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Influence of Temperature Acclimatization on the Ionic Activation of Goldfish Intestinal Adenosine Triphosphatase

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1. An adenosine triphosphatase membrane system, dependent on Mg^{2+} and activated further by $Na^+ + K^+$, was prepared from goldfish anterior intestine by differential centrifugation of homogenized intestinal scrapings. 2. The affinity of this preparation for Na^+ in the presence of $K^+ + Mg^{2+}$, for K^+ in the presence of $Na^+ + Mg^{2+}$ and for Mg^{2+} alone, measured at 37°, did not depend on the previous environmental temperature of the fish. When $Na^+ + K^+$ were added to preparations from 8°-acclimatized fish the affinity for Mg^{2+} increased; this was not seen with preparations from 30°-acclimatized fish. 3. Part of the Mg^{2+}-activated adenosine triphosphatase was inhibited by Na^+ and the amount of inhibition appeared to increase at high acclimatization temperatures. 4. This Na^+-inhibited adenosine triphosphatase was separated from the $(Na^+ + K^+)$-activated enzyme by centrifugation on sucrose density gradients. 5. Preparations from 8°-acclimatized fish contained less Mg^{2+}-activated and more $(Na^+ + K^+)$-activated adenosine triphosphatase than did similar fractions from 30°-acclimatized fish. 6. Acclimatization to different environmental temperatures might involve one form of adenosine triphosphatase changing to another. The origin of various membranes seen in microsomal fractions must, however, be established before this hypothesis can be tested further.

The microsomal ATPase† system of goldfish intestinal mucosa can be inhibited by ouabain to an extent that depends on the previous environmental temperature of the fish (Smith, 1967). The total ATPase activity of these microsomal fractions remains apparently unaffected by changes in acclimatization temperature and this raises the possibility that some of the Mg^{2+}-activated enzyme might become sensitive to inhibition by ouabain when fish are exposed to a cold environment. It is still not certain whether Mg^{2+}-activated and $(Na^+ + K^+)$-activated ATPase(s) are the same or different enzymes (see the review by Skou, 1965) and the present work was undertaken partly to study this question. Although ouabain inhibits specifically the $(Na^+ + K^+)$-activation of ATPase and can be used to measure the activity of this enzyme, the correlation between the two activities is not always perfect (Järnefelt, 1964) and it was therefore decided to measure directly the activating effects of these ions on microsomal ATPase(s) prepared from the anterior intestines of goldfish acclimatized to different temperatures. It soon became obvious that some microsomal fractions contained an ATPase activity that was inhibited by Na^+ in the presence of Mg^{2+}, and part of the work then involved separating this enzyme from the $(Na^+ + K^+)$-activated enzyme by subfractionation on sucrose density gradients.

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† Abbreviation: ATPase, adenosine triphosphatase.

EXPERIMENTAL

Materials

Fish. Goldfish weighing 30–60g. were obtained over a period of 5 months (February to July 1967) from two sources: Pet-Reks (Anglia) Ltd., Melbourn, Cambs., and Perry's Hardy Plant Farm, Enfield, Middx. The fish were kept for the first week in a large aquarium containing aerated water at room temperature and later transferred in groups of six to acclimatization tanks where the water was maintained at a constant temperature of 8° or 30°. Fish were kept in these tanks for a further 2–3 weeks before being used for experiments. The whole series of experiments took 5 months to complete. There was a suspicion that the amount of intestinal $(Na^+ + K^+)$-activated ATPase fell slightly for both cold- and warm-adapted fish in late spring and early summer. Groups of 8°-acclimatized fish were alternated with 30°-acclimatized fish throughout, making comparisons between the two acclimatization temperatures independent of seasonal changes.

Reagents. ATP (disodium salt) was obtained from Sigma

(London) Chemical Co., London, S.W. 6. Tris ATP was prepared from the disodium salt by passing 200mM-ATP (disodium salt) through a cation-exchange column (Dowex 50) in the H^+ form, adjusting the pH of the effluent to 7·1 with tris and then diluting this solution with 10mM-histidine to give 100mM-tris ATP. This solution was divided and stored at −18°. All other reagents were of A.R. grade.

Methods

Enzyme preparation. The procedure for killing fish and for collecting mucosal scrapings from the anterior intestine has already been described in detail (Smith, 1967). Homogenization was in 10 parts (v/w) of 0·25M-sucrose solution containing 5mM-EDTA, 60mM-histidine and 0·1% sodium deoxycholate buffered to pH 7·1 with HCl. The resulting homogenate was centrifuged in the cold in a Spinco model L preparative ultracentrifuge (rotor AH 50) for 15min. at 10000*g*. The supernatant was collected and centrifuged a further 60min. at 20000*g* to produce a sediment, which was dispersed in, and recovered from, 10ml. of 10mM-histidine–HCl buffer, pH 7·1, by final centrifugation at 20000*g* for 60min. The 20000*g* sediment was resuspended in 7ml. of 10mM-histidine–HCl buffer, pH 7·1, and stored at −18° until required. The concentration of protein in these suspensions was slightly less than 1mg./ml. Occasionally samples of the original homogenate, the 10000*g* sediment and the final supernatant fraction were also saved and assayed to determine the overall recovery of ATPase activity after differential centrifugation.

Density-gradient centrifugation. Linear gradients (28ml.) of 15–60% (w/v) sucrose in 60mM-histidine–HCl buffer, pH 7·1, were prepared in the cold with the apparatus described by Greville, Munn & Smith (1965). Suspensions of 20000*g* sediments from goldfish intestinal mucosa in 1·0ml. of 10mM-histidine buffer, pH 7·1, were layered over the gradients. Centrifugation was carried out in the cold in the SW 25 rotor of the Spinco model L centrifuge at 63500*g* (25000rev./min.) for 9hr. The brake was not applied during deceleration. Gradients were fractionated after centrifugation by collecting drops from a hole pierced in the bottom of each tube. Fractions were frozen and assayed later the same day.

Assay of ATPase activity. Initially the incubation medium consisted of 3mM-$MgCl_2$, 100mM-NaCl, 10mM-KCl, 10mM-tris ATP and 10mM-histidine adjusted to pH 7·1 with HCl. Normally 25–100μg. of enzyme protein was used, the final volume of the incubation medium being always 1·0ml. Considerably less than 25μg. of enzyme protein had to be used when certain of the fractions collected from sucrose gradients were assayed. Incubation media were equilibrated at 37° for 10min. before addition of ATP solution and hydrolysis was stopped after 15min. incubation by the addition of 0·1ml. of 50% (w/v) trichloroacetic acid. Suspensions were centrifuged (1400*g* for 5min.) and 0·5ml. samples of the supernatant taken for determination of P by the method of Fiske & Subbarow (1925). Exactly 20min. was allowed for the development of colour and a correction made for the P_i liberated non-enzymically. The ionic composition of the incubation media varied as the experiments progressed; the final optimum concentrations of $MgCl_2$ and KCl were judged to be 6mM and 20mM respectively. The concentration of NaCl giving maximal total ATPase activity was 40mM for 8°-acclimatized fish and 10mM for 30°-acclimatized fish. All assays were completed within 3 days of preparation.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard.

Electron microscopy. Fractions were spread on carbon-coated colloidin or on holey-carbon films (Huxley & Zubay, 1960), washed free from sucrose and examined by the negative staining technique with 2% (w/v) sodium phosphotungstate or 5% (w/v) ammonium molybdate in a Siemens Elmiskop I instrument (generously provided by the Wellcome Trust) at a magnification of ×40000.

RESULTS

Control experiments. Microsomal pellets normally contain some Na^+ and K^+ and it has been suggested that their presence might be responsible for partial activation of the membrane ATPase system (Järnefelt, 1964). Deoxycholate removes much of the bound Na^+ and K^+ from microsomal preparations (Järnefelt, 1964) and it has been used for this purpose in the present work. The microsomal pellets from ten preparations, resuspended in 7ml. of histidine–hydrochloric acid buffer as described above, were analysed for Na^+ and K^+, usually without further dilution, by emission spectrophotometry. Each preparation used material from three fish: six preparations were from fish acclimatized to 30° and four from fish acclimatized to 8°. The amounts found did not depend on the previous acclimatization temperature of the fish and results were therefore pooled. The concentrations of Na^+ and K^+ were $0·29 \pm 0·05$ and $0·1 \pm 0·03$ μmole/ml. of microsomal suspension respectively (means ± S.E.M. of ten experiments). The preparations were diluted ten- or 20-fold in 10mM-histidine–hydrochloric acid buffer before use, giving maximal final concentrations of Na^+ and K^+ of 0·03mM and 0·01mM respectively. These concentrations are extremely low, but could conceivably exert an activating effect if bound to membranes near to an ATPase system.

The near-total dependence of ATPase activity on Mg^{2+} was established by incubation of microsomal fractions in 10mM - histidine – hydrochloric acid buffer, pH 7·1. Eight preparations were used from 24 fish, 15 of which had been acclimatized to 30° and nine acclimatized to 8°. The rate of hydrolysis in the absence of any added ions was very low and independent of the previous environmental temperature of the fish; the mean activity found was 0·34μmole of P_i/hr./mg. of protein. This represented less than 1% of the ATPase activity shown by the same preparations in the presence of 3mM-magnesium chloride. No correction was made for hydrolysis measured in the absence of Mg^{2+} when

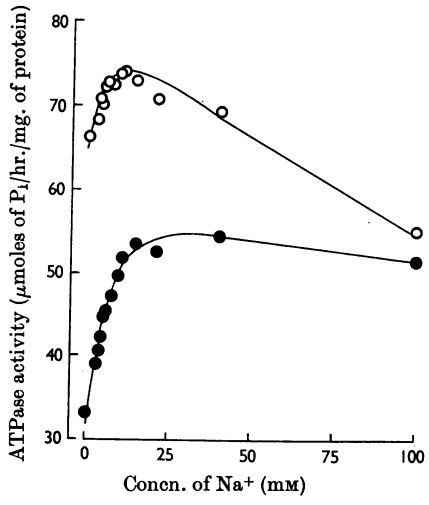

Fig. 1. Activation of goldfish intestinal ATPase by Na^+ in the presence of $K^+ + Mg^{2+}$. The enzyme preparation consisted of a 20000*g* sediment of homogenized goldfish mucosa washed once in Na^+-free buffer as described in the text. Each point gives the mean of four determinations and each determination used an ATPase preparation derived from the pooled mucosal scrapings of three fish. Incubation was at 37° for 15min. in media containing 10mM-tris ATP, 3mM-$MgCl_2$, 10mM-KCl and 10mM-histidine–HCl buffer, pH 7·1; NaCl was present in these media at different known concentrations and the final osmolality depended on the concentration of NaCl used. Goldfish had been maintained at 8° (●) or 30° (○) for 2–3 weeks before the experiment.

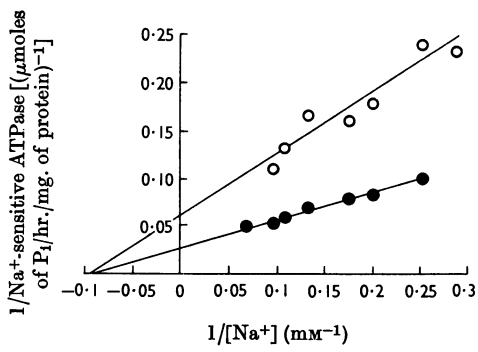

Fig. 2. Double-reciprocal plots showing the dependence of $(Na^+ + K^+)$-activated ATPase on the concentration of NaCl. The enzyme preparations and conditions of incubation were those described in Fig. 1. Points were plotted over a limited range of Na^+ concentration (3–15mM) where Na^+ stimulation of ATPase activity was seen for both warm- and cold-adapted fish. The Na^+-sensitive portion of total ATPase activity was obtained by subtracting the amount of hydrolysis recorded in media containing only 3mM-$MgCl_2$ and 10mM-histidine–HCl buffer from that found in similar media containing, in addition, various concentrations of NaCl with 10mM-KCl. ●, Fish previously acclimatized to 8°; ○, fish acclimatized to 30°.

the specific activities of subsequent ATPase preparations were calculated.

Dependence of microsomal ATPase activity on Na^+. Microsomal fractions prepared from the intestines of goldfish acclimatized to 8° or 30° were incubated in 10mM-histidine–hydrochloric acid buffer, pH 7·1, containing 3mM-magnesium chloride, 10mM-potassium chloride and various known concentrations of sodium chloride. ATPase activities were stimulated by the addition of small amounts of sodium chloride (Fig. 1). High concentrations of sodium chloride inhibited the total ATPase activity of preparations from warm-adapted fish and there was also a slight inhibition of microsomal ATPase from cold-adapted fish. The specific activities of ATPase from warm- and cold-adapted fish measured without sodium chloride added to the incubation media were about 65 and 35μmoles of P_i/hr./mg. of protein respectively. With 100mM-sodium chloride present the specific activities became nearly equal at about 55μmoles of P_i/hr./mg. of protein. This agreement between the total ATPase activities of 8°- and 30°-adapted fish measured in the presence of 100mM-sodium chloride verifies previous results and the twofold difference found when sodium chloride was absent is the same as that found when the ouabain-insensitive ATPase activities are compared in fractions prepared from fish acclimatized to these two temperatures (Smith, 1967).

Low concentrations (3–15mM) of sodium chloride stimulated the ATPase activities of preparations from both cold- and warm-adapted fish (Fig. 1), and reciprocal plots for the Na^+-stimulated hydrolysis, shown in Fig. 2, gave K_m values that were very similar, 11·7mM- and 11·0mM-Na^+ with microsomal fractions from cold- and warm-adapted fish respectively. The $V_{max.}$ for preparations from cold-adapted fish, 38·5μmoles of P_i/hr./mg. of protein, was considerably higher than for preparations from warm-adapted fish, 16·4μmoles of P_i/hr./mg. of protein, suggesting that the amount of Na^+-sensitive enzyme was increasing in response to a cold environment.

The inhibition of ATPase activity by sodium chloride first became noticeable when the concentration of sodium chloride was raised to 20mM with preparations from warm-adapted fish. Fig. 3 shows the Na^+-sensitive hydrolysis of ATP with fractions prepared from warm-adapted fish incubated in the presence and absence of 10mM-potassium chloride. Magnesium chloride was present throughout at a concentration of 3mM. Removal of potassium chloride from the incubation

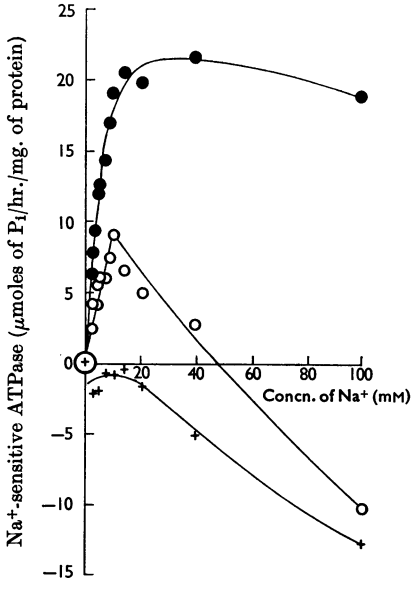

Fig. 3. Influence of temperature acclimatization on the Na^+-sensitive hydrolysis of ATP showing inhibition of ATPase activity by Na^+ to be independent of added K^+. Fish had been acclimatized to 8° (●) or 30° (○ and +). ○ and ●, Na^+-sensitive hydrolysis measured in the presence of 10mM-KCl; +, hydrolysis in the absence of KCl. Conditions of incubation were otherwise as described in Fig. 1. Each point gives the mean of four determinations and for each determination a pooled preparation of ATPase taken from three fish was used.

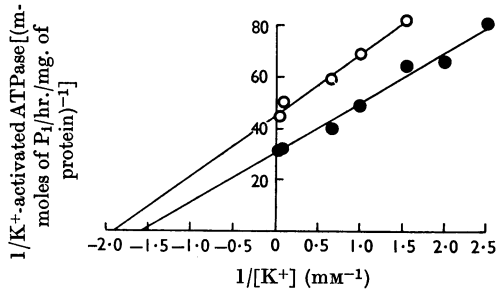

Fig. 4. Activation of goldfish intestinal ATPase by K^+ in the presence of $Na^+ + Mg^{2+}$. The 20000*g* fractions of mucosal scrapings, prepared as described in the text, were incubated for 15min. at 37° in media containing 10mM-tris ATP, 3mM-$MgCl_2$ and either 40mM-NaCl (8°-acclimatized fish) or 10mM-NaCl (30°-acclimatized fish); KCl was present in different concentrations, and the reciprocal of the K^+-activated ATPase activity was plotted over the concentration range 0·4–20mM-KCl. Each point gives the mean of four determinations, each determination being made on a pooled ATPase fraction prepared from three fish. Fish had been acclimatized to 8° (●) or 30° (○) for 2–3 weeks beforehand.

medium eliminated the Na^+-activated hydrolysis of ATP, emphasizing that this activation was ($Na^+ + K^+$)-dependent. However, sodium chloride continued to inhibit ATPase activity in the absence of potassium chloride, the lower limit for detecting this inhibition being in the region of 20mM-sodium chloride.

Activation of microsomal ATPase by K^+ *in the presence of* $Na^+ + Mg^{2+}$. Activation of different microsomal fractions by K^+ was measured in the presence of 40mM-sodium chloride (for cold-adapted fish) or 10mM-sodium chloride (for warm-adapted fish). These concentrations of sodium chloride caused maximal stimulation of ATPase activity in the presence of 10mM-potassium chloride (Fig. 3) and were chosen for this reason. Magnesium chloride was present in all media at a concentration of 3mM. The ATPase activity of both types of preparation increased with increase in the concentration of potassium chloride. Reciprocal plots of the K^+-activated ATPase activities are shown in Fig. 4. The K_m values for activation by K^+ were 0·64mM and 0·53mM for microsomal fractions prepared from cold- and warm-adapted fish respectively. The calculated $V_{max.}$ for fractions from cold-adapted fish was 32·3 μmoles of P_i/hr./mg. of protein, a value close to that found for Na^+-activated ATPase measured in the presence of 10mM-potassium chloride (see above) and much higher than that found for warm-adapted fish (22·0 μmoles of P_i/hr./mg. of protein). There was no inhibition of ATPase activity by high concentrations of potassium chloride; the K^+-activated hydrolysis increased by an average of 8% when the concentration of potassium chloride was raised from 10mM to 20mM. It was therefore decided to use 20mM-potassium chloride in all subsequent experiments.

Activation of microsomal ATPase by Mg^{2+} *in the presence and absence of* $Na^+ + K^+$. Microsomal preparations from the intestines of 8°- and 30°-acclimatized goldfish were incubated in 10mM-histidine–hydrochloric acid buffer, pH 7·1, containing 0–6mM-magnesium chloride. Part of each preparation was incubated with sodium chloride and potassium chloride present in addition to magnesium chloride, so that the effect of these univalent ions could be measured at each chosen concentration of magnesium chloride. Sodium chloride was used at a concentration of 40mM or 10mM, depending on whether the fish had been acclimatized previously to 8° or 30°. Potassium chloride was used at a concentration of 20mM. Reciprocal plots showing the rate of hydrolysis of ATP under these various conditions are shown in Fig. 5. The curves in Fig. 5(*a*) were obtained with microsomal prepara-

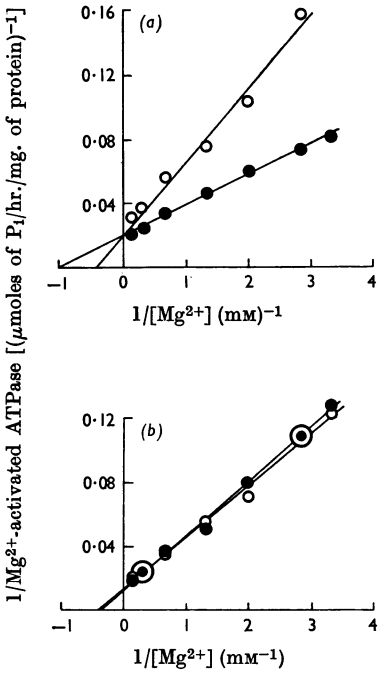

Fig. 5. Activation of goldfish intestinal ATPase by Mg^{2+} in the presence and absence of $Na^+ + K^+$. The preparation and incubation of ATPase fractions was as described in the text. Incubation media contained 10mM-tris ATP with no KCl or NaCl (○) or 20mM-KCl with either 40mM-NaCl (8°-acclimatized fish, ● in *a*) or 10mM-NaCl (30°-acclimatized fish, ● in *b*). (*a*) Mg^{2+}-activated ATPase of preparations from 8°-acclimatized fish; (*b*) similar plot for 30°-acclimatized fish. The ATPase activity of each enzyme preparation was determined in the presence and absence of $Na^+ + K^+$ at all concentrations of Mg^{2+}. Each point is the mean of four determinations, each determination being made on pooled material from three fish.

tions from 8°-acclimatized fish and those in Fig. 5(*b*) with fractions from 30°-acclimatized fish. In both cases the ATPase activity, measured with no Na^+ or K^+ present, showed a similar dependence on the Mg^{2+} concentration; the K_m values were 2·3mM and 2·5mM for 8°- and 30°-acclimatized fish respectively. The addition of Na^+ and K^+ to incubation media containing preparations from 30°-acclimatized fish did not change significantly the affinity for Mg^{2+} (K_m 3·1mM compared with 2·5mM). However, preparations from 8°-acclimatized fish showed a striking increase in affinity for Mg^{2+} when Na^+ and K^+ were present, the K_m falling from 2·3mM to 1·0mM. These changes took place with no detectable change in $V_{max.}$. There was, however, a change in $V_{max.}$ associated with the different acclimatization temperatures. The mean $V_{max.}$ for the whole ATPase system was 50·3 μmoles of P_i/hr./mg. of protein for cold-adapted fish and 82·4 μmoles of P_i/hr./mg. of protein for warm-adapted fish. This suggests that a real increase in the total amount of Mg^{2+}-activated enzyme had taken place in response to the warm environment, but with the bulk of this enzyme(s) remaining insensitive to the activating effect of $Na^+ + K^+$.

In a series of experiments not shown in Fig. 5 the hydrolysis of ATP was measured with 6mM- or 10mM-magnesium chloride with preparations from goldfish acclimatized to 8° or 30°. Magnesium chloride at 10mM concentration was no more effective than 6mM-magnesium chloride as an activator of the ATPase system. This was true whether or not $Na^+ + K^+$ were present in the incubation medium. The mean specific activities from six preparations from 18 fish were 43·5 and 43·1 μmoles of P_i/hr./mg. of protein with 6mM- and 10mM-magnesium chloride respectively. The optimum concentration of magnesium chloride was therefore taken to be 6mM when the concentration of ATP used was 10mM.

Separation of a Na^+*-inhibited ATPase from microsomal fractions of goldfish intestinal mucosa.* Potassium chloride (20mM) and 6mM-magnesium chloride caused maximal stimulation of microsomal ATPase activity irrespective of the temperature to which the fish had been previously acclimatized, but the concentration of sodium chloride used had been kept at 10mM when warm-adapted preparations were used, because of the obvious inhibition seen when higher concentrations of sodium chloride were used. It seemed unlikely that the Na^+-activated ATPase with K_m 11mM would be inhibited by as little as 20mM-sodium chloride, and so microsomal fractions, prepared by the usual method of differential centrifugation, were subfractionated on 15–60% (w/v) sucrose density gradients to test for the presence of more than one ATPase. Recoveries of ATPase activity were calculated for each stage of the preparation. The overall recovery of ATPase activity after differential centrifugation was 61% (mean for cold- and warm-adapted fish; compare with the 59–63% recoveries found previously; Smith, 1967). There was no further loss of ATPase activity during centrifugation through the sucrose density gradient, the mean recovery at the end of this stage being 117% of the amounts added.

By coincidence, the microsomal fractions prepared from cold- and warm-adapted fish contained the same amount of ATPase activity (590 μmoles of P_i/hr.), representing 35·8% of the total ATPase recovered after differential centrifugation. Fractions collected from these gradients were frozen and later assayed for ATPase activity by using 6mM-magnesium chloride and 20mM-potassium chloride with and without 100mM-sodium chloride. The

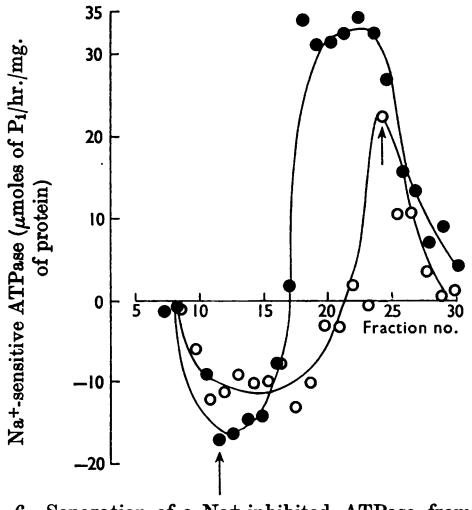

Fig. 6. Separation of a Na^+-inhibited ATPase from the ($Na^+ + K^+$)-activated enzyme by means of sucrose-density-gradient centrifugation. Washed microsomal fractions of goldfish intestinal mucosa in 1·0 ml. of 10 mM-histidine–HCl buffer, pH 7·1, were layered over linear gradients (28 ml.) of 15–60% (w/v) sucrose in 60 mM-histidine–HCl buffer, pH 7·1. Centrifugation was in the cold at 63 500*g* for 9 hr. Fractions (0·85 ml.) were collected and stored at −18° before being incubated for 15 min. at 37° in media containing 10 mM-tris ATP, 6 mM-$MgCl_2$, 20 mM-KCl and 10 mM-histidine–HCl buffer, pH 7·1. Duplicate samples were incubated in the same media plus 100 mM-NaCl. Fish had been acclimatized previously to 8° (●) or 30° (○). The arrows indicate fractions of both cold- and warm-adapted preparations, which were taken for examination under an electron microscope.

Na^+-sensitive hydrolysis of ATP by two preparations, one from cold-adapted fish and one from warm-adapted fish, is shown in Fig. 6. ATPase activities measured in the denser parts of the gradient (fractions 10–20 with the warm-adapted preparation and fractions 10–15 for the cold-adapted preparation) were inhibited by 100 mM-sodium chloride, whereas the lighter fractions, 22–28 and 17–30 for preparations from warm- and cold-adapted fish respectively, were stimulated by Na^+ in the presence of K^+. The stimulation by Na^+ was less with fractions prepared from fish acclimatized to the higher temperature. This represented a real difference since the recovery of ATPase activity at each stage was comparable and not dependent on the previous environmental temperature of the fish. The pattern of distribution was reproducible though the specific activity of Na^+-inhibited ATPase varied in different experiments, usually being greater for the 30°-acclimatized fish. Fig. 6 illustrates two experiments where the inhibition by Na^+ was clearly seen with both types of preparation.

Electron-microscopic appearance of separated membrane fragments. The change from inhibition to stimulation by Na^+ shown in Fig. 6 was abrupt with preparations from both cold- and warm-adapted fish, suggesting a good separation of the two ATPase activities. Fractions 12 and 23, shown by arrows in Fig. 6, were chosen to represent peak activities of Na^+-inhibited and ($Na^+ + K^+$)-activated ATPase respectively.

Fraction 12 showed two major types of membrane structure: (1) more or less disrupted inner membranes of mitochondria identified by their characteristic 90 Å particles (first described by Fernández-Morán, 1962); smooth membranes, thought to be portions of mitochondrial outer membranes, were often closely associated with these inner membranes; (2) sheet-like membranes roughly circular in shape (0·2–0·65 μ diam.), sometimes ribbonlike membranes (0·07–0·2 μ in width) coated with globular particles 40–70 Å across.

Fraction 23 consisted almost exclusively of one kind of membrane coated with particles measuring 30–50 Å across and about 40 Å apart. The membranes ranged from wispy strands up to 1 μ long to larger more sheetlike material from which the strands appeared to be derived. Descriptions for fractions 12 and 23 fitted preparations from both 8°- and 30°-acclimatized fish. The pretreatment with deoxycholate precluded positive identification of these various membrane fragments.

DISCUSSION

Microsomal preparations used in the present work consisted of small fragments presumed to come from the cell membrane and endoplasmic reticulum. They also possibly contained pieces of ruptured mitochondria. When the total ATPase activity of these relatively crude preparations was divided into Mg^{2+} and ($Na^+ + K^+$)-activated portions, it became immediately obvious that there was an inverse relation between the two, with a low environmental temperature favouring the ($Na^+ + K^+$)-activated ATPase at the expense of the Mg^{2+}-activated enzyme(s). This was a direct confirmation of previous work where ouabain-sensitive ATPase activity had been used as an indirect measure of ($Na^+ + K^+$)-activated enzyme (Smith, 1967). It has previously been shown that the ($Na^+ + K^+$)-activated/Mg^{2+}-activated ATPase ratio can be increased by storage (Schwartz, 1962), by treatment with deoxycholate (Skou, 1962), by addition of sodium iodide (Nakao, Nagano, Adachi & Nakao, 1963) or by addition of heated supernatant fractions (Skou, 1964). It may be relatively easy therefore to change from one form of the enzyme to another, but this cannot be proved because of the net loss of total ATPase activity associated with these various treatments. The total ATPase activity of

goldfish preparations depends very much on the Na^+ concentration chosen for the comparison because of the Mg^{2+}-activated Na^+-inhibited ATPase present in certain of the fractions. A rough comparison between the two acclimatization temperatures can, however, be made by adding together the $V_{max.}$ values for the Mg^{2+}-activated and the $(Na^+ + K^+)$-activated enzyme(s). The maximal specific activities for 30°- and 8°-acclimatized fish then become 99 and 89 μmoles of P_i/hr./mg. of protein respectively. These values are close and would presumably become nearly equal if a correction could be made for the Mg^{2+}-activated Na^+-inhibited ATPase present mainly in samples from 30°-acclimatized fish. There is, then, some reason to suppose that a common ATPase might change its form permanently in response to a changed environment, but it could be a mistake to regard the Mg^{2+}-activated ATPase merely as a potential reservoir for the $(Na^+ + K^+)$-activated enzyme. Both enzymes (or forms of the same enzyme) are situated in the same type of membrane and could have equally important functions to perform in controlling the transport of different substances across these membranes.

The subcellular distribution of goldfish intestinal ATPase is very different from that of mammals. The highest specific activity of $(Na^+ + K^+)$-activated ATPase from goldfish mucosa was obtained from a 20000*g* membrane fraction (Smith, 1967). Taylor (1962), using guinea-pig intestine, found $(Na^+ + K^+)$-activated ATPase in a low-speed sediment, and a similar distribution has since been confirmed for rat and hamster intestine (Berg & Chapman, 1965; Eichholtz & Crane, 1966). The normal variation in distribution of membrane ATPase(s) found by Skou (1962) would be too small to account for the above discrepancy. The intestinal mucosa of goldfish probably breaks up more or less completely during homogenization to produce elements of both apical and basal mucosal membranes, which can then be recovered in the 20000*g* fraction. A rather similar distribution of $(Na^+ + K^+)$-activated ATPase has been obtained with the colonic mucosa of toads (H. G. Ferreira & M. W. Smith, unpublished work). The presence of deoxycholate in the original homogenization medium might be expected to aid this process of membrane disruption.

The concentration of ouabain needed to cause half-maximal inhibition of intestinal $(Na^+ + K^+)$-activated ATPase was species-dependent, the goldfish (Smith, 1967) being eight times as sensitive as the guinea pig (Taylor, 1962) and 200 times as sensitive as the rat (Berg & Szekerczes, 1966). Apart from this difference, however, there was great similarity in the way different ions activated ATPase separated from the three species. The concentrations of Na^+ and K^+ needed to cause half-maximal stimulation of guinea-pig $(Na^+ + K^+)$-activated ATPase, each being measured with an excess of the other ion present, were 10mM and 0·5mM respectively (Taylor, 1962). The corresponding values for goldfish $(Na^+ + K^+)$-activated ATPase were 11·3mM and 0·58mM (mean values for fish acclimatized to 8° and 30°). The corresponding concentrations for Na^+ with rat preparations may have been higher (Berg & Szekerczes, 1966), but the pattern of activation by K^+ in the presence of Na^+ was very similar for all three species. The concentration of Mg^{2+} needed to cause maximal activation of the whole ATPase system depended on the concentration of ATP used, there being virtually no further increase in ATPase activity when the Mg^{2+}/ATP concentration ratio was increased from 0·5 to 1·0. The emphasis of species difference is concerned therefore with subcellular distribution and not with the properties of the separated ATPase system. In saying this, however, it should be remembered that the activity of goldfish $(Na^+ + K^+)$-activated ATPase was measured at 37°, which ensured that the $(Na^+ + K^+)$-activated ATPase systems of both 8°- and 30°-acclimatized fish were in their highest state of activation (Smith, 1967). The affinities for various ions measured under these conditions probably bear no relation to those measured at the environmental temperature of the fish.

Part of the ATPase recovered from microsomal fractions of homogenized goldfish intestine is inhibited by Na^+. This inhibition is most pronounced with preparations from 30°-acclimatized fish. One explanation, that Na^+ can inhibit the $(Na^+ + K^+)$-activated ATPase by saturating the Na^+ site or by competing with K^+ for the site with high affinity for K^+, could not apply in this case because the total ATPase activity was inhibited to a level below that found in the absence of Na^+. The concentration of Na^+ causing inhibition was too low to exert non-specific osmotic effects. The separation of a Mg^{2+}-activated Na^+-inhibited ATPase from the $(Na^+ + K^+)$-activated enzyme confirmed that the inhibitory activity was caused by a different enzyme. This enzyme might function as a repressor of Na^+ transport across cells kept at a high environmental temperature. However, fractions containing this activity also contained mitochondrial membranes, and their importance as a source of ATPase activity must be established before defining more accurately the possible role for the Na^+-inhibited enzyme. Membranes prepared from deoxycholate-disrupted rat liver mitochondria also possess on ATPase inhibited by low concentrations of Na^+ (Ulrich, 1963).

Acclimatization of Na^+ transport across the goldfish intestine seems therefore to be a complicated process involving changes in the amount

of $(Na^+ + K^+)$-activated ATPase, changes in the ease by which this enzyme can be activated at different incubation temperatures (Smith, 1967) and possibly the synthesis of a Na^+-inhibited ATPase. There are reports that the amount of $(Na^+ + K^+)$-activated ATPase can change in the eel intestine (Oide, 1967), in the toad colon (H. G. Ferreira & M. W. Smith, unpublished work) and in the gills of killifish (Epstein, Katz & Pickford, 1967) after acclimatization to salt water. The two types of acclimatization might involve similar control processes, but in none of these cases is it possible to identify the membrane in which changes take place and this is essential before the control of Na^+ transport across epithelial tissues can be fully understood.

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