Influence of Temperature Acclimatization on the Temperature-Dependence and Ouabain-Sensitivity of Goldfish Intestinal Adenosine Triphosphatase

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1. Homogenates of goldfish intestinal mucosa were separated into various fractions by differential centrifugation. Both adenosine-triphosphatase and β -glycerophosphatase activities were found to be concentrated mainly in a membrane fraction which sedimented after 120000g-min. 2. This membrane adenosine-triphosphatase system was activated by $\text{Na}^+ + \text{K}^+$ and inhibited by ouabain. 3. The ouabain-sensitive adenosine-triphosphatase activity was high and the ouabain-insensitive activity low in membrane fractions prepared from fish acclimatized previously to 8°. The opposite was true for fish acclimatized to 30°. 4. The Arrhenius plots of ouabain-sensitive and ouabain-insensitive adenosine-triphosphatase activities, measured from 5° to 30°, showed discontinuities at incubation temperatures that varied with the previous acclimatization temperature of the fish. 5. It is considered that modification of the membrane adenosine-triphosphatase system in goldfish intestinal mucosa may serve to regulate Na^+ transport at different environmental temperatures.

There is evidence that suggests that the transport of Na^+ ions across the intestine of goldfish is regulated by means of allosteric proteins responding to the effector action of glucose (Smith, 1966a) and amino acids (Mephram & Smith, 1966a,b). Transport of Na^+ , judged indirectly by measuring the transmural potential, is much increased if cold-adapted fish are placed in warm water and the intestines then taken and incubated at the new environmental temperature. This situation still persists 15 hr. after raising the environmental temperature, but after 20 hr. the permeability of the mucosal membranes changes dramatically and the effector action of glucose again has the characteristics previously recorded at the original temperature (Smith, 1966b). Other work has shown that an increase in protein synthesis precedes the changes in membrane permeability and that injection of puromycin will stop the subsequent change in permeability mechanisms (Smith & Morris, 1966; Smith, 1966c). Some of the induced protein synthesis is probably directly concerned with the regulation of Na^+ entry and passage through the mucosal cell, but the identity of this protein remains unknown.

One possible point of control is through the enzyme system thought to be most intimately connected with Na^+ transport across cell membranes, the $(\text{Na}^+ + \text{K}^+)\text{-activated ATPase}^*$ system

first extracted from peripheral nerve by Skou (1957) and later identified in many different types of tissue (Bonting, 1964). More recently a similar ouabain-sensitive ATPase system has been found in the intestinal mucosa of goldfish (Smith, 1964). The object of the present work therefore was to study the temperature-sensitivity of mucosal ATPase preparations taken from goldfish acclimatized to different temperatures, in the hope that this would reveal changes connected with the regulation of Na^+ transport in that tissue.

EXPERIMENTAL

Materials

Fish. Goldfish weighing 40–60 g. were obtained in one batch from Pet-Reks (Anglia) Ltd., Melbourn, Cambs. They were kept in large aquaria at room temperature and later transferred in groups of six to acclimatization tanks containing aerated water maintained at 8°, 19° or 30°. The period of acclimatization was 2–3 weeks. The whole series of experiments was complete within 2 months of receiving the fish; during this time, the acclimatization temperature selected for individual groups of fish was varied in a random manner, to avoid time-dependent changes. Fish were fed daily up to the time of the experiment; their food intake was dependent on the environmental temperature, 30°-acclimatized fish eating much more than those acclimatized to 8°.

Reagents. ATP (disodium salt) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP (tris salt) was Bioch. 1967, 105

* Abbreviation: ATPase, adenosine triphosphatase.

prepared by Dr D. C. Hardwick by passing a solution of ATP (disodium salt) through a cation-exchange column (Dowex 50) in the H^+ form, then adjusting the pH to 7.5 with tris (Rodnight & Lavin, 1966). Sodium β -glycerophosphate and strophanthin-G (ouabain) were purchased from the British Drug Houses Ltd., Poole, Dorset. All other reagents were of A.R. grade.

Methods

Enzyme preparation. Goldfish were killed by decapitation and, after removal of adhering tissues, the anterior intestine was rinsed inside and out with ice-cold 0.9% NaCl. Excess of fluid was removed on filter paper and the mucosa then scraped away from the muscle layers with a metal spatula. The mucosal scrapings were weighed and homogenized in 10 parts (v/w) of 0.25 M-sucrose containing EDTA (5 mM), sodium deoxycholate (0.1%) and histidine (60 mM) buffered to pH 7.1 with HCl. The initial dissection was carried out at 6° and the homogenization at 0°. The subsequent preparation of membrane ATPase was similar to that described by Skou (1962). The homogenate was first centrifuged at 10000g for 15 min. and the resulting supernatant centrifuged at 20000g for 60 min. to give a sediment that corresponded to the membrane ATPase fraction described by Skou (1962). A high-speed pellet was obtained finally by further centrifugation of the second supernatant fraction at 85000g for 30 min. The low-speed, medium-speed and high-speed pellets were each resuspended in histidine buffer (10 mM) at pH 7.1 before being stored, together with a sample of the high-speed supernatant fraction and part of the original homogenate, at -20° until required. All centrifugations were performed at 0° in a Spinco model L preparative ultracentrifuge (rotor AH 50).

Assay of ATPase activity. The standard incubation medium consisted of 3 mM-MgCl₂, 100 mM-NaCl, 20 mM-KCl, 3 mM-ATP and 30 mM-histidine adjusted to pH 7.1 with HCl. Enzyme preparations were added in amounts between 0.1 and 1.0 mg.; the final volume was 1.0 ml. Incubation media were equilibrated at the desired temperature for 20 min. before addition of ATP and hydrolysis was stopped after 15 min. incubation by adding 0.1 ml. of 50% (w/v) trichloroacetic acid. The suspensions were centrifuged (1400g for 5 min.) and 0.5 ml. samples of the supernatants taken for estimation of P_i by the method of Fiske & Subbarow (1925). Exactly 20 min. was allowed for the development of colour. A correction was made for the small amount of P_i liberated non-enzymically. The maximum amount of enzymically liberated P_i was equivalent to 65% of the ATP terminal phosphate and the rate at which the P_i was produced appeared to be approximately linear up to this value.

The ATPase activity of membrane preparations changes with time (Skou, 1965). For this reason estimations of ATPase activity were carried out on all fractions after storage for 1 night only. Subsequent estimations, with the 20000g membrane ATPase preparation incubated at different temperatures, were completed within 3 days of the original preparation.

Assay of β -glycerophosphatase activity. The procedure used was similar to that described for the assay of ATPase, but the concentration of histidine was decreased to 10 mM and the time of incubation increased to 30 min. The initial concentration of β -glycerophosphate was 3 mM; 0.9 ml. samples of the incubated solutions were taken for determina-

tion of P_i . For both the assay of ATPase and β -glycerophosphatase activities, results were expressed as μ moles of P_i produced/hr./mg. of protein.

Estimation of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard.

Temperature gradient. The apparatus was similar to that described by Selwyn (1961). It consisted of an ice bath connected to a heated bath by insulated copper rods having holes drilled in them to receive incubation tubes. Two tubes, one of which contained the standard incubation medium and the other standard medium plus ouabain in a final concentration of 0.1 mM, were incubated at each of ten different temperatures (5–30°), 20 tubes being incubated in all.

Electron microscopy. The fraction of goldfish mucosal homogenate that sedimented at 20000g was washed free from salt and negatively stained with 2% (w/v) ammonium molybdate, and the structure then examined at magnifications between $\times 70000$ and $\times 140000$.

RESULTS

Activation of membrane ATPase by Na^+ + K^+ and inhibition by ouabain. A 20000g sediment, prepared from the homogenized mucosa of goldfish previously acclimatized to 8°, was suspended in 10 ml. of 0.25 M-sucrose containing 60 mM-histidine (pH 7.1) and recentrifuged at 20000g for 60 min. to remove traces of Na^+ and K^+ associated with the first sediment. Portions of this second sediment, each containing 100 μ g. of protein, were then incubated in a series of media containing 3 mM-magnesium chloride, 3 mM-tris ATP, 60 mM-histidine and various amounts of sodium chloride and potassium chloride. Fig. 1 shows a pattern of (Na^+ + K^+)-activation very similar to that reported by Skou (1962) for other membrane ATPase preparations. The ATPase was more sensitive to activation by K^+ in the presence of Na^+ than to activation by Na^+ in the presence of K^+ and showed a two- to three-fold increase in activity over the concentration range 100–140 mM for Na^+ and 50–15 mM for K^+ . It was therefore decided to use 100 mM-sodium chloride and 20 mM-potassium chloride in all subsequent incubation media. Ouabain (0.1 mM) abolished the activation caused by 100 mM-sodium chloride plus 50 mM-potassium chloride; the residual ATPase activity was not appreciably different from that measured in the absence of Na^+ or K^+ . Three fish acclimatized to 19° were next killed to provide one mucosal preparation sedimenting at 20000g. Samples of this fraction were incubated under standard conditions with different concentrations of ouabain. Fig. 2 shows that in the presence of 0.01 M-ouabain the maximum ATPase activity was about half that recorded with no ouabain present. This was the maximum inhibition that could be obtained. The concentration of ouabain needed to produce half-maximal inhibition was 0.5 μ M.

Fig. 1. Activation of goldfish mucosal ATPase with $\text{Na}^+ + \text{K}^+$. The enzyme preparation consisted of a 20000g sediment of homogenized goldfish mucosa that had been resuspended in 10 ml. of 0.25M-sucrose containing 60 mm-histidine and then recentrifuged at 20000g for 60 min. Mucosal scrapings were pooled from three fish previously acclimatized to 8°. Incubation was at 37° in media containing 3 mm-tris ATP, 3 mm- MgCl_2 , 30 mm-histidine and various amounts of NaCl and KCl at pH 7.1. The procedure for incubation was as described in the text. ○, ATPase activity at different concentrations of $\text{Na}^+ + \text{K}^+$; ●, ATPase activity at 100 mm-NaCl + 50 mm-KCl in the presence of 0.1 mm-ouabain.

Fig. 2. Ouabain inhibition of goldfish mucosal membrane ATPase. The enzyme preparation was a 20000g sediment made from the pooled mucosal scrapings of three fish previously acclimatized to 19°. The ATPase activity was measured at 37° by using the incubation procedure described in the text. The reaction mixture was the standard one plus ouabain. The molar concentration of ouabain has been plotted as the negative logarithm.

Table 1. *Distribution of protein in fractions separated by differential centrifugation*

Scrapings of goldfish mucosa (1g.) were homogenized in 0.25M-sucrose containing 5 mm-EDTA, 0.1% sodium deoxycholate and 60 mm-histidine, final pH 7.1. Values give the means of 15 determinations.

Fraction	Protein (mg.)	Preparation
Homogenate (H)	99.2	—
Sediment 1	38.1	10000g for 15 min.
Sediment 2	7.8	20000g for 60 min.
Sediment 3	3.0	85000g for 30 min.
Final supernatant	42.4	85000g for 30 min.

Distribution of phosphatase activity in fractions of goldfish mucosa prepared by differential centrifugation. The centrifugation of goldfish intestine homogenates, described in the previous section, gave the characteristic distribution of protein shown in Table 1. Total protein represented 10% of the tissue wet weight; over 80% of the recovered protein came from the low-speed sediment and final supernatant fractions. This pattern of distribution did not depend on the previous acclimatization temperature of the fish. The phosphatase activities associated with these fractions, obtained from fish acclimatized to 19°, are shown in Table 2. Homogenates of goldfish mucosa hydrolysed ATP much more rapidly than β -glycerophosphate, but both phosphatase activities were concentrated in the fraction sedimenting at 20000g. β -Glycerophosphatase activity appeared to be more stable: 96% of the activity assayed in the homogenate could be recovered from the centrifuged fractions compared with 56% of the ATPase activity. The ouabain-sensitive ATPase in the homogenate was also found to be concentrated in the 20000g sediment. Electron microscopy of this fraction showed membranous structures, some of which were large and convoluted, while others were much smaller and rounded in appearance. Ruptured mitochondria were occasionally seen.

ATPase activity in the intestinal mucosa of goldfish acclimatized to different temperatures. The mucosa of goldfish acclimatized to 8°, 19° or 30° as homogenized and centrifuged to give different fractions in the usual way. The total and ouabain-sensitive hydrolysis of ATP by these fractions is shown in Table 3. The total ATPase activity in the 20000g fraction did not change with the previous acclimatization temperature of the fish ($P > 0.1$). This was also true for the original homogenates and other fractions with the one exception of the 85000g sediment, where preparations from 8°-acclimatized fish had a lower activity than preparations from 30°-acclimatized fish ($P < 0.02$). The recoveries of

Table 2. *Phosphatase activity of different centrifugal fractions of goldfish mucosal homogenates*

The procedure for fractionation is described in the text. All fish had been acclimatized to 19° for 2–3 weeks beforehand and each value is the mean of five estimations. Each estimation used pooled material from three fish. Ouabain concentration was 0.1 mM and the temperature of incubation was 37°.

Substrate	Liberation of P _i (μmoles/hr./mg. of protein)					Overall recovery (as % of H ± s.e.m.)
	Homogenate (H)	10000g sediment	20000g sediment	85000g sediment	85000g supernatant	
β-Glycerophosphate	1.24	1.31	2.25	0.80	1.16	96.4 ± 4.9
ATP (no ouabain)	21.1	21.1	34.5	11.9	2.17	56.1 ± 1.6
ATP (with ouabain)	17.1	18.4	22.1	7.0	2.08	56.5 ± 1.8
ATP hydrolysis inhibited by ouabain	4.0	2.7	12.4	4.9	0.09	58.1 ± 6.3

Table 3. *ATPase activity of different centrifugal fractions of goldfish mucosal homogenates*

Fish had been acclimatized to one of three different temperatures for 2–3 weeks beforehand and each value gives the mean of five estimations. Each estimation used pooled material collected from three fish. Ouabain concentration was 0.1 mM and the incubation temperature was 37°.

Acclimatization temp. ...	ATPase activity (μmoles of P _i /hr./mg. of protein)					
	Total hydrolysis			Ouabain-sensitive hydrolysis		
	8°	19°	30°	8°	19°	30°
Homogenate (H)	20.4	21.1	21.3	4.38	4.05	4.23
10000g sediment	20.0	21.1	23.1	2.90	2.71	4.30
20000g sediment	36.8	34.5	42.2	20.1	12.3	11.4
85000g sediment	8.03	11.9	11.7	3.24	4.86	3.70
85000g supernatant	2.17	2.17	2.27	0.16	0.09	0.05
Overall recovery	59.0	56.1	63.2	81.0	58.1	62.4
(as % of H ± s.e.m.)	± 2.5	± 1.6	± 6.6	± 21.0	± 6.3	± 8.6

ATPase were independent of environmental temperature. In contrast with these findings, the amount of ATPase activity that could be inhibited by ouabain did vary, the membrane fraction from 30°-acclimatized fish being only half as sensitive to ouabain as that obtained from preparations from 8°-acclimatized fish. This was a significant difference found only in the fraction sedimenting at 20000g. Subsequent experiments used this fraction to determine the temperature-sensitivity of ATPase.

Effect of incubation temperature on ATPase activity in goldfish intestinal mucosa. The activity of goldfish mucosal ATPase (20000g sediment) increased with increase in incubation temperature in a way that did not appear to depend on the previous acclimatization temperature of the fish (Fig. 3). The Arrhenius plots were non-linear, but the error of individual points and the complex appearance of the plots made it difficult to decide whether clear discontinuities occurred. The nature of these curves became more obvious when experiments were repeated in the presence of 0.1 mM-ouabain. The ouabain-insensitive and ouabain-sensitive

ATPase activities have been plotted in Figs. 4 and 5 respectively. There was an inverse relation between the ouabain-sensitive and ouabain-insensitive ATPase activities, with the ouabain-sensitive activity low and the ouabain-insensitive activity high in 30°-acclimatized fish. Adaptation of the fish to a low temperature favoured the ouabain-sensitive ATPase, but apparently only at the expense of the ouabain-insensitive enzyme. This relation held over the whole of the Arrhenius plot, so that adding up the total ATPase activity at any one incubation temperature always gave a total ATPase activity that was independent of acclimatization temperature (Fig. 3).

The separation of total ATPase in this way made more obvious the discontinuities in the Arrhenius plots. A first discontinuity in the ouabain-insensitive ATPase (Fig. 4) occurred at about 14°, 17° and 19° and a second discontinuity at about 22°, 26° and 28° for fish acclimatized previously to 8°, 19° and 30° respectively. The main discontinuity in the ouabain-sensitive ATPase (Fig. 5) was at about 12° and 21° for fish acclimatized to 8° and 30° respectively.

Fig. 3. Effect of incubation temperature on the total ATPase activity of goldfish mucosal membranes. The enzyme preparations were 20000g sediments of goldfish mucosal homogenates used in amounts equivalent to 150 μ g. of protein/ml. All incubation media contained 100mM-NaCl, 20mM-KCl, 3mM-MgCl₂, 3mM-ATP (disodium salt) and 30mM-histidine (pH 7.1). The procedure for incubation is described in the text. Each point is the mean of five determinations and each determination uses the ATPase preparation pooled from three fish. ●, □ and ○, ATPase activities prepared from goldfish acclimatized to 30°, 19° and 8° respectively. The vertical bars measure twice the S.E.M.

Fig. 4. Effect of incubation temperature on mucosal ATPase activity measured in the presence of 0.1 mM-ouabain. The enzyme preparations and conditions for incubation are the same as those used to measure total ATPase activity and are described in the legend to Fig. 3. Each point is the mean of five determinations, each determination being on one ATPase preparation pooled from three fish. ●, □ and ○, Ouabain-insensitive ATPase activities of membrane fractions prepared from goldfish acclimatized to 30°, 19° and 8° respectively.

Fig. 5. Ouabain-sensitive hydrolysis of ATP by membrane preparations of goldfish mucosa incubated at different temperatures. The enzyme preparations used and conditions of incubation have been described in the legend to Fig. 3. Values were obtained by subtraction of the ouabain-insensitive ATPase activities (Fig. 4) from the total ATPase activities (Fig. 3). ● and ○, ATPase activities of membrane fractions prepared from fish acclimatized to 30° and 8° respectively.

For fish acclimatized to 19° (not shown in Fig. 5) the hydrolysis at low incubation temperatures was similar to that for 8°-acclimatized fish but here the discontinuity occurred at about 16°. The similarity between these temperatures and those for the first discontinuity of the ouabain-insensitive enzyme may mean that the two activities are closely related.

DISCUSSION

Recent work has led several investigators to conclude that $\text{Na}^+ + \text{K}^+$ can together induce changes in the structure of membrane ATPase and that these changes might control the movement of Na^+ and K^+ across cell membranes (Whittam & Ager, 1965; Opit & Charnock, 1965; Squires, 1965). The degree of $(\text{Na}^+ + \text{K}^+)$ -activation decreases as the incubation temperature is lowered and no activation is found at 5° (Swanson, 1966). Moreover the decrease in $(\text{Na}^+ + \text{K}^+)$ -activation takes place in a non-linear way with two discontinuities in the Arrhenius plot at 6° and 20°, suggesting that the nature of the supposed conformational change might itself be dependent on the temperature at which it is measured (Gruener & Avi-Dor, 1966).

These results have particular relevance when applied to an animal such as the goldfish, which can live in equilibrium with its environment at the same temperatures as those where changes in ATPase have been shown to take place.

The present results show temperature-dependent discontinuities in ATPase activity that are similar in some ways to those reported by Gruener & Avi-Dor (1966) for rat-brain ATPase, but the mucosal ATPase shows changes that depend on the previous environmental temperature of the fish. This variation moves the major break in the Arrhenius plot for ouabain-sensitive ATPase activity in the same direction as the environmental temperature, though the shift is never as great as the change in environmental temperature (30°-acclimatized fish show a break at about 21°; 8°-acclimatized fish show a break at about 12°). These results suggest that the goldfish intestine is using an ATPase system in a different form (a high-temperature form) when living at 19° or 30° from that when living at 8°. Two assumptions have been made in order to reach this conclusion: first, that the sensitivity of ATPase to ouabain is equivalent to (Na⁺ + K⁺)-activation of the enzyme; secondly, that discontinuities in Arrhenius plots reflect changes in conformational structure. The first assumption is justified both by control experiments showing hydrolysis of ATP to be inhibited to an equal extent by removal of Na⁺ and K⁺ or by the addition of ouabain, and by the work of Bonting, Caravaggio & Hawkins (1963) who found a similar close correlation for many different tissues. The second assumption, however, can only be proved to be correct when it is established that the enzyme used was reasonably pure and only involved in one discrete reaction (Dixon & Webb, 1964). Unfortunately, these criteria cannot be applied to membrane ATPase systems, where attempts at purification have invariably resulted in a loss of specificity to (Na⁺ + K⁺)-activation (Skou, 1965). The detailed nature of the changes taking place with temperature must therefore remain unknown, but this does not hinder consideration of the possible effects such changes might have on Na⁺ transport in the goldfish intestine.

Until now changes in Na⁺ transport across this tissue have been examined indirectly by measuring the changes in transmural potential that take place at different incubation temperatures (Smith, 1966*a*, *b*). The recorded shifts in (Na⁺ + K⁺)-activated ATPase could provide a possible biochemical explanation for these physiological findings. The lowest incubation temperature at which glucose causes a permanent increase in the transmural potential is known to increase with acclimatization temperature over the range 15–30°, but this is not true for acclimatization temperatures below 12° (Smith, 1966*b*). Suppose glucose were to increase

Na⁺ transport by making Na⁺ more accessible to the ouabain-sensitive ATPase system. Then the significance of 12° as the lower limit for recording a glucose effect becomes clear since the ATPase system could never exist in its 'high-temperature form' at incubation temperatures below 12°. According to this reasoning acclimatization of Na⁺ transport to a high environmental temperature involves the modification or synthesis of a (Na⁺ + K⁺)-activated ATPase system that is more resistant to the effector action of glucose. There could, however, be several steps between the action of glucose and the end effect on ATPase. It is possible for instance to imagine glucose facilitating the entry of Na⁺ across the luminal membrane of the mucosal cell in a way that does not depend on ATPase, and that it is the increase in intracellular Na⁺ that produces changes in the final transmural potential through stimulation of the ATPase system known to be present in the serosal membrane of these cells (Hollands & Smith, 1964). It is impossible to distinguish between these two possibilities with preparations that contain fragments of both luminal and serosal membranes.

Histidine was always present in the incubation media used in the work reported here and it is known that histidine acts like glucose on everted sacs of goldfish intestine, stimulating the transfer of Na⁺ (Mepharm & Smith, 1966*a*). Histidine also activates ATPase in an unknown way (Skou, 1965). The discontinuities in the Arrhenius plots may have been made more obvious in the presence of histidine by activation of the membrane ATPase, but this was not checked thoroughly with other types of buffering systems.

The changes in ouabain-sensitive ATPase activity all took place within a virtually constant amount of total membrane ATPase activity. The relation between Mg²⁺-activated enzyme and (Mg²⁺ + Na⁺ + K⁺)-activated enzyme is still poorly understood (Skou, 1965), but the similarities between the Arrhenius plots for both enzyme activities [Gruener & Avi-Dor (1966) and this paper, with ouabain-insensitive ATPase as a measure of the Mg²⁺-activated enzyme] suggest that the differences between the two may be small. Increase in the ouabain-sensitive enzyme activity could result from changes in the Mg²⁺-activated ATPase already present in the membrane, but if this were so then the modification probably depends on newly formed protein because inhibition of protein synthesis with puromycin stops acclimatization to a new temperature (Smith, 1966*c*).

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