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The effect of amino acids and dipeptides on sodium-ion transport in rat enterocytes

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Sodium efflux from isolated intestinal epithelial cells was measured during incubation with several different free amino acids and dipeptides. L-Leucine, which is cotransported with sodium across the brush border membrane, significantly stimulated the total sodium efflux and almost all of this increase involved the ouabain-sensitive flux, i.e., the active component. In contrast, glycyl-L-leucine had little or no effect on active sodium efflux either in the presence or absence of 0.1 mM bestatin, a peptide hydrolase inhibitor. A second dipeptide L-carnosine (β-alanyl-L-histidine) which is poorly hydrolysed by enterocytes also had no effect upon sodium efflux. However, glycylglycine, which has been shown to be cotransported with sodium, did stimulate the ionic efflux. In addition, measurement of sodium uptake by sheets of small intestine showed that glycyl-L-leucine, carnosine and glycyl-L-proline failed to increase the uptake of the ion, while glycylglycine did significantly stimulate sodium uptake. These data indicate that some dipeptides are not cotransported with sodium, while others are. This suggests that there may well be multiple peptide transporters with very different characteristics in the brush border membrane of enterocytes.

Introduction

The movement of small peptides across the brush-border membrane of intestinal epithelial cells has been investigated with a variety of techniques in many different species and this has lead to a rather confused picture as to the specificity and characteristics of the transporter or transporters involved [1]. Failure in some instances to account for superficial hydrolysis has also made interpretation of data difficult.

Early experiments which did not eliminate superficial peptide hydrolysis indicated that some peptides appeared to have a partial requirement for sodium in the lumen and that removal of the ion from the incubation medium would reduce the uptake, but not to the same degree seen for free amino acids [2,3]. Subsequently, it was shown that when uptake of intact peptide alone was measured at least for some peptides the presence of sodium was not necessary [4–6]. However, the recent work of Himukai et al. [7] has shown that a peptide like glycylglycine can apparently stimulate the entry of sodium, although the ion has no effect on the entry of the peptide. This has lead them to coin the term of 'non-mutual stimulation' of transport. However, they did not control for the effect of the uptake of free amino acid liberated either by superficial or intracellular hydrolysis, which could have stimulated the sodium uptake.

Consequently, it appeared to be necessary that a reassessment be made of the data for glycylglycine and some other peptides, most notably glycyl-L-leucine, which does not require sodium for its uptake. This study was undertaken to look at the effect of these peptides on the flux of sodium

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across the intestinal epithelial cell to see whether they all exhibited 'non-mutual stimulation'.

Materials and Methods

Preparation of isolated cells. Male Sprague-Dawley (200–250 g) rats were anaesthetised with sodium pentobarbitol, and the entire small intestine was removed, everted, washed, and incubated in gassed isolation medium at 37°C for 20 min. Every 5 min during the incubation the solution was agitated vigorously with a plastic pipette tip, a procedure which greatly increased the cell yield. The resulting suspension was then filtered through a nylon mesh to remove mucus and tissue debris before being centrifuged for 2 min at 1200 rpm in a DYNAC II centrifuge (Clay Adams Co.) The cell pellet was resuspended in 30 ml of Krebs solution of 4°C, mixed thoroughly and recentrifuged for 2 min at 1200 rpm. This wash procedure was repeated to give a final yield of 0.5 to 1.0 ml packed cells. Histological examination of the cells and the remaining tissue showed that the cells came only from the villi, not the crypts, and that the enterocytes themselves exhibited very good structural integrity. Trypan blue exclusion by these cells was routinely better than 90% and often exceeded 95%.

At all stages of the preparation, plastic beakers and test tubes were employed to minimise damage to the cells and to reduce clumping.

Na^+ efflux procedure. The method of measuring Na^+ efflux from the enterocytes was very similar to that employed by Gall et al. [8]. The cell pellet was divided into two, and one half was added to the incubation medium containing 100 μCi $^{24}Na^+$ for preloading. The cells were added to the solution in a ratio of 1 to 6 (v/v) and incubated at 37°C for 20 min while being shaken at 100 strokes per min. The cells were then pelletted at 1200 rpm for 2 min and washed by resuspending them in ice-cold $MgCl_2$-Tris-buffer twice. The remaining half of the cells were treated in the same manner but 1.0 mM ouabain was added to the preloading medium.

The washed cells were then added to tracer-free incubation medium at a dilution of at least 1 to 60 (v/v). Those cells which had been preincubated with ouabain were suspended in incubation medium containing 1.0 mM ouabain for the efflux measurements. In this way a comparison could always be made between cells from the same animal to assess the active and passive sodium fluxes. To measure the sodium efflux a 500 μl aliquot was taken at the start and at the end of the incubation for the estimation of total initial radiolabelled sodium and 300 μl samples were taken at time zero and every 60 or 120 s for the measurement of appearance of sodium in the incubation medium. The 300 μl samples were centrifuged for 30 s at 1200 rpm to pellet the cells and 200 μl of the supernatant was drawn off for counting. All samples were counted on Beckman Gamma 4000 counter for 60 s.

Efflux rate constants were calculated by the method of Hoffman [9], which assumes that the efflux is a first-order mechanism. A dilution of 1 vol. of cells in 30 vol. of medium will satisfy that assumption with red cells; however, we found a greater dilution was necessary for enterocytes, because they transport sodium much more rapidly.

To obtain the efflux rate constants $\ln(1 - \text{supernatant sodium}/\text{total sodium}) \times 100$ was plotted against time and the slope of the line was obtained by linear regression analysis. All rate constants were then expressed in h^{-1}. That portion which could be inhibited by 1.0 mM ouabain was considered to be the actively mediated component.

Solutions. The isolation medium was Krebs bicarbonate saline which contained in addition 2.5 mg/ml bovine serum albumin fraction V, 1.5 mg/ml hyaluronidase and 10 mM sucrose. This solution was continuously gassed with 95% O_2/5% CO_2 during the cell isolation. The $MgCl_2$-Tris wash solution contained 110 mM $MgCl_2$ and 2 mM Tris-HCl adjusted to pH 7.4. The incubation medium was Krebs bicarbonate saline containing 1.0 mg/ml albumin.

Amino acids and peptides were present at the concentrations given below, and mannitol was used to adjust the osmolarity of the solutions. Substrates were always present in both the sodium-loading solution and the unloading solution. The amino acids and peptides were always present in the same concentrations during the sodium-loading efflux periods. Similarly, ouabain was present in both the loading and unloading medium and

was normally present at a concentration of 1.0 mM. Some experiments were performed with 5.0 mM ouabain, but the higher concentration had no significantly greater effect on sodium efflux. The peptide hydrolase inhibitor bestatin was also present in both the loading and unloading solutions at a concentration of 0.1 mM, and the sodium/proton exchange inhibitor amiloride was similarly employed at a concentration of 0.1 mM.

Estimation of peptide hydrolysis. The hydrolysis of glycyl-L-leucine and L-carnosine was assayed by measuring the appearance of free amino acids with L-amino acid oxidase [10]. In the case of glycyl-L-leucine, the enzyme was used to measure the appearance of free leucine, and in the case of carnosine the appearance of histidine. Carnosine hydrolysis was found to be negligible in this system when 0.5 ml of packed cells were incubated in 30 ml of 20 mM carnosine solution for up to 25 min. No histidine could be detected in the incubation medium when the cells were pelletted. In contrast, there was significant hydrolysis of glycyl-L-leucine. When 0.5 ml of cells were incubated with 30 ml of 20 mM glycyl-L-leucine, the concentration of free leucine in the incubation medium reached 1.5 mM after 25 min. The average rate of hydrolysis from four such experiments was 105.1 nmol peptide per mg cell protein per min. In the presence of 0.1 mM bestatin, the rate of hydrolysis was significantly reduced, and the difference in the slopes of the lines over a 20 min period gave an average inhibition of 90.6% $\pm$ 0.3 ($n = 4$).

Sodium uptake experiments. To measure the entry of sodium across the brush border of the enterocytes, the rapid stirring technique of Sepúlveda and Smith was used [11]. Sheets of the middle fifth of the small intestine were mounted in the chamber which exposed 12 separate ports, each of 0.196 cm^2 area. After a 10 min equilibration period, during which Krebs bicarbonate saline was superfused over the surface of the tissue, the ports were filled with incubation medium containing $^{24}Na^+$ and 20 mM substrate. The incubation solution was stirred at 900 rpm with a small propeller and then drained by suction after 15, 30, 45, 60, 90 or 120 s. The surface of the tissue was then rapidly rinsed with 5 ml of ice-cold $MgCl_2$-Tris buffer and punched out with a metal punch. Each piece of tissue was then immersed in 300 μl of 0.05 M nitric acid and counted. The remaining extracellular sodium was estimated from the zero time intercept when the uptake curve was plotted, and this value was subtracted from the subsequent uptake values.

Materials. The peptides and amino acids were all obtained from Sigma Chemical Co., St. Louis, MO. The $^{24}Na^+$ was supplied by the University of Alberta Slowpoke Nuclear Reactor, Department of Pharmacy, University of Alberta. Bestatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanol-L-leucine) was a generous gift from Prof. Hamao Umezawa, Institute of Microbial Chemistry, Microbial Chemistry Research Foundation, Tokyo, Japan.

Results

Effect of actively transported substrates

Fig. 1 shows data from typical experiments under control conditions (30 mM mannitol) in the

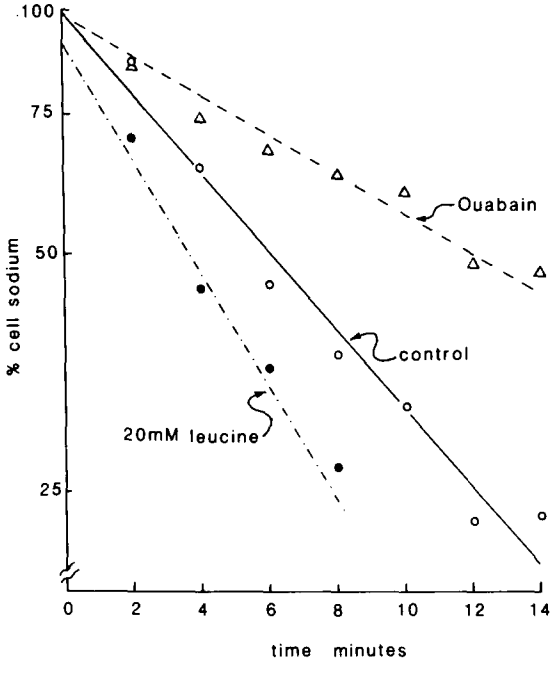

Fig. 1. Na^+ efflux from isolated intestinal epithelial cells. O——O, the percentage of sodium remaining in the cells incubated in 30 mM mannitol; Δ- - - - -Δ, 30 mM mannitol + 1.0 mM ouabain; ●–·–·–●, 20 mM leucine + 10 mM mannitol. Lines were fitted by linear regression analysis. Cells were incubated at 37°C at a dilution of at least 1:60 (v/v).

TABLE I

EFFECT OF SUBSTRATES ON Na^+ EFFLUX FROM ISOLATED ENTEROCYTES

Sodium efflux rate constants h^{-1}. Values are means ± S.E. with the number of observations in parentheses. $\Delta(a-b)$ represents the ouabain-insensitive flux, and percentage change represents increase in this component.

	Total (a)	+1 mM Ouabain (b)	$\Delta(a-b)$	% change
Control	6.25 ± 0.30 (18)	3.84 ± 0.14 (20)	2.41	–
10 mM D-glucose	8.28 ± 0.70 (9)	4.01 ± 0.34 (9)	4.27	77
20 mM L-leucine	9.64 ± 0.41 (12)	5.60 ± 0.37 (12)	4.04	68
30 mM L-leucine	12.45 ± 1.02 (8)	6.03 ± 0.91 (8)	6.42	166
20 mM L-leucine + 10 mM L-proline	12.08 ± 0.76 (7)	5.14 ± 0.17 (8)	6.94	188
30 mM glycine	5.47 ± 0.42 (5)	3.51 ± 0.38 (6)	1.96	–

presence (1 mM) and absence of ouabain and the effect of the addition of 20 mM L-leucine to the incubation medium.

Table I shows the rate constants for Na^+ efflux when the cells were incubated with free amino acids and D-glucose, which was included for comparison with previously published data. 10 mM glucose stimulated the ouabain-sensitive sodium efflux by 77%. 20 mM L-leucine similarly promoted the active sodium efflux and 30 mM had an even greater effect. The addition of 10 mM L-proline to 20 mM L-leucine caused an increase in sodium efflux comparable to simply raising the leucine concentration by 10 mM. 30 mM glycine had no effect at all on sodium flux. Although the major effect of the free amino acids with the exception of glycine was to increase the ouabain-sensitive flux, it should be noted that in addition the ouabain-insensitive flux was increased. This effect could not be blocked by increasing the ouabain concentration to 5 mM. Also, this effect could still be seen in the presence of 0.1 mM amiloride (Table II).

Effect of glycyl-L-leucine

Table III shows the rate constants for sodium efflux when glycyl-L-leucine was present in the incubation medium. When present on its own, the peptide had no effect on the ouabain-sensitive sodium flux but did have a small stimulatory effect on the ouabain-insensitive efflux. 20 mM glycyl-L-leucine with 0.1 mM bestatin did cause a significant increase in sodium efflux, but this was almost all ouabain-insensitive. Bestatin on its own significantly raised the ouabain-insensitive flux, but had no effect on the ouabain-sensitive component.

The addition of 10 mM glycyl-L-leucine to 20 mM leucine in the incubation medium did not increase either component of the sodium exit compared to leucine on its own.

Effect of poorly hydrolysed peptides

The effects of carnosine and glycylglycine are summarised in Table IV.

20 mM carnosine had no effect at all on the sodium efflux, while glycylglycine had a significant effect on both components of the sodium exit when present on its own. The ouabain-sensitive component of this stimulation was not affected by presence of 30 mM free glycine when compared to the 50 mM mannitol control, although the total fluxes were reduced at this high osmolarity.

TABLE II

EFFECT OF INCREASING OUABAIN CONCENTRATION TO 5 mM AND PRESENCE OF AMILORIDE (0.1 mM) ON OUABAIN-INSENSITIVE Na^+ EFFLUX FROM ISOLATED ENTEROCYTES

Values are means ± S.E. Numbers of observations are given in parentheses.

	30 mM mannitol	30 mM L-leucine
1 mM ouabain [a]	3.84 ± 0.14 (20)	6.03 ± 0.91 (8)
5 mM ouabain	3.97 ± 0.72 (3)	7.17 ± 0.86 (3)
1 mM ouabain + 0.1 mM amiloride	4.57 ± 0.38 (5)	5.67 ± 0.35 (5)

[a] Data taken from Table I.

TABLE III

EFFECT OF GLYCYL-L-LEUCINE ON Na^+ EFFLUX FROM ISOLATED ENTEROCYTES

Values are means ± S.E. Numbers of observations are given in parentheses.

	Total (a)	+1 mM ouabain (b)	$\Delta(a-b)$	% change
20 mM mannitol	6.25 ± 0.30 (18)	3.84 ± 0.14 (20)	2.41	–
20 mM GlyLeu	7.52 ± 0.82 (13)	5.00 ± 0.46 (13)	2.52	4
20 mM L-leucine + 10 mM GlyLeu	9.30 ± 0.82 (8)	4.31 ± 0.30 (8)	4.99	107
20 mM GlyLeu + 0.1 mM bestatin	8.67 ± 0.43 (7)	5.55 ± 0.32 (6)	3.12	29
20 mM mannitol + 0.1 mM bestatin	6.73 ± 0.28 (4)	4.85 ± 0.29 (4)	1.88	–

TABLE IV

EFFECT OF POORLY HYDROLYSED PEPTIDES ON Na^+ EFFLUX FROM ISOLATED ENTEROCYTES

Values are means ± S.e. Numbers of observations are given in parentheses.

	Total (a)	+1 mM ouabain (b)	$\Delta(a-b)$	% change
30 mM mannitol	6.25 ± 0.30 (18)	3.84 ± 0.14 (20)	2.41	–
50 mM mannitol	5.06 ± 0.47 (7)	2.92 ± 0.40 (6)	2.14	–
20 M GlyGly	10.50 ± 0.73 (10)	5.33 ± 0.47 (10)	5.17	115 [a]
20 mM GlyGly + 30 mM glycine	7.69 ± 0.42 (7)	2.83 ± 0.24 (8)	4.86	127 [b]
20 mM carnosine	6.50 ± 0.86 (8)	3.93 ± 0.31 (8)	2.57	7

[a] % increase in Δ compared to 30 mM mannitol control.
[b] % increase in Δ compared to 50 mM mannitol control.

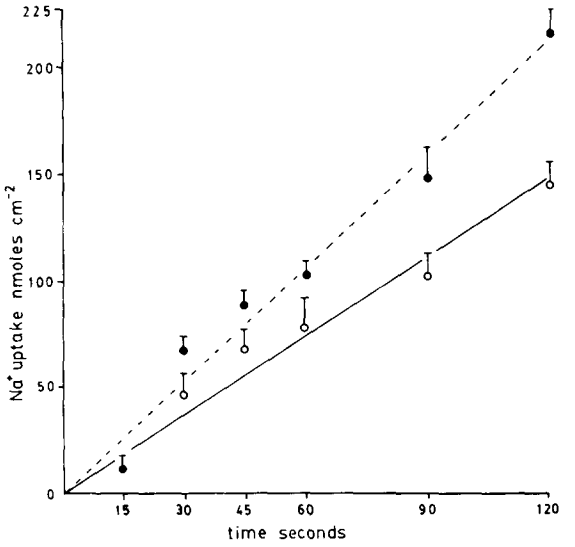

Fig. 2. Sodium uptake by sheets of mid small intestine. ○——○, uptake in the presence of 20 mM PEG 400; ●- - - - -●, uptake in the presence of 20 mM L-leucine. Incubation medium was stirred at 900 RPM. Bars represent S.E. of mean and each value is the mean of at least 11 determinations made in 6 animals. Lines were fitted by linear regression analysis.

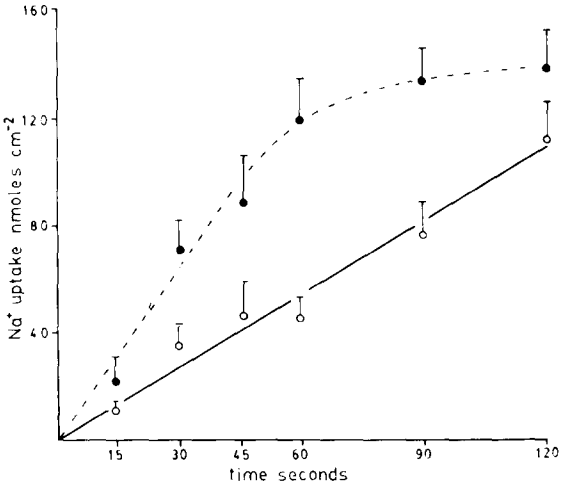

Fig. 3. Sodium uptake by sheets of mid small intestine. ○——○, uptake of sodium in the presence of 20 mM glycyl-L-leucine; ●- - -●, uptake in the presence of 20 mM glycylglycine. The incubation medium was stirred at 900 rpm. Bars represent S.E. of mean and each value is the mean of at least 11 determinations made in six animals. Lines were fitted by linear regression analysis.

Effect of substrates on sodium uptake

Figs. 2 and 3 show the time course of the uptake of sodium across the luminal surface of mid-small intestinal sheets. The data is expressed in nmol of sodium taken up per cm^2 of tissue. Leucine and glycylglycine caused a marked stimulation of sodium uptake over controls (20 mM P.E.G. 400). The difference between the curves was assessed by analysis of covariance, and in both cases the difference between the control sodium uptake and that in the presence of substrate was significant at the 1% level. Conversely, glycyl-L-leucine, carnosine and glycyl-L-proline did not stimulate the uptake of sodium.

Discussion

The isolated cells used in this study appear to be in good condition for several reasons. Firstly, they excluded Trypan blue to a large degree indicating good morphological integrity. Secondly, under control conditions nearly 50% of the total sodium efflux was ouabain-sensitive, i.e., actively mediated and this proportion was much greater when the additional substrates were present. Thirdly, despite being prepared with an enzyme digestion technique the efflux rate constants under control conditions and in the presence of D-glucose were almost identical to those observed by Gall et al. [8], with cells prepared by a vibration technique. Finally the sodium efflux followed first-order kinetics for at least 8 min.

D-Glucose, L-leucine and L-proline have all been shown previously with a variety of techniques to be cotransported across the brush border membrane of enterocytes with sodium [12–15]. The data presented here confirm that these substrates can stimulate the pumping of sodium out of these epithelial cells, indicating an initial increased influx of sodium on the cotransporter. This technique can apparently detect quite large changes in the sodium flux, as indicated by the substantial extra increase in the sodium efflux rate constant when the leucine concentration was raised from 20 to 30 mM or when 10 mM proline was added to 20 mM leucine. Evidently, the presence of sucrose in the incubation medium provided sufficient metabolic energy for the $(Na^+ + K^+)$-ATPase and this was not a limiting factor.

Not all of the increase in the sodium efflux in the presence of the amino acids was apparently via the ouabain-sensitive route however. In contrast to the glucose stimulation, leucine and the leucine/proline mixture significantly increased the flux in the presence of 1 mM ouabain. This response was not abolished when the ouabain concentration was raised to 5 mM, suggesting that this increased flux was not mediated by $(Na^+ + K^+)$-ATPase. Another explanation could be that sodium/proton exchange was being stimulated in response to an altered intracellular pH [16]. However, two factors argue against such a mechanism. Firstly, the response was not abolished by 0.1 mM amiloride, which blocks the sodium/proton exchange mechanism [17], and secondly, the raised intracellular amino acid concentration would most likely lower the intracellular pH and stimulate sodium uptake by the exchange mechanism.

At present, the data do not allow for a conclusion to be reached on the mechanism involved, and further work is required to resolve this problem, but it should be borne in mind that $(Na^+ + K^+)$-ATPase systems in the rat are relatively insensitive, to ouabain.

The failure of the dipeptide glycyl-L-leucine to stimulate the sodium efflux argues strongly in favour of the hypothesis that this peptide is not cotransported with sodium across the brush-border membrane. Earlier studies have shown that the uptake of this peptide is not reduced when sodium is eliminated from the luminal solution, but these experiments clearly show that the entry of this peptide does not exhibit 'non-mutual stimulation' with sodium. This statement is supported by the more direct observation that sodium uptake into the sheets of tissue was not stimulated by the presence of this dipeptide. Similarly, carnosine had no effect on either the efflux or the uptake of sodium, indicating that it too is not cotransported with sodium.

In contrast, the poorly hydrolysed peptide glycylglycine, which has previously been shown to exhibit 'non-mutual stimulation' of sodium uptake in the small intestine [7], did stimulate both the sodium efflux from the isolated cells and the uptake of sodium by the tissue sheets. The presence of 30 mM free glycine did not change this effect, so it is highly unlikely that glycine liberated by

hydrolysis is responsible for this increased sodium flux. Glycyl-L-proline was only used in the sodium uptake experiments, and although it too is relatively poorly hydrolysed by intestinal epithelial cells [18], it did not stimulate the uptake of sodium.

Various competition studies have suggested that peptides can cross the brush-border membrane by more than one entry route [19,20–23], however, the complications of surface hydrolysis and the poor availability of radiolabelled peptides have made such studies tedious and difficult to interpret. This study provides clear evidence that there do indeed exist several peptide-transport mechanisms in the enterocyte only some of which involve some form of stimulation of sodium entry at the same time. Recently, Ganapathy et al. [24] have provided evidence with brush-border membrane vesicles that glycyl-L-proline may be cotransported with protons, not sodium ions, as glycylglycine appears to be.

At present, it is not clear what role, if any, these ions play in energising the uptake of small peptides by enterocytes, but it is evident that this important group of compounds can cross the brush-border membrane by several distinct routes, whose properties appear to be quite divergent.

References

1 Matthews, D.M. (1975) Physiol. Rev. 55, 537–608
2 Rubino, A., Field, M. and Schwachman, H. (1971) J. Biol. Chem. 246, 3542–3548
3 Ganapathy, V. and Radhakrishnan, A.N. (1980) Biochem. Pharmacol. 29, 713–716
4 Cheeseman, C.I. and Johnston, G. (1982) Can. J. Physiol. Pharmacol. 60, 1177–1184
5 Cheeseman, C.I. and Parsons, D.S. (1976) J. Physiol. 262, 459–476
6 Berteloot, A., Khan, A.H. and Ramaswamy, K. (1982) Biochim. Biophys. Acta 686, 47–54
7 Himukai, M., Kameyama, A. and Hoshi, T. (1983) Biochim. Biophys. Acta 732, 659–667
8 Gall, D.G., Butler, D.G., Tepperman, F. and Hamilton, J.R. (1974) Biochim. Biophys. Acta 339, 291–302
9 Hoffman, J.R. (1962) J. Gen. Physiol. 45, 837–859
10 Fujita, M., Parsons, D.S. and Wojnarowska, F. (1972) J. Physiol. 225, 377–394
11 Sepúlveda, F.V. and Smith, M.W. (1978) J. Physiol. 282, 73–90
12 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637–718
13 Smith, M.W. and Sepulveda, F.V. (1979) Biochim. Biophys. Acta 555, 374–378
14 Kimmich, G.A. and Randles, J. (1980) Biochim. Biophys. Acta 596, 439–444
15 Corcelli, A., Prezioso, G., Palmieri, F. and Storelli, C. (1982) Biochim. Biophys. Acta 689, 97–105
16 Murer, H., Hopfer, U. and Kinne, R. (1976) Biochem. J. 154, 597–604
17 Kinsella, J.L. and Arousen, P.S. (1981) Am. J. Physiol. 241, F374–F379
18 Ganapathy, V., Mendicino, J.F. and Leibach, F.H. (1981) J. Biol. Chem. 256, 118–1124
19 Edwards, K.D.G. (1970) Med. J. Aust. 2, 1213
20 Gupta, V.J. and Edwards, K.D.G. (1976) Clin. Exp. Pharmacol. Physiol. 3, 511–521
21 Berteloot, A., Khan, A.H. and Ramaswamy, K. (1981) Biochim. Biophys. Acta 649, 179–188
22 Himukai, M., Kano-Kameyama, A. and Hoshi, T. (1982) Biochim. Biophys. Acta 687, 170–78
23 Burston, D., Wapnir, R.A., Taylor, E. and Matthews, D.M. (1982) Clin. Sci. 62, 617–626
24 Ganapathy, V. and Leibach, F.H. (1983) J. Biol. Chem. 258, 14189–14192