Protein Synthesis in the Intestine of Goldfish Acclimatized to Different Temperatures

By D. MORRIS* AND M. W. SMITH

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(Received 20 July 1966)

1. The incorporation of [^{14}C]valine and [^3H]threonine has been studied in mucosa of goldfish acclimatized to different temperatures. 2. [^{14}C]Valine was incorporated more rapidly than [^3H]threonine at acclimatization temperatures below 15°; this distinction disappeared at higher temperatures. 3. The rate of amino acid incorporation rose as the environmental temperature was increased. 4. The number of valine residues in mucosal protein fell and the number of methionine residues rose as the acclimatization temperature was raised. 5. The concentration of free methionine was closely related to the amount in protein in goldfish mucosa; both increased at higher acclimatization temperatures. 6. An attempt is made to show that these changes are integrated with possible homeostatic mechanisms in the goldfish intestine.

The rate at which radioactively labelled amino acids are incorporated into goldfish mucosal protein reaches a maximum 10 hr. after the transfer of cold-adapted fish to warm water (Smith & Morris, 1966). Then 5–10 hr. later the transmural potential of the intestine changes to resemble that recorded across the intestines of fish fully acclimatized to warm water (Smith, 1966b). This delayed increase in protein synthesis and the subsequent change in cell-membrane characteristics can be prevented by the previous injection of actinomycin D and puromycin respectively (Smith & Morris, 1966; Smith, 1966c). An ability to reorganize protein synthesis appears a prerequisite for the completion of acclimatization.

Recent information suggests that acclimatization might be associated with the permanent modification of amino acid transport mechanisms; valine transport is consistently greater than threonine at low acclimatization temperatures (Mephram & Smith, 1966). Permanent changes in protein metabolism may be produced by a change in acclimatization temperature. The purpose of the present work was to relate these qualitative shifts in the transport of amino acids with possible changes in pool sizes and rates of incorporation into protein. The amino acid composition of mucosal protein was also determined and an attempt made to integrate observed changes with possible mechanisms of cellular homeostasis.

* Present address: Department of Physiology and Biochemistry, University of Southampton.

EXPERIMENTAL

Materials

Fish. Goldfish weighing about 50 g. were obtained from Pet-Reks (Anglia) Ltd., Melbourn, Cambs. They were kept in a large aquarium at room temperature for 1 week and then transferred to acclimatization tanks where the water was stirred, aerated and maintained at a number of constant temperatures for 2 weeks. Fish were fed daily up to the time of the experiments.

Chemicals. DL-[^{14}C]Valine (33.9 $\mu\text{C}/\mu\text{mole}$) and DL-[^3H]threonine (66.1 $\mu\text{C}/\mu\text{mole}$), obtained from The Radiochemical Centre, Amersham, Bucks., were combined and diluted with 0.9% NaCl so that 0.1 ml. of final solution contained 20 μC of each amino acid. D.E.M. dioxan-based scintillation fluid was purchased from Panax Equipment Ltd., Redhill, Surrey. Hyamine hydroxide [*p*-(di-isobutyl-cresoxyethoxyethyl)dimethylbenzylammonium hydroxide] was supplied as a 2M solution in methanol by Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from the Packard Instrument Co. Ltd., Downers Grove, Ill., U.S.A.

Methods

Administration of labelled amino acids and removal of mucosa. Fish, acclimatized to 3°, 8°, 15°, 20°, 25°, 30° and 35°, were injected intraperitoneally with 0.1 ml. of the mixture of radioactive amino acids (40 μC) and returned within 10 sec. to the acclimatization tanks. Then 30 min. later each was decapitated and the anterior intestine dissected free from adhering tissue. The intestine was rinsed inside and out with 0.9% NaCl, lightly blotted on filter

paper and the mucosa separated from the muscle by drawing a spatula along the intestine. The mucosal scrapings were quickly weighed on a torsion balance, homogenized in 1 ml. of ice-cold 0.9% NaCl and re-homogenized after addition of 1 ml. of 24% (w/v) trichloroacetic acid. Experiments were performed in triplicate for each acclimatization temperature.

Isolation of mucosal protein. The trichloroacetic acid homogenate was quantitatively transferred to a centrifuge tube with the aid of two successive 2 ml. washings with 12% (w/v) trichloroacetic acid and heated at 90° for 10 min. After cooling to room temperature the homogenate was centrifuged (1400g for 5 min.) and the centrifugate washed at the centrifuge with two successive portions (5 ml.) of 12% (w/v) trichloroacetic acid. All supernatants were combined and stored at 0°. The crude protein precipitate was then extracted with 5 ml. of chloroform-methanol (1:1, v/v) containing 0.25% (v/v) of conc. HCl and left for 20 min. before centrifugation. This procedure was repeated with acidified chloroform-methanol (2:1, v/v). Finally the protein was washed twice at the centrifuge with sodium-dried ether and dried *in vacuo* over P₂O₅. Control experiments, in which 1 μ C of radioactive amino acid in trichloroacetic acid was added to a saline mucosal homogenate obtained from fish not previously injected, showed that all contaminating radioactivity was removed by this procedure.

Radioactivity measurements. For estimation of protein radioactivity, weighed amounts of protein (approx. 5 mg.) were placed in 20 ml. counting vials and 1 ml. of 2M-Hyamine hydroxide in methanol was added. After they had stood overnight the vials were heated at 60° for 1 hr. and 14 ml. of AnalaR toluene containing 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene was added. The ³H and ¹⁴C content of the samples was measured in a two-channel Packard scintillation spectrometer. A minimum of 10000 counts was recorded in each channel and corrections were made for background with Hyamine-containing vials. The mean efficiency of detection of the two isotopes, obtained by the successive addition and counting of known amounts of ³H- and ¹⁴C-labelled toluene was, in the red channel: ¹⁴C, 37%, ³H, 2%; in the green channel: ¹⁴C, 35%; ³H, 11%. The results were analysed by the discriminator-ratio method (Okita, Kabara, Richardson & LeRoy, 1957) and, after correction for efficiency, expressed as counts/min./mg. of protein.

For estimation of the threonine and valine radioactivity in the mucosal amino acid pool, the combined trichloroacetic acid supernatants (see above) were extracted six times with 20 ml. portions of ether to remove trichloroacetic acid and the aqueous phases were evaporated to dryness in a rotary evaporator at 35°. The residue was dissolved in sufficient 0.2M-sodium citrate buffer (Moore & Stein, 1954) to give a solution equivalent to 100 mg. wet wt. of mucosa/ml. Samples (0.1 ml.) from each extract were placed in vials containing 10 ml. of D.E.M. phosphor and counted as described above. The mean efficiencies, with [³H] water and [¹⁴C]acetic acid as internal standards were, in the red channel: ¹⁴C, 43%; ³H, 2%; in the green channel: ¹⁴C, 35%; ³H, 11%. The results were analysed as before and expressed as counts/min./g. wet wt. of mucosa.

Amino acid analysis. The amino acid composition of mucosal protein (after hydrolysis in 6N-HCl in a sealed tube at 100° for 18 hr.) and of mucosal amino acid pool was determined by the method of Spackman, Stein & Moore

(1958). One estimation was made for each acclimatization temperature with combined triplicate samples for both protein hydrolysates and pool amino acids. Methionine estimations do not allow for small amounts of methionine sulphoxides formed during hydrolysis and they therefore represent minimum values.

RESULTS

Incorporation of valine and threonine into goldfish mucosal protein. The specific activities (counts/min./ μ mole) of valine and threonine in the mucosal protein were calculated for each of the environmental temperatures. These results are compared in Fig. 1 with the corresponding values for the mucosal valine and threonine pools. The specific activities of both valine and threonine in mucosal protein increased markedly as the environmental temperature increased. The specific activities of threonine were lower and more temperature-dependent than those of valine below 15°, but at higher temperatures these differences became insignificant. Estimates of the incorporation of radioactivity into protein amino acids necessarily represent the integrated incorporation over 30 min., during which time the specific activity of the amino acid pool is almost certainly changing. This makes it impossible to calculate the absolute rate of

Fig. 1. Specific activities (counts/min./ μ mole) of valine and threonine in amino acid pool and mucosal protein of goldfish acclimatized to different temperatures. A mixture of DL-[1-¹⁴C]valine and DL-[³H]threonine were injected intraperitoneally into goldfish. After 30 min. incorporation the radioactivity and concentration of these amino acids in the protein and amino acid pool were determined. For details see the text. ○, Pool threonine; ●, pool valine; □, protein threonine; ■, protein valine.

Table 1. *Amino acid composition of bulk mucosal protein from goldfish acclimatized to different temperatures*

The protein from three fish at each temperature was combined, hydrolysed with 6N-HCl at 100° in a sealed tube for 18 hr. and the amino acids were estimated on an automatic amino acid analyser. Mean concentrations are for the environmental temperatures 3–35° and the range about the mean is also given. The variance ratio is the variance due to regression divided by the variance due to all other causes and the probability gives the significance of regression with environmental temperatures. Numbers underlined show ratios that are significantly different from zero.

Amino acid	Concn. (μ moles/m-mole of total N)		Variance ratio	Probability
	Mean	Range		
Asp	82.9	77.1–88.6	0.02	> 0.2
Thr	45.7	43.6–47.1	0.00	> 0.2
Ser	46.4	43.6–50.0	0.00	> 0.2
Pro	32.1	27.8–37.1	2.12	> 0.2
Glu	86.4	77.1–94.3	0.24	> 0.2
Gly	47.1	45.7–47.9	0.86	> 0.2
Ala	60.0	55.0–74.3	0.44	> 0.2
Val	49.3	38.6–62.1	7.37	< 0.05 > 0.01
Met	7.9	4.3–14.3	<u>13.50</u>	< 0.05 > 0.01
Ile	33.6	32.1–35.0	1.57	> 0.2
Leu	55.7	52.1–57.1	0.11	> 0.2
Tyr	17.9	15.7–20.7	1.20	> 0.2
Phe	31.4	28.6–36.4	1.28	> 0.2
Lys	53.6	52.1–55.0	0.34	> 0.2
His	15.0	10.7–18.6	2.58	< 0.2 > 0.1
Arg	39.3	35.0–43.6	0.18	> 0.2

incorporation of valine and threonine into protein. Nevertheless, as is shown in Fig. 1, the specific activities of the two amino acids determined at the end of the incorporation period were similar to each other at each acclimatization temperature, and relative differences between incorporated valine and threonine below 15° are therefore likely to be significant.

Amino acid composition of goldfish mucosal protein. The amino acid composition was expressed as μ moles of amino acid/m-mole of total recovered nitrogen. This enabled us to make comparisons between the amounts of a single amino acid at different acclimatization temperatures or between different amino acids at the same acclimatization temperature. Table 1 shows the mean amino acid composition of mucosal protein isolated from fish acclimatized to temperatures ranging from 3° to 35°. Only valine, methionine and histidine, out of the 16 amino acids analysed, showed marked variation with environmental temperature. The variance of the slope of the temperature–amino acid concentration plot was calculated and compared with the variance due to all other causes, to test whether this variation was linearly related to temperature. There was a significant correlation between environmental temperature and concentration of amino acid for valine and methionine ($P < 0.05$ in both cases), but the variation in histidine was not significant

Fig. 2. Amino acid composition of goldfish mucosal protein extracted from fish acclimatized to different temperatures. ●, Valine; ○, threonine; ■, methionine. For experimental details see the text.

($P > 0.1$). The actual concentrations of valine and methionine are plotted at the different environmental temperatures and compared with those for threonine in Fig. 2. The concentration of valine in mucosal protein was higher than that of threonine for most acclimatization temperatures, but the

Table 2. *Free amino acid pools of goldfish mucosa*

Each estimate is obtained from the pooled extracts of three goldfish mucosae. The amino acids were separated and estimated on an automatic amino acid analyser.

Acclimatization temp.	Concn. of amino acids (μ moles/10g. wet wt. of mucosa)			Val/Thr ratios
	Met	Val	Thr	
3°	1.0	5.3	4.6	1.2
8	1.1	4.4	3.5	1.3
15	1.5	6.6	3.7	1.8
20	2.2	3.5	3.4	1.0
25	2.3	3.6	5.8	0.6
30	1.8	4.9	5.0	1.0
35	2.9	4.3	4.8	0.9

difference decreased as the environmental temperature was raised until at 35° the valine concentration was less than that of threonine. The methionine content of mucosal protein, plotted on a different scale because of the small amounts present, showed an overall increase at higher acclimatization temperatures.

Free pools of amino acids in goldfish mucosa and their relation to protein composition. The free mucosal pools of valine, threonine and methionine were determined by estimating their concentrations in the trichloroacetic acid supernatant fractions, saved after the original precipitation of mucosal proteins (see the Methods section). The pools for these three amino acids are shown in Table 2. The methionine pool increased with the acclimatization temperature but the changes in valine and threonine pools were not consistent. However, the valine pool was always larger than that for threonine over the temperature range 3–15° and the opposite was true for the range 20–35°. These changes were similar to those found previously in the mucosal protein.

The free pools are plotted against the amounts found in mucosal protein in Fig. 3 to determine more exactly the relation between pool size and protein composition. The amount of methionine in protein appeared to be directly related to the size of the free-methionine pool. No similar correlation was apparent for valine or threonine, but this may be because large amounts of valine and threonine are normally present free in mucosal cells.

DISCUSSION

Goldfish show a marked individual variation in the rate at which their intestinal mucosae synthesize protein when acclimatizing to a new temperature (Smith & Morris, 1966). Much of this variation has been controlled in the present work by the use of

Fig. 3. Relation between the free amino acid pools of goldfish mucosa and the amino acid composition of goldfish mucosal protein. Each point is one estimate on pooled samples from three fish. The fish were acclimatized to different temperatures between 3° and 35°. ●, Valine; ○, threonine; ■, methionine. For experimental details see the text.

fish fully acclimatized to different temperatures. The rate at which valine and threonine are incorporated into protein has been related to their specific activities, determined in the mucosa at the end of each experiment, and all values are based on the mean of three experiments. The concentrations of free amino acids in fish tissue vary independently of each other with the time of year (Jones, 1959), but our experiments, performed in one day, cannot be subject to this type of variation. Valine and threonine were injected simultaneously into each fish to eliminate differences in efficiency of absorption after injection. The amino acid analyses showed no consistent change for most amino acids and this constancy gives added significance to the changes in valine and methionine concentrations that did take place.

The change in methionine pools within mucosal cells might have occurred by chance through fish eating more at the higher environmental temperatures. There is also evidence to suggest that the transport of amino acids across these cells is modified by acclimatization (Mephram & Smith, 1966) and, if methionine were similarly affected, this could also lead to the maintenance of different methionine pools at the different temperatures. The amount of methionine in protein was proportional to the free-methionine pool in the mucosa. Protein synthesis in cultured mammalian

cells can depend on the intracellular concentration of amino acids, any amino acid limiting its own incorporation into protein at very low concentrations (Eagle, Piez & Levy, 1961). This experimental situation may have become a physiological reality for the goldfish, but this could not by itself explain the changes in amino acid composition that took place.

The number of valine residues in mucosal protein increased as those of methionine fell. A similar change takes place when bacteria are cultured in increasing concentrations of valine; it was suggested that valine substituted directly for methionine (Cohen, 1958). More recently two s-RNA (transfer RNA) species have been isolated from *Escherichia coli*, one for valine and one for methionine, both recognizing the same codon GUG (Kellogg, Doctor, Loebel & Nirenberg, 1966). Substitution of methionine for valine in mucosal protein might be explained by the predominance of methionine-s-RNA (methionine-s-RNA I of Kellogg *et al.* 1966; methionine-s-RNA₂ of Clark & Marcker, 1966) at the higher environmental temperatures. It has been suggested that cellular homeostasis might be controlled by allosteric enzymes (Monod, Changeux & Jacob, 1963) and adaptation to different temperatures may well involve the new synthesis of different allosteric proteins (Smith, 1966a). The increase of

methionine in mucosal protein, at the expense of valine, might reflect part of this change.

The rate of incorporation of valine into bacterial protein, or into the protein of cultured mammalian cells, can be a function of its availability in the free pool (Britten & McClure, 1962; Eagle *et al.* 1961) and it is difficult in these cases to assess the importance of transport mechanisms in controlling the pool size (Riggs & Walker, 1963). The interrelation between the transport of amino acids, their free pools and the amino acid composition of hydrolysed protein, together with their overall dependence on the environmental temperature of the fish, is shown in Fig. 4. Data are presented in terms of valine/threonine ratios to allow comparison with previous results on the transport of these two amino acids across the mucosal cell (Mephram & Smith, 1966). The valine/threonine ratios of the amino acid pools and those of protein are generally lower at high environmental temperatures. This trend is also seen with the valine/threonine ratios for the transport across the mucosa, but there are differences in the slopes of the three curves, the transport of amino acids being more sensitive to acclimatization than the free pools or protein composition. Nevertheless valine is incorporated into protein much more rapidly than threonine at low environmental temperatures, suggesting a more rapid turnover of valine-enriched protein at these low temperatures.

The thermal adaptability of the goldfish mucosa provides an unusual opportunity for studying cellular control mechanisms. These involve the modification of amino acid transport mechanisms and the ability to reorganize protein synthesis. Previous evidence suggested that an initial reorganization of protein synthesis led to a change in transport mechanisms (Smith & Morris, 1966) and present work implies that changes in amino acid transport mechanisms permanently modify protein synthesis within the mucosa. The exact nature of these interactions remains to be defined.

It is a pleasure to thank Mr T. B. Mephram and Dr J. L. Mangan for the help they provided, both practical and theoretical, in the analysis of amino acids. We are also grateful to Mr K. Burton for his technical assistance in this work.

REFERENCES

- Britten, R. J. & McClure, F. T. (1962). *Bact. Rev.* **26**, 292.
- Clark, B. F. C. & Marcker, K. A. (1966). *J. molec. Biol.* **17**, 394.
- Cohen, G. N. (1958). *Annls Inst. Pasteur*, **94**, 15.
- Eagle, H., Piez, K. A. & Levy, M. (1961). *J. biol. Chem.* **236**, 2039.
- Jones, N. R. (1959). *J. Sci. Fd Agric.* **10**, 282.
- Kellogg, D. A., Doctor, B. P., Loebel, J. E. & Nirenberg, M. W. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 912.

Fig. 4. Comparison of free pools, protein composition and serosal transport of valine and threonine obtained from the mucosa of goldfish acclimatized to different temperatures. O, Free pools; ■, amino acid composition of mucosal protein; ●, transport across the goldfish intestine (from Mephram & Smith, 1966).

- Mephram, T. B. & Smith, M. W. (1966). *J. Physiol.* **186**, 619.
- Monod, J., Changeux, J.-P. & Jacob, F. (1963). *J. molec. Biol.* **6**, 306.
- Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
- Okita, G. T., Kabara, J. J., Richardson, F. & LeRoy, G. V. (1957). *Nucleonics*, **15**, 111.
- Riggs, T. R. & Walker, L. M. (1963). *J. biol. Chem.* **238**, 2663.
- Smith, M. W. (1966a). *J. Physiol.* **183**, 574.
- Smith, M. W. (1966b). *J. Physiol.* **183**, 649.
- Smith, M. W. (1966c). *Experientia*, **22**, 252.
- Smith, M. W. & Morris, D. (1966). *Experientia*, **22**, 678.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.