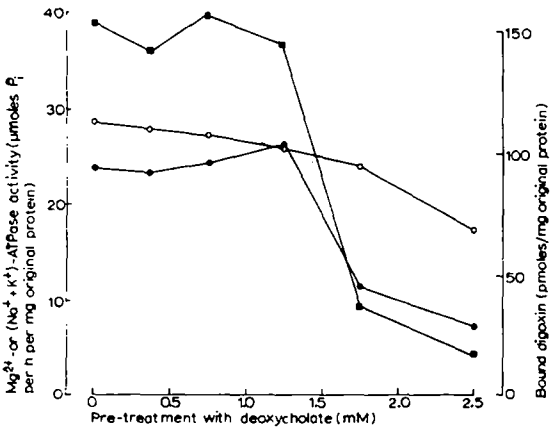

Fig. 3. Effect of deoxycholate on the subsequent digoxin binding and ATPase activity of goldfish intestinal membranes. The conditions for estimating ATPase activity are as described in the text. ○——○, Mg^{2+}-ATPase; ●——●, $(Na^+ + K^+)$-ATPase; ■——■, digoxin binding. Each point represents the mean of three determinations, each determination using a membrane fraction prepared from the pooled mucosal scrapings of four 8°-acclimatised fish.

small apparent increases with the lower deoxycholate concentrations were not significant ($t = 0.47$, $P > 0.5$). Concentrations of deoxycholate higher than 1.25 mM caused a further inhibition of Mg^{2+}-ATPase and a fall both in the $(Na^+ + K^+)$-ATPase activity and in the amount of digoxin bound. Both the ATPase activities and the amount of bound digoxin have been plotted per mg of protein added to the deoxycholate-containing incubation media. Any deoxycholate-dependent loss of protein by solubilisation would change the shape of these curves. Nevertheless it was decided to plot results in this way at first to demonstrate that deoxycholate did not increase the amount of digoxin bound. This was important since if there had been an increase, this would have provided strong evidence that deoxycholate had increased the number of K^+ sites present in the membrane.

Fig. 4 shows the recovery of membrane protein following incubation with deoxycholate and digoxin. The interrupted line represents the amount of protein added to the various histidine–deoxycholate mixtures. Less protein was recovered from solutions containing high concentrations of deoxycholate, though the highest concentration of deoxycholate used (2.5 mM) was less than that normally needed to solubilise membranes. The loss of protein during the subsequent incubation with digoxin and washing procedures was small provided $Na^+ + Mg^{2+} + ATP$ was present, but a further loss occurred when histidine was used as the sole suspending medium. This second loss of protein appeared independent of the previous treatment with deoxycholate.

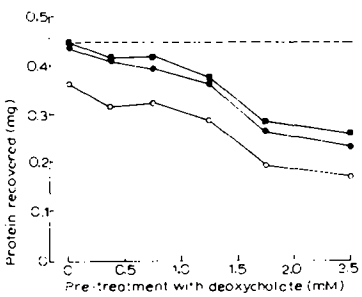

Fig. 4. Recovery of membrane protein following incubation with deoxycholate and digoxin. The broken line shows the amount of protein present at the start of incubation. ■——■, protein recovered after 15 min incubation at 37° with different concentrations of deoxycholate; ○——○, protein recovered after a further incubation of the deoxycholate-treated membranes with 3 μM digoxin in 10 mM histidine; ●——●, digoxin incubation as above, the medium containing 3 mM Tris–ATP, 40 mM NaCl, 6 mM $MgCl_2$ and 10 mM histidine. Each point is the mean of determinations on three separate membrane fractions.

The amount of digoxin bound to K^+-sites in the membrane is calculated by subtraction of the amount bound in the presence of histidine from that bound in the presence of $Na^+ + Mg^{2+} + ATP$. It was intended to compare this figure with the $(Na^+ + K^+)$-ATPase activity determined immediately after treatment with deoxycholate. A membrane suspension, treated in the normal way with different concentrations of deoxycholate, was used to determine what correction should be applied to account for this protein loss. Protein and ATPase determinations were made after deoxycholate treatment and again after a further incubation for 15 min at 37° in 3 mM Tris–ATP, 40 mM NaCl, 6 mM $MgCl_2$ and 10 mM histidine. The suspensions were centrifuged, and the pellets were washed as in the digoxin-labelling experiments. The loss of protein which

occurred during this second incubation was accompanied by a fall in both Mg^{2+}- and $(Na^+ + K^+)$-ATPase activities. This loss did not depend on the concentration of deoxycholate used beforehand. The fall in $(Na^+ + K^+)$-ATPase activity was 87 ± 8% of the protein loss (mean ± S.D. for 6 different deoxycholate concentrations). The amount of digoxin bound to deoxycholate-treated membranes was therefore corrected on the assumption that the small amount of protein lost during incubation with tritiated digoxin had the same specific activity as that found in the recovered protein.

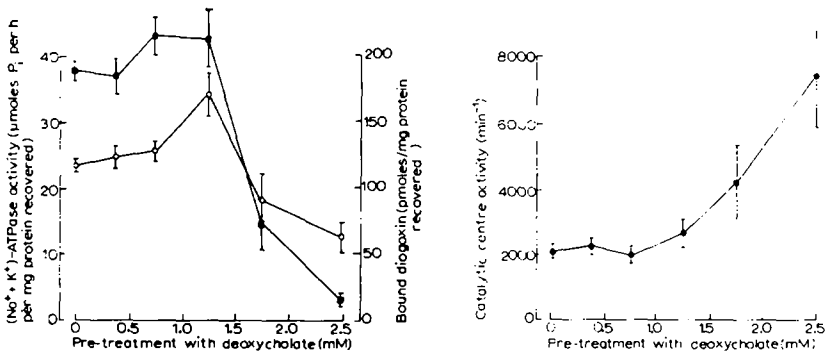

Fig. 5. Relation between bound digoxin and $(Na^+ + K^+)$-ATPase activity recovered after treatment with different concentrations of deoxycholate. O——O, $(Na^+ + K^+)$-ATPase activity; ■——■, bound digoxin. Each value is the mean of three determinations ± S.E.

Fig. 6. Changes in the catalytic centre activity of goldfish intestinal $(Na^+ + K^+)$-ATPase induced by the treatment of membranes with various concentrations of deoxycholate. Each result derived from the data in Fig. 5, represents the mean for three preparations ± S.E.

Fig. 5 shows the relation between digoxin binding and $(Na^+ + K^+)$-ATPase activity measured after deoxycholate treatment and corrected for protein loss. Both increased as the concentration of deoxycholate was raised to 1.25 mM, the proportional increase in $(Na^+ + K^+)$-ATPase exceeding that for digoxin binding. Higher concentrations of deoxycholate decreased the binding of digoxin and inhibited the $(Na^+ + K^+)$-ATPase, the decrease in binding being more pronounced than the fall in enzyme activity. The catalytic centre activity of the enzyme, defined as the number of molecules of substrate hydrolysed per enzyme site per min at 37°, has been plotted in Fig. 6. This remains essentially unchanged using concentrations of deoxycholate up to 0.75 mM but rises rapidly when higher concentrations of deoxycholate are used. Deoxycholate appears therefore to increase the efficiency with which the $(Na^+ + K^+)$-activated enzyme hydrolyses ATP.

DISCUSSION

The sequential operation of membrane-bound $(Na^+ + K^+)$-ATPase has been shown to consist of an initial Na^+-dependent transfer of phosphate from ATP to membrane intermediates followed by a K^+-dependent dephosphorylation[11]. The number of Na^+-sites present in any given preparation can be inferred by measuring the Na^+-dependent incorporation of ^{32}P from [^{32}P]ATP[12] and the number of K^+-sites estimated from the amount of bound cardiac glycoside[6]. Although simple in principle, in practice the nonspecific labelling encountered with either [^{32}P]ATP or tritiated glycoside must

be carefully controlled before an accurate estimate can be made of the number of sites sensitive to these monovalent ions. Under controlled conditions, however, there is little doubt that isotope techniques can be used to show selective labelling of Na^+- or K^+-activated sites in the membrane. The agreement which is found between Na^+-dependent phosphorylation, amount of cardiac glycoside bound and the $(Na^+ + K^+)$-dependent hydrolysis of ATP holds true for several ATPase preparations of widely different specific activities[5,12]. The specific activity of a $(Na^+ + K^+)$-ATPase preparation can be increased in a number of ways ranging from simple storage to treatment with NaI or detergents[13–15]. The present work has shown that in one such instance, in which membranes have been treated with deoxycholate prior to reaction with digoxin, the increase in $(Na^+ + K^+)$-ATPase activity which occurs is caused by each site having a faster effective turnover rate and not by the production of a greater number of sites sensitive to digoxin and hence to K^-. This special case should be distinguished from others where an increase in specific activity represents merely a partial purification of the enzyme complex. The requirement that the bound digoxin truly measures the number of K^+-sites under all conditions must also be met for the results to be valid. In assessing the amount of nonselective binding, control conditions were chosen both from the present experimental evidence and from the previously published work of others. The catalytic centre activities of the deoxycholate-treated mucosal membranes were within the range of values reported for other preparations[5,8,12]. The time-course of digoxin binding and the increase in amount bound when $Na^+ + Mg^{2+} + ATP$ were present are also characteristics common to other ATPase preparations[5–7]. This evidence would suggest therefore that the experiments had been adequately controlled. Only under the unlikely circumstance where deoxycholate inhibited preferentially the ability of membranes to bind digoxin would the values be in error. It seems justified to ignore this possibility until definitive experiments can be designed to test the point further.

The $(Na^+ + K^+)$-ATPase of goldfish intestine can change its activity in response to a change in environmental temperature[2], but it cannot be assumed from the present work that the mechanism for this adaptation *in vivo* resembles the changes caused by deoxycholate. There appear to be several ways by which an individual cell can change its $(Na^+ + K^+)$-ATPase activity. LEE *et al.*[16] have spoken about the maturation of membrane transport in red blood cells, and it is now known that the $(Na^+ + K^+)$-ATPase of low-K^+ sheep red blood cells can be increased dramatically by a specific antigen–antibody reaction[17]. The mechanism for this type of change remains unknown, but it would appear to be an alteration which is not organised directly by the cell itself. It seems more likely in the goldfish mucosa that each cell synthesises its own modifier of ATPase activity. Adaptation in this tissue, as witnessed through changes in protein synthesis and Na^+ transport, can be stopped by the injection of puromycin or actinomycin D[18,19]. How such modifiers might work—whether they remove hindrances to normal ATPase operation increasing the maximum rate of turnover or whether they create new sites for ATP hydrolysis — still remains an open question.

Membrane fractions prepared from the goldfish intestinal epithelium differ in many respects from those prepared from other species. Apart from the effect of adaptation on membrane ATPase and the action of deoxycholate, the sensitivity to glycoside inhibition is high relative to that of other intestinal epithelia[20,21] and the membrane protein is more easily solubilised. The reasons for these are probably 2-fold; first the intestinal lipids of cold-adapted fish contain a high proportion of polyenoic unsatu-

rated fatty acids (P. KEMP AND M. W. SMITH, unpublished observations) and, second, the temperature at which these studies were carried out (37°) was nearly 30° higher than the environmental temperature of the fish. Both these circumstances would tend to increase the fluidity of the membranes, making them more susceptible to detergent action. Deoxycholate taken into the membrane lipids could stimulate the hydrolysis of ATP either by easing a steric constraint normally imposed on the $(Na^+ + K^+)$-ATPase or by increasing the mobility of phosphorylated intermediates within the membrane. Kinetic studies to determine the K_m of ATP hydrolysis by $(Na^+ + K^+)$-ATPase in the presence of various deoxycholate concentrations might help to distinguish between these two possibilities.

REFERENCES

1 M. W. SMITH, *Biochem. J.*, 105 (1967) 65.
2 M. W. SMITH, V. E. COLOMBO AND E. A. MUNN, *Biochem. J.*, 107 (1968) 691.
3 M. W. SMITH AND P. BROWN, *Experientia*, 24 (1968) 1005.
4 I. M. GLYNN, *J. Physiol. London*, 136 (1957) 148.
5 R. W. ALBERS, G. J. KOVAL AND G. J. SIEGEL, *Mol. Pharmacol.*, 4 (1968) 324.
6 A. SCHWARTZ, H. MATSUI AND A. H. LAUGHTER, *Science*, 159 (1968) 323.
7 H. MATSUI AND A. SCHWARTZ, *Biochim. Biophys. Acta*, 151 (1968) 655.
8 J. C. ELLORY AND R. D. KEYNES, *Nature*, 221 (1969) 776.
9 C. H. FISKE AND Y. SUBBAROW, *J. Biol Chem.*, 66 (1925) 375.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
12 H. BADER, R. L. POST AND G. H. BOND, *Biochim. Biophys. Acta*, 150 (1968) 41.
13 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
14 T. NAKAO, K. NAGANO, K. ADACHI AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 13 (1963) 444.
15 F. MEDZIHRADSKY, M. H. KLINE AND L. E. HOKIN, *Arch. Biochem. Biophys.*, 121 (1967) 311.
16 P. LEE, A. WOO AND D. C. TOSTESON, *J. Gen. Physiol.*, 50 (1966) 379.
17 J. C. ELLORY AND E. M. TUCKER, *Nature*, 222 (1969) 477.
18 M. W. SMITH AND D. MORRIS, *Experientia*, 22 (1966) 678.
19 M. W. SMITH, *Experientia*, 22 (1966) 252.
20 C. B. TAYLOR, *Biochim. Biophys. Acta*, 60 (1962) 437.
21 G. G. BERG AND J. SZERKERCZES, *J. Cellular Comp. Physiol.*, 67 (1966) 487.