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REGULATION OF AMINO ACID TRANSPORT ACROSS INTESTINES OF GOLDFISH ACCLIMATIZED TO DIFFERENT ENVIRONMENTAL TEMPERATURES

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SUMMARY

1. Serosal transfers of valine and threonine were measured using everted sacs of anterior intestine taken from goldfish acclimatized to different temperatures.

2. Both valine and threonine were actively transported at incubation temperatures equal to or greater than the previous environmental temperature of the fish. There was also a positive serosal transfer of valine, but not threonine, at incubation temperatures below the previous environmental temperature of the fish.

3. The mean stable transmural potentials and amino-acid-evoked potentials depended both on the temperature to which the fish had been acclimatized and on the temperature at which the sacs were incubated.

4. There was a linear relation between the transmural potential and the serosal transfer of amino acid, one additional μmole of valine or threonine being transferred/2 hr incubation period for each 3 mV rise in potential. There was a less obvious correlation between the amino-acid-evoked potential and on serosal transfer of amino acid.

5. Acclimatization of the goldfish intestine from 8 to 25° C, assessed by changes occurring in the transmural potential and serosal transfer of amino acids, tended to stabilize both parameters, but the compensation in each case was only partial.

6. It is possible that the imbalance in transfer of valine-like and threonine-like amino acids, seen at incubation temperatures below the previous acclimatization temperature of the fish, has a special function in initiating the process of acclimatization to the new environmental temperature.

INTRODUCTION

Goldfish intestines, taken from fish acclimatized to 8° C and then incubated *in vitro* at 25° C, transport several amino acids against their concentration gradients, the net transfer being correlated with the steady

transmural potential measured during the period of incubation (Mepham & Smith, 1966). Acclimatization of goldfish to different environmental temperatures changes the transmural potential measured subsequently at any one incubation temperature, the potential falling as the environmental temperature increases (Smith, 1966*b*). Assuming that the correlation between transport of neutral amino acids and the transmural potential is not due to chance, then the serosal transfer of amino acids should also fall in conjunction with the transmural potential, as the acclimatization temperature is increased. The present work sets out to test this simple prediction.

Certain amino acids (e.g. valine) are transported by the goldfish intestine more readily than would be expected from a comparison with analogous data obtained for the hamster intestine (Mepham & Smith, 1966; Wiseman, 1956). Akedo & Christensen (1962) have postulated the presence of two mechanisms to explain the transport of neutral amino acids by the rat intestine; Oxender & Christensen (1963*a*, *b*) have made a detailed investigation of similar transport mechanisms in the Ehrlich ascites tumour cell. Two transport mechanisms may also operate for different neutral amino acids in the goldfish intestine and, to test this possibility, the serosal transfers of valine and threonine were compared at different incubation temperatures, using intestines taken from fish acclimatized to different temperatures.

The thermal sensitivity of the goldfish is subject to seasonal variation (Hoar, 1955). For this reason the results reported here were obtained during a single 5-week period and groups of experiments were designed so that the first and last series of experiments were duplicates. In this way any marked change due to seasonal factors could be detected immediately. In fact no changes with time were seen so that the effects of acclimatization to different temperatures could be assessed with greater confidence.

METHODS

Goldfish, weighing approximately 50 g, were obtained from the Chess Fish Farm, Chorley Wood, Herts. They were stored for 1 week in a large aquarium at room temperature and then transferred to acclimatization tanks where the water was aerated, stirred and the temperature maintained at 8, 15 or 25° C. All fish were killed during the 3rd week of acclimatization and the anterior intestine isolated and everted as described previously (Mepham & Smith, 1966). Two sacs, formed from the anterior intestine of each fish, were incubated at 8, 15 or 25° C in bicarbonate saline (Krebs & Henseleit, 1932) containing 5·6 mM D-glucose and either 10 mM L-valine or 10 mM L-threonine. Each group of experiments consisted of tests done on twelve sacs taken from six fish. Six of these sacs were incubated in bicarbonate saline containing threonine, the other six in bicarbonate saline with valine. The order in which sacs were incubated was alternated so that, on completing each group of experiments, both proximal and distal parts of the anterior intestine had been incubated an equal number of times with each amino acid. This design was followed to

minimize variations between individual fish and between local regions of each anterior intestine. Three incubation temperatures were used for intestines acclimatized to each of three environmental temperatures.

Sacs were incubated for 2 hr in 15 ml. of bicarbonate saline containing 10 mM valine or threonine and gassed with a mixture of 95 % O_2 + 5 % CO_2. The same amino-acid-containing medium (0·1 ml.) was placed in each everted sac at the start of incubation. The transmural potential was recorded during incubation through agar bridges and calomel electrodes, using a Vibron electrometer and a Xactrol pen recorder. Values were corrected for the small junction potential measured between the two calomel electrodes when both agar bridges dipped into the same solution of bicarbonate saline. Each sac was transferred, during the 2nd hr of incubation, for a period of 5 min, into bicarbonate saline with no added amino acid. The potential increased immediately the sac was replaced in amino-acid-containing medium and this increase has been called the 'amino-acid-evoked potential'.

The serosal solutions for any group of experiments, recovered at the end of incubation, were weighed individually and then pooled. Protein was removed from the pooled sample by precipitation with picric acid (Moore & Stein, 1954) and the individual amino acids estimated on an automatic amino acid analyser of the type described by Spackman, Stein & Moore (1958). The serosal transfers of threonine and valine were expressed as μmoles transferred by 100 mg of intestine in a 2 hr incubation. These values were corrected for the estimated amounts of valine and threonine which have been found to come from the intestine in the absence of any added amino acid.

RESULTS

Control experiments. Small amounts of different amino acids are found in the serosal fluid of goldfish intestinal sacs after incubation for 2 hr at 25° C in bicarbonate saline (Mepham & Smith, 1966). It was possible that the previous acclimatization temperature of the fish, and the temperature at which sacs were incubated, might change the rate at which tissue amino acids leached into the serosal fluids. To test this possibility, the quantity of alanine and leucine which appeared in the serosal fluid of sacs incubated in bicarbonate saline containing only valine or threonine was estimated at three different incubation temperatures. Alanine and leucine were chosen because they showed high control effluxes. Table 1 shows that alanine appeared at rates which varied from 0·09 to 0·26 μmoles/100 mg intestine . 2 hr and threonine from 0·05 to 0·30 μmoles/100 mg intestine . 2 hr. The rate at which these amino acids appeared seemed to increase both with the temperature of incubation and of acclimatization, but there were several exceptions to this general pattern. Changing the incubation or acclimatization temperature of the intestine altered the efflux of endogenous alanine and leucine in the same direction and it was assumed that changes in valine and threonine transfers would likewise parallel changes of the other two amino acids. The relation between control effluxes of all four amino acids was known (Mepham & Smith, 1966) and, in experiments recorded in Table 1, the estimated serosal appearance of valine and threonine has been calculated from the determined values for alanine and leucine. These estimated values agreed closely whether estimated from

the amount of alanine or of leucine. For incubation temperatures above the previous acclimatization temperature of the fish, the estimated contribution of endogenous valine and threonine to the total serosal transfer of these amino acids was negligible; at incubation temperatures equal to the previous acclimatization temperature of the fish, the estimated contribution varied from 10 to 30% of the total serosal transfer. The estimated control efflux of threonine, at incubation temperatures below the previous acclimatization temperature of the fish, exceeded the total serosal transfer. The serosal transfers of valine and threonine have been corrected throughout for the estimated contribution of endogenous amino acids. This corrected transfer is probably still only an approximation of the true transfer where the total transfer was low, because of the assumptions made in calculating control effluxes.

TABLE 1. Appearance of endogenous amino acids in the serosal fluid of everted sacs of goldfish anterior intestine measured after a 2 hr incubation in bicarbonate saline containing 5·6 mM glucose and 10 mM valine or threonine. The amounts of endogenous valine and threonine were estimated from the experimentally determined content of alanine and leucine, assuming that the efflux of amino acids was in the same proportion as found previously (Mepham & Smith, 1966). Each value is from a pooled sample of twelve experiments

Temperature (°C)		Serosal appearance of amino acids (μmole/100 mg intestine. 2 hr)			
		Determined		Estimated	
Incub.	Acclim.	Alanine	Leucine	Valine	Threonine
8	8	0·09	0·06	0·04	0·02
15	8	0·13	0·14	0·08	0·04
25	8	0·10	0·05	0·04	0·02
8	15	0·14	0·10	0·06	0·03
15	15	0·19	0·13	0·09	0·05
25	15	0·26	0·30	0·16	0·08
8	25	0·13	0·17	0·08	0·04
15	25	0·20	0·18	0·10	0·05
25	25	0·15	0·16	0·09	0·05

Effect of temperature acclimatization on the transmural potential of goldfish intestine incubated in the presence of valine or threonine. Figure 1 shows potential differences measured across goldfish anterior intestines incubated at 8, 15 and 25° C. Valine or threonine was present in the serosal fluids throughout the experiments but absent from mucosal fluids during the time period shown between the arrows. Changes in transmural potential were similar for both amino acids. Potentials increased as the temperature of incubation was raised, for all acclimatization temperatures. Previous acclimatization of the fish to a high environmental temperature reduced the transmural potential measured at any one incubation temperature, except for intestines incubated at 8° C from '15° C-acclimatized' fish. Threonine or valine inhibited the transmural potential measured at 8° C

with intestines acclimatized previously to 25° C. These findings are similar to others reported previously for the effects of glucose on transmural potentials (Smith, 1966*b*).

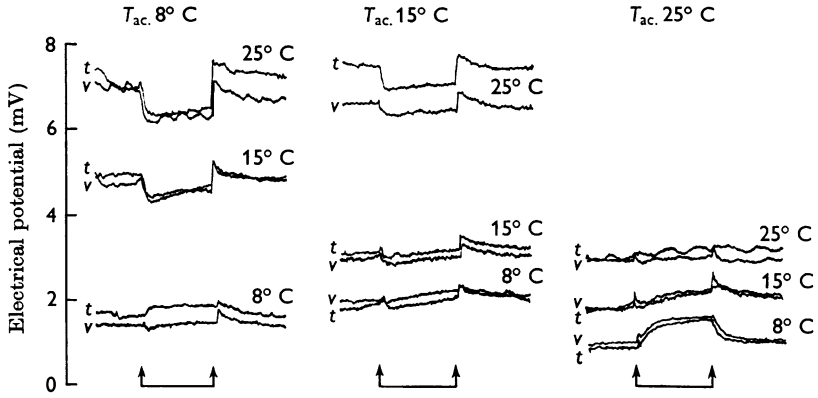

Fig. 1. Effect of valine and threonine on the transmural potentials of goldfish intestine measured at different incubation temperatures. $T_{ac.}$ acclimatization temperature; *v*, L-valine; *t*, L-threonine; incubation temperatures are shown to the right of each pair of braces. Everted sacs were placed in amino-acid-free bicarbonate saline for 5 min, shown in the figure by the arrows.

The mean steady transmural potentials obtained at three incubation temperatures, using intestines from fish acclimatized to different environmental temperatures, are shown in Fig. 2. The potentials using valine or threonine, measured at any one incubation temperature, were similar. The mean transmural potentials, measured at incubation temperatures equal to the previous environmental temperature of the fish, doubled as the acclimatization temperature changed from 8 to 25° C. If no acclimatization had taken place the steady potential would have increased about four times (8° C intestines compared at incubation temperatures of 8 and 25° C). These compensating changes were clearly evident but not sufficient to stabilize the transmural potential completely. The first group of experiments, 8° C intestines incubated at 25° C, was repeated at the end of the series. Figure 2 shows no significant difference between these two sets of figures, eliminating the possibility that changes within the series were due to seasonal variations.

Part of the transmural potential is normally dependent on valine or threonine coming in contact with the mucosa (Fig. 1), and changes in the magnitude of the amino-acid-evoked potentials are therefore reflected as changes in the steady potential. Figure 3 shows valine- and threonine-evoked potentials measured across intestines taken from fish acclimatized to 8 and 25° C, measured at incubation temperatures of 8, 15 and 25° C. Threonine-evoked and valine-evoked potentials changed in the same way

as their respective steady transmural potentials, increasing as the incubation temperature was raised or the environmental temperature lowered. Compensation of this mechanism was complete, about 0·3 mV for an 8° C intestine incubated at 8° C and 0·2 mV for a 25° C intestine incubated at 25° C. Full compensation of an amino-acid-evoked potential at different environmental temperatures, associated with only partial regulation of steady potentials, implies that the steady potential in the absence of amino

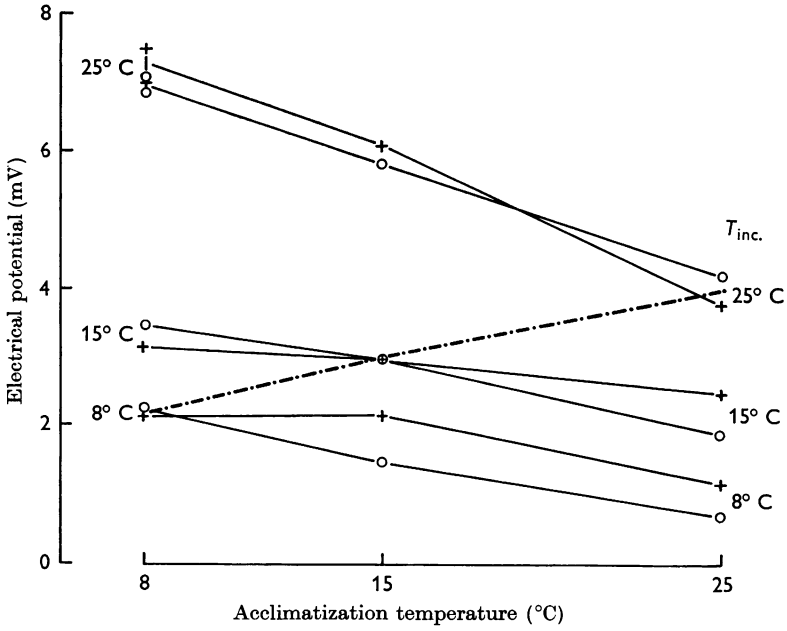

Fig. 2. The effect of temperature acclimatization on stable transmural potentials of goldfish intestine measured at different incubation temperatures in the presence of D-glucose (5·6 mM) and either 10 mM L-valine —+—, or 10 mM L-threonine —○—. The heavy interrupted line shows the transmural potentials of everted sacs incubated at their previous environmental temperature. $T_{inc.}$, incubation temperature. Each point is the mean of six determinations.

acids, but with glucose present, is not changing with alterations in environmental temperature. But compensation of steady potentials in the presence of glucose alone has been shown previously to be complete under similar conditions (Smith, 1966*b*). Obviously other factors are operating when glucose and amino acids are presented together to the luminal membrane of the mucosa.

Effect of temperature acclimatization on the serosal transfers of valine and threonine measured at different incubation temperatures. The serosal transfers of valine and threonine, corrected for the estimated contribution of endogenous amino acids, are shown in Fig. 4. For each of the three acclima-

tization temperatures used, the transfer of valine and threonine increased as the incubation temperature was raised. A difference between threonine and valine transport was detected at incubation temperatures below the previous acclimatization temperature of the fish (to the right of the interrupted line, Fig. 4) where valine transfer was positive and threonine transfer negative. Intestines acclimatized to 15° C transported valine and

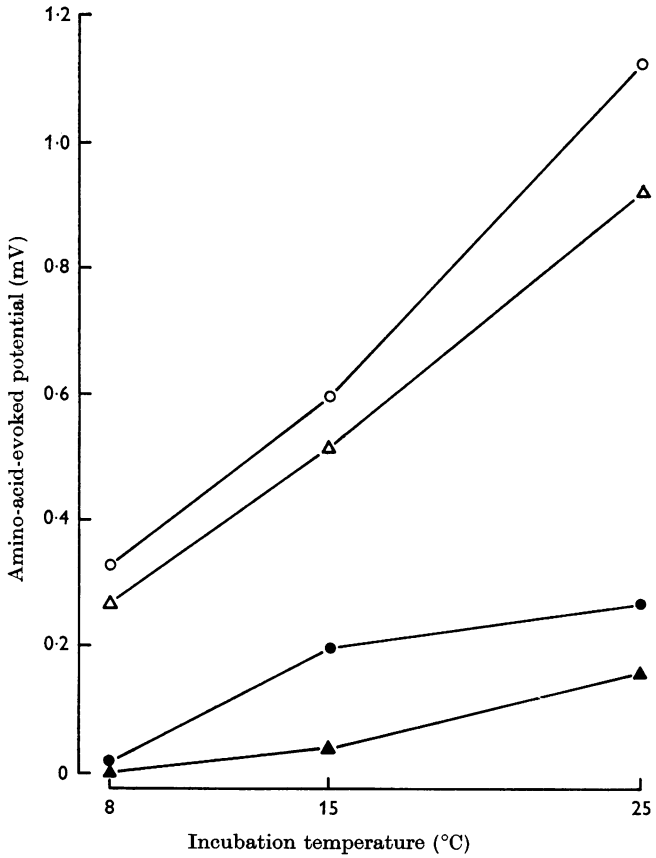

Fig. 3. Amino-acid-evoked potentials for valine (triangles), and threonine (circles) determined at different incubation temperatures. The fish had been acclimatized to 8° C (open symbols) or 25° C (filled symbols) for at least 2 weeks.

threonine at similar rates to 8° C intestines when measured at incubation temperatures of 15 and 25° C, and acclimatization of amino acid transport was more easily seen with 25° C intestines incubated at 8, 15 or 25° C. The serosal transfers of valine and threonine, measured at incubation temperatures equal to the previous acclimatization temperature of the fish, rose as the acclimatization temperature increased from 8 to 25° C (see Fig. 4),

showing that acclimatization was not perfect (type 3 regulation—Precht, 1958).

Comparison of serosal transfers of amino acids with steady and amino-acid-evoked potentials of goldfish intestine. The serosal transfers of valine and threonine, measured at three incubation temperatures using intestines from fish acclimatized to three different temperatures, have been collected and plotted against the corresponding steady transmural potentials in Fig. 5. There is a clear and close correlation between the rate of serosal

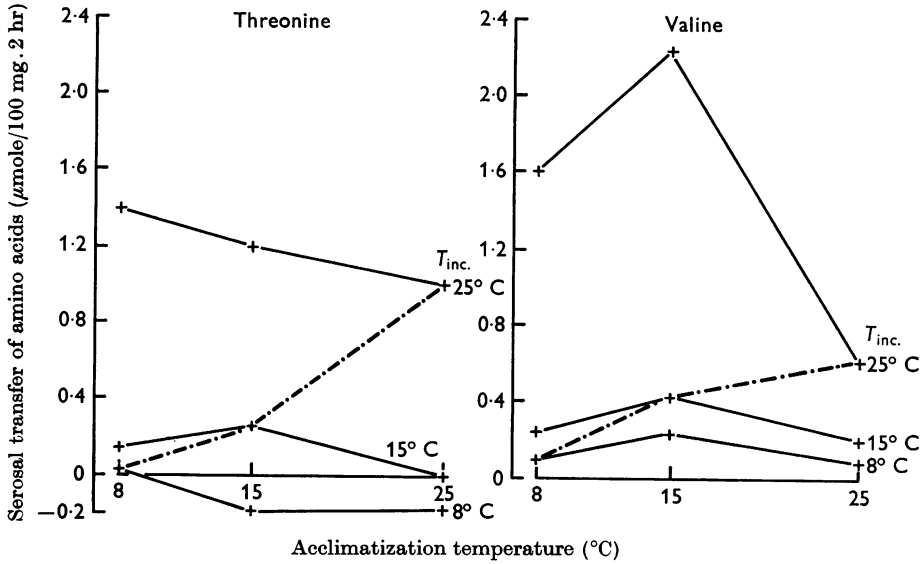

Fig. 4. Serosal transfers of threonine and valine across everted sacs of goldfish anterior intestine measured at different incubation temperatures. Sacs were incubated for 2 hr in bicarbonate saline containing D-glucose (5·6 mM) and the amino acid under test in a concentration of 10 mM. The heavy interrupted line shows the serosal transfers of these amino acids measured at incubation temperatures equal to the previous environmental temperature of the fish. $T_{inc.}$, incubation temperature. Each point is one determination made on a pooled sample from six experiments.

appearance of amino acids and the recorded transmural potentials. The best straight line for all points, excluding those obtained with threonine at incubation temperatures below the acclimatization temperature of the fish, has a slope of 1 μmole/3 mV. There is a residual potential of about 2 mV which has to be exceeded before this relation becomes established. The amino-acid-evoked potentials also increased when amino acids were transferred at high rates (Fig. 6) but the correlation was not as close as when the comparison was made with the steady potential. About 2 μmoles of amino acid were transferred/1 mV increase in the amino-acid-evoked

potential (cf. 1 μmole/3 mV steady potential). A small evoked potential appears under some circumstances to be multiplied into a large steady potential, a phenomenon which had been suspected from earlier studies (Mepham & Smith, 1966).

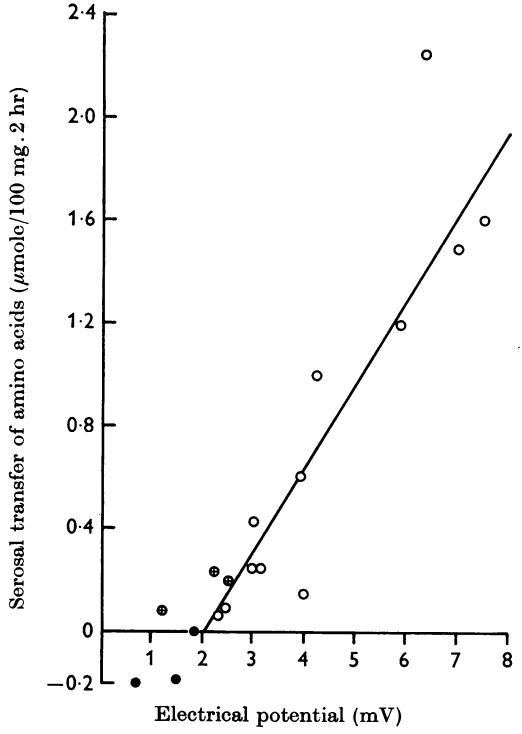

Fig. 5. Serosal transfers of threonine and valine across everted sacs of goldfish anterior intestine plotted against the corresponding transmural potentials. The fish had been acclimatized to different temperatures and the intestines were incubated for 2 hr at different temperatures in bicarbonate saline containing 10 mM L-valine or 10 mM L-threonine. ○, valine or threonine transfer measured at an incubation temperature equal to or higher than the previous environmental temperature of the fish; ⊕, valine transfer and, ●, threonine transfer measured at incubation temperatures below the previous environmental temperature of the fish. Each potential is the mean of six determinations and each serosal transfer one estimate of a pooled sample taken fron the same six experiments.

DISCUSSION

The present results confirm that the serosal transfer of amino acids is related to the transmural potential. The exact relation between the two is obtained by first subtracting the residual potential, caused partly by the presence of glucose, from the total potential measured with an amino acid present, then comparing this corrected potential with the amount of

amino acid transferred. Provided that the incubation temperature has exceeded the previous temperature of acclimatization, one additional μmole of amino acid is transferred/2 hr incubation, for each 3 mV rise in potential. This ratio of 1 to 3 remains constant when the potential is varied by changing either the temperature of incubation or acclimatization and the constant also applies for any one of eight actively transported amino acids tested (Mepham & Smith, 1966). The origin of the transmural potential appears to be the basal membrane of the mucosal cell when measured under

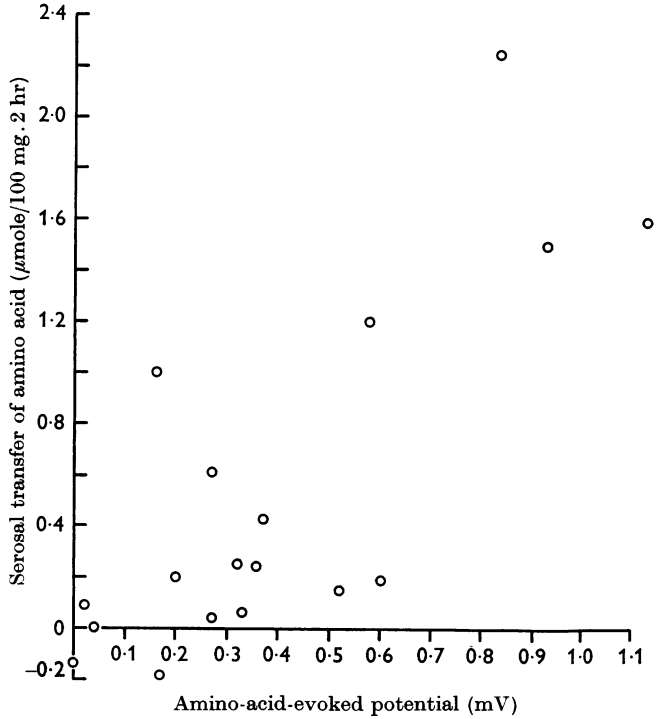

Fig. 6. Serosal transfers of valine and threonine by goldfish anterior intestine plotted against the corresponding amino-acid-evoked potential. The fish had been acclimatized to different temperatures and the conditions of incubation were as stated in Fig. 5. Each amino-acid-evoked potential is the mean of six determinations and each serosal transfer one estimate of a pooled sample taken from the same six experiments.

steady-state conditions (Gilles-Baillien & Schoffeniels, 1965) and the accumulation of actively transported amino acids, shown to take place within mucosal cells (Agar, Hird & Sidhu, 1954; Rosenberg, Coleman & Rosenberg, 1965), suggests that this membrane also acts as the rate-limiting barrier to the transfer of amino acids.

Efflux of amino acids may be mediated by a carrier (Akedo & Christen-

sen, 1962) and diffusion of amino acids into and out of mucosal cells is known to be affected by changes in the concentration of sodium ions (Rosenberg *et al.* 1965). Actively transported amino acids raise the transmural potential, the short-circuit current, and the active transport of sodium across rabbit ileum (Schultz & Zalusky, 1965), and it is possibly this fraction of the sodium transport, induced by amino acids and recorded in the present experiments as an increase in the transmural potential, which is directly linked to the efflux of amino acids. In a recent review, Curran (1965) has pointed out the difficulties of equating changes in potential difference with changes in sodium transport, because, although sodium fluxes across the small intestine have usually been accounted for by the current needed to abolish this potential (Schultz & Zalusky, 1964; Asano, 1964), this has not been invariably so (Barry, Smyth & Wright, 1965). In view of these findings, the assumption that potential is caused by sodium transport must be made with some reservation.

The serosal transfer of valine and threonine, measured at one incubation temperature, fell as the acclimatization temperature increased. Compensation of the transfer mechanism was clearly evident, but not complete (type 3 regulation—Precht, 1958). A similar partial regulation was shown for the transmural potential. Stabilization of transmural potential with only glucose in the bicarbonate saline had been shown previously to be perfect over the same 8–25° C range of environmental temperature (Smith, 1966*b*). In the present experiments, sodium transport was being stimulated by both glucose and an amino acid and it may be that the efficiency of regulation is reduced slightly as the number of substances transported across the intestine, at any one time, increases.

The process of acclimatization in the goldfish intestine, explained on the basis of a previous hypothesis (Smith, 1966*a*, *b*) and now extended to explain regulation of amino acid transfer, suggests that the goldfish mucosa synthesizes new carrier molecules under the stimulus of a changed body temperature. It is thought that these carrier molecules facilitate the entry of sodium ions into the mucosa and that they can be activated by certain sugars and amino acids, but only at temperatures equal to or greater than the environmental temperature of the fish. Puromycin retards these changes, suggesting that acclimatization is dependent on the normal functioning of protein synthesis (Smith, 1966*c*). So far it has not been necessary to postulate that activation of sodium influx is directly linked to the influx of amino acids (Mepham & Smith, 1966) because sodium reaching the basal membrane of the mucosal cell would itself stimulate the sodium pump, which could then determine the rate of efflux (and thereby the entry) of amino acids. However, Rosenberg *et al.* (1965) showed that sodium directly affects the entry of amino acids into mucosal cells and

both may enter the cell together. But, whether or not this occurs in the goldfish, the ability of the mucosal cell to regulate its sodium transport at widely different body temperatures appears to be the key to the subsequent control of amino acid transport.

This discussion has so far dealt with points of similarity between the transports of valine and threonine across the goldfish intestine. There was one important difference between these two amino acids. Valine was still transferred from the mucosa to serosa of intestines incubated at a temperature 17° C below the previous environmental temperature of the fish. Under these same conditions the net transfer of threonine was negative. In fact it seems likely that threonine transport failed immediately the incubation temperature fell below the acclimatization temperature. For other tissues it has been suggested that amino acids like valine or leucine move across membranes partly by mediated diffusion (with a low temperature dependence), while other neutral amino acids like alanine or threonine are more actively concentrated within the cell (with a high temperature dependence) (Oxender & Christensen, 1963*a*, *b*; Christensen, 1964). Probably valine moves into and across goldfish mucosal cells by some form of diffusion at low incubation temperatures and, in addition, is transported like threonine by an alanine-preferring mechanism at high incubation temperatures. There is already evidence that goldfish intestines transport relatively more valine than the hamster intestine (Mepham & Smith, 1966). This may have a particular relevance to acclimatization processes within the goldfish mucosa. Valine transported into *Escherichia coli* stimulates RNA synthesis and the protein synthesized subsequently contains an increased amount of valine (Cohen, 1958). The goldfish, when subjected to either a rise or a fall in environmental temperature, will transfer valine into mucosal cells relatively more easily than other amino acids. If this imbalance between valine and other amino acids were to stimulate RNA synthesis in the goldfish intestine, this could trigger the synthesis of new carrier molecules specifically designed to restore homeostasis at the new environmental temperature. This is of course highly speculative, but such control would be consistent with the finding of a lag period before acclimatization (Smith, 1966*d*) and a greatly increased rate of protein synthesis at the time of acclimatization (D. Morris & M. W. Smith, unpublished results) and would explain why puromycin extends the time-lag before acclimatization is completed (Smith, 1966*c*).

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