Biochimica et Biophysica Acta, 603 (1980) 288—297

BBA 79027

STOICHIOMETRY VERSUS COUPLING RATIO IN THE COTRANSPORT OF Na AND DIFFERENT NEUTRAL AMINO ACIDS

J.Y.F. PATERSON, F.V. SEPÚLVEDA and M.W. SMITH

A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT (U.K.)

(Received June 23rd, 1980)

Key words: Na^+/amino acid coupling; Amino acid uptake; Kinetic analysis; Coupling ratio; Cotransport; (Ileum)

Summary

The stoichiometry of Na coupling to amino acid movement across the brush border membrane of the rabbit distal ileum has been determined under initial rate conditions.

The coupling ratio, defined as the amino acid-dependent Na influx/the Na-dependent amino acid influx, was equal to unity for alanine, measured over a 10-fold range of Na and alanine concentrations. Coupling ratio values determined under a single set of conditions for a number of amino acids varied from 1 for serine to 4.6 for methionine. Reducing the methionine concentration from 12.5 to 1.5 mM caused the coupling ratio value to fall from 4.6 to 1.2.

These results are explained by assuming a fixed stoichiometry of 1 : 1 under all conditions, with initial binding of the amino acid (A) to the Na-dependent carrier (E) but with some amino acids being able to cross on the Na-dependent carrier in the absence of Na.

The variation in coupling ratio values can be used to calculate K_A, the apparent dissociation constant of amino acid from the Na-dependent carrier in the absence of Na, and the ratio k_1/k_2, where k_1 and k_2 are first-order rate constants for translocation of the complexes EA and EANa, respectively. This method of processing results has been defined as delta analysis. The value of K_A for methionine is 3.6 ± 1.1 mM and the k_1/k_2 ratio is 1.01 ± 0.07. The constant coupling ratio value of 1 for alanine indicates that the value for K_A is extremely high or that the k_1 value is extremely low.

Introduction

It has been shown recently that neutral amino acids use two mediated systems when entering the intestinal mucosa of the rabbit [1]. Only one

of these systems, having high affinity but low transport capacity for different amino acids, was found to be Na-dependent [2,3]. Previous work, carried out assuming only one transport system to be present, suggested a variable coupling of Na and alanine influxes with a maximum value of unity [4]. Re-evaluation of this work is needed because of the contribution of the Na-independent carrier to total alanine influx.

Numerous papers have been published recently showing a multiplicity of amino acid transport systems in cells other than enterocytes [5,6]. In this work it has been widely recognized that only some of these processes are Na-dependent. It is important to realize, however, that the term Na-dependent is operational only. It arises from an inability to detect transport taking place through an Na-dependent carrier in the absence of Na, when an Na-independent transport system is also present. This problem has been overcome in the present work by using a new method of analysis based on the varying ability of Na and amino acid to stimulate the influx of one another.

Materials and Methods

Animals. Rabbits of the New Zealand White strain weighing 2—3 kg were purchased from Morton Commercial Rabbits, Stansted, Essex, U.K. They were deprived of food but not water for 24 h before each experiment.

Rapid uptake measurements. Distal ileum taken from rabbits killed by intravenous injection of a solution of sodium pentobarbitone was rinsed in bicarbonate/saline [7] before being mounted in an influx apparatus identical to that described previously [1]. All subsequent operations were carried out at 37°C.

Each tissue was superfused initially with choline-substituted Na-free bicarbonate medium, gassed with 95% O_2/5% CO_2, for 10 min. Uptake of radioactively labelled substrates across the brush border membrane [^{14}C-labelled amino acid or ^{22}Na) was then measured, from solutions stirred at 700 rev./min, for 45 s. Autoradiography performed on rabbit ileum incubated under identical conditions showed amino acids to be retained within the mucosa during this short period of incubation [8]. The process of tissue washing at the end of incubation, subsequent disruption of the intestinal mucosa in acid and determination of radioactivity, were as described previously [1]. ^{3}H-labelled poly-(ethylene glycol) of molecular weight 900 was present in all solutions as a marker of the extracellular space.

Radioactive isotopes. The following radioactive amino acids were purchased from the Radiochemical Centre, Amersham, Bucks., U.K.: L-[U-^{14}C]serine, L-[U-^{14}C]threonine, L-[U-^{14}C]alanine, L-[U-^{14}C]isoleucine, L-[1-^{14}C]leucine and L-[*methyl*-^{14}C]methionine. ^{22}Na was purchased from the Radiochemical Centre, Amersham, as a solution of ^{22}NaCl (greater than 100 mCi/mg). Poly-([1,2-^{3}H]ethylene glycol), molecular weight 900 (2—10 mCi/g), was obtained from New England Nuclear Chemicals GmbH, D-6072 Dreieichenhain, F.R.G.

Results and Discussion

Control experiments

The final protocol adopted for the present experiments was based on the

TABLE I

ANALYSIS OF VARIANCE OF NATURAL LOGARITHMS OF ALANINE AND Na INFLUX INTO RABBIT ILEAL MUCOSA

The experimental conditions were as described in the text. J_{Ala} and J_{Na} were 20.0 ± 1.6 and 74.0 ± 3.7 nmol · cm^{-2} · min^{-1}, respectively; mean ± S.E. of 48 observations using 3 mM alanine and 25 mM Na. n.s., not significant; ss, sum of squares; ms, mean squares.

Substrate	Source of variation	ss	d.f.	ms	F	P
Alanine	Rabbits	10.08	7	1.44	55.6	<0.001
	Ports	0.16	5	0.03	1.2	n.s.
	Residuals	0.91	35	0.03		
Na	Rabbits	4.15	7	0.59	38.7	<0.001
	Ports	0.15	5	0.03	2.0	n.s.
	Residuals	0.54	35	0.02		

assumption that there was no within-tissue variation in substrate influx. This was investigated in a first series of experiments. Each piece of intestine provided 12 areas of tissue for analysis. Six of these were used to estimate alanine influx and six to estimate the influx of Na, in alternate ports. The concentrations of alanine and Na were 3 and 25 mM, respectively, and the experiment was repeated on eight rabbits.

There was, as reported previously, inhomogeneity of variance between rabbits, with variance proportional to the square of mean influx [3,9], so that transformation to logarithms was necessary before analysis of variance for a two-factor design (rabbits and distance along the ileum). The results are shown in Table I. It can be seen that between-rabbit differences were the only significant source of variation in influxes of both alanine and Na. There was no correlation between alanine or Na influx with distance along the ileum (Alanine r = 0.61, Na r = 0.63, 4 d.f. *).

Na-coupled substrate influx

There are large differences in amino acid and Na influxes between animals and it is essential to minimize the effect of this variation on the estimation of coupling. Our solution to this problem was to estimate four fluxes in each animal. Na uptake was measured in the absence and presence of substrate amino acid and the difference between these values (ΔJ_{Na}) was taken to be the Na uptake specifically due to substrate influx. Similarly, the uptake of amino acid was measured in the absence and presence of Na and the difference between these values (ΔJ_A) was taken to be the amino acid uptake specifically due to Na influx. Each influx was estimated in triplicate to minimize further error.

ΔJ_{Na} and ΔJ_A will still show between-animal differences and they must, therefore, be treated as paired observations. The best treatment for such results is regression analysis where, since only differences in flux are involved,

* d.f., degrees of freedom.

TABLE II

STOICHIOMETRY OF COUPLING OF Na TO ALANINE INFLUX IN RABBIT ILEUM

Alanine influx in the absence of Na is assumed to take place on an Na-independent transport process (system 2 [3]). Alanine stimulation of Na influx and Na stimulation of alanine influx are assumed to take place on an Na-dependent process (system 1 [2,3]). The experimental conditions are as described in the text. Each value represents the mean of estimates carried out on eight rabbits. Mean values of the coupling ratio (R) are calculated (±S.E.) from the slopes of individual ΔJ_{Na} vs. ΔJ_{Ala} influx plots passing through the origin.

Concentration (mM)		Influx (nmol · cm^{-2} · min^{-1})				R ($\Delta J_{Na}/\Delta J_{Ala}$)
Na	Alanine	Alanine		Na		
		Na absent	Na present	Alanine absent	Alanine present	
12.5	25	87.4	114.7	51.5	78.0	0.9 ± 0.1
25	25	67.8	117.6	81.6	116.1	0.6 ± 0.1
50	25	85.6	131.6	149.9	188.4	0.7 ± 0.2
90	25	90.4	140.0	233.6	276.7	0.8 ± 0.2
143	25	77.4	139.4	302.4	349.2	1.0 ± 0.1
25	6	23.6	46.4	73.2	96.0	1.0 ± 0.1
25	12.5	29.2	60.1	75.7	96.9	0.7 ± 0.1
25	40	108.7	169.2	68.9	145.5	1.2 ± 0.1
25	50	165.0	223.0	87.8	146.4	1.0 ± 0.1

no intercept would be expected. We then define the slopes of such plots through the origin as coupling ratios.

Na-coupled alanine influx

The coupling ratios for alanine and Na influx were determined over a wide range of concentrations (6—50 mM for alanine, 12.5—143 mM for Na). The results obtained are shown in Table II.

The reproducibility of results was good, judged from measurement of alanine influx in the absence of Na (67.8—90.4 nmol · cm^{-2} · min^{-1} for 25 mM alanine) and of Na influx in the absence of alanine (68.9—87.8 nmol · cm^{-2} · min^{-1} for 25 mM Na; range of means obtained from five groups of rabbits in both cases). The additional presence of alanine or Na invariably caused a significant increase in the influx of its co-substrate. The coupling ratio, Na : Ala, calculated for these dependent fluxes using the method described above, ranged from 0.6 to 1.2. There was no significant trend between the coupling ratio and alanine or Na concentration (r 0.38 and 0.63, respectively; 3 d.f.). It was therefore decided to pool all results to obtain an overall coupling ratio for Na and alanine influx. A plot of these individual results is shown in Fig. 1.

The dependent fluxes for any one set of experimental conditions showed a 2- to 3-fold variation. This presumably reflects the normal variation seen in the amount of the Na-dependent system found between individual rabbits [9]. The slope passing through the origin is 0.94 ± 0.04 with a correlation coefficient of 0.94 ($P < 0.001$; 68 d.f.). That this slope is not significantly different from unity was verified using a paired Student's t-test of ΔJ_{Na} and ΔJ_{Ala} ($t = 0.25$).

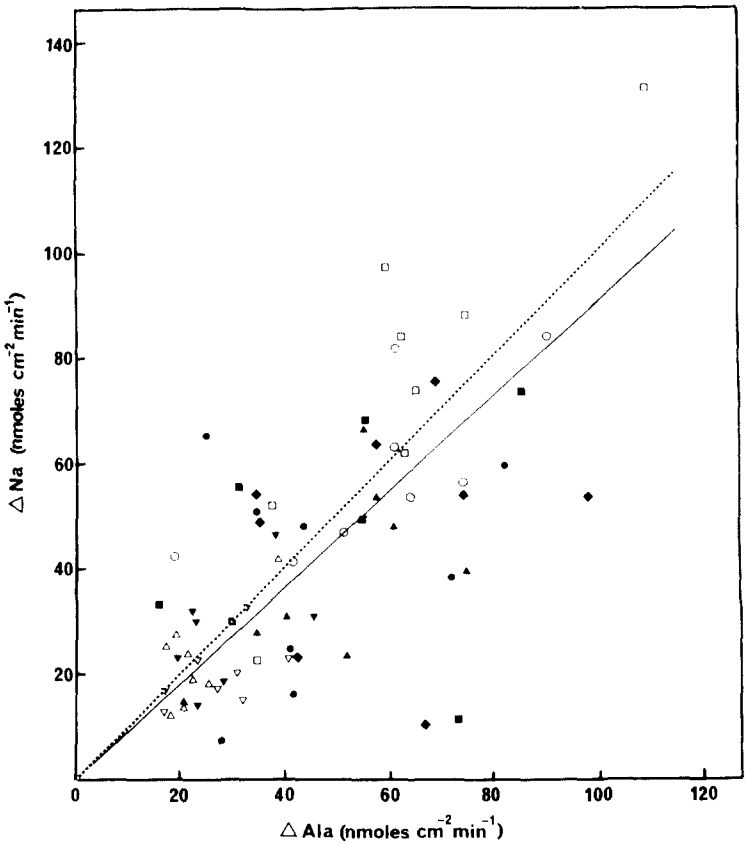

Fig. 1. Alanine-dependent Na influx (ΔNa) and Na-dependent alanine influx (ΔAla) in rabbit ileal mucosa. Uptake of alanine and Na was measured, in the same rabbit ileum, in the absence and presence of Na and alanine, respectively. Open symbols show uptakes measured at an Na concentration of 25 mM and alanine concentrations of 6, 12.5, 40 and 50 mM (△, ▽, □ and ○, respectively). Filled symbols show uptakes measured at an alanine concentration of 25 mM and Na concentrations of 12.5, 25, 50, 90 and 143 mM (▼, ▲, ●, ■ and ◆, respectively). The solid lines gives the least-squares fit through the origin with a slope of 0.94 ± 0.04. The broken line is the line of identity, shown for comparison.

There is a clear discrepancy between our observations that the coupling ratio = 1 at all alanine and Na concentrations and the earlier observation that the coupling ratio was a function of Na concentration [4]. We have already justified our method of analysis whereby correction is made for uptakes taking place through pathways other than the Na-dependent amino acid transport system.

The question remains as to why a recent re-interpretation of earlier influx data should have led to the suggestion that the stoichiometry of coupling was 2 : 1 [10]. The most likely explanation for this is that it arises from an overestimation of the amino acid-dependent Na influx measured at 140 mM Na in the original series of experiments [4]. The standard error of individual coupling coefficients was not provided in these earlier experiments, but it is possible to make some comparison from similar measurements carried out in the rabbit jejunum [11]. The coupling coefficient for Na and alanine influx,

TABLE III

STOICHIOMETRY OF COUPLING OF Na TO AMINO ACID INFLUX IN RABBIT ILEUM

The conditions involved in calculating coupling ratios (R) are as stated in Table II. Amino acids were used at a concentration of 12.5 mM. The Na concentration used was 25 mM. Each value represents the mean carried out on seven or eight rabbits.

Amino acid	Influx (nmol · cm^{-2} · min^{-1})				R ($\Delta J_{Na}/\Delta J_{Amino\ acid}$)
	Amino acid		Na		
	Na absent	Na present	Amino acid absent	Amino acid present	
Serine	37.3	64.5	93.3	121.0	0.9 ± 0.1
Threonine	34.6	51.7	62.8	95.0	1.5 ± 0.4
Isoleucine	51.0	60.7	77.7	109.9	2.8 ± 0.3
Leucine	41.5	47.5	80.2	105.4	3.6 ± 1.0
Methionine	51.5	56.4	86.5	109.9	4.7 ± 0.7

calculated as the ratio of the alanine-dependent Na influx to the total alanine influx, was 0.7 ± 0.16 at 140 mM Na. This value could just as well approximate to 0.5 as to 1.0. Similar low coupling coefficients have been reported when measuring Na : phenylalanine coupling in guinea-pig ileum [12].

Na-coupled influx of amino acids other than alanine

The ability of amino acids other than alanine to use the two carrier-mediated entry systems is well established [2,3]. It follows that the stoichiometry for alanine entry on the Na-dependent system should apply to other neutral amino acids. The following experiments were designed to test this prediction.

The influx of five neutral amino acids into rabbit ileum was measured in the presence and absence of 25 mM Na. Each amino acid (serine, threonine, isoleucine, leucine and methionine) was used at a concentration of 12.5 mM. The results obtained are shown in Table III. Na influx in the absence of amino

TABLE IV

STOICHIOMETRY OF COUPLING OF Na TO METHIONINE INFLUX IN RABBIT ILEUM

Assumptions involved in calculating coupling ratios (R) are as stated in Table II. The concentration of Na used in these experiments was 25 mM. Each value represents the mean of estimates carried out on seven to nine rabbits. Results using methionine at a concentration of 12.5 mM have been taken from Table III for comparison.

Methionine concentration (nmol · cm^{-2} · min^{-1})	Influx (mM)				R ($\Delta J_{Na}/\Delta J_{Met}$)
	Methionine		Na		
	Na absent	Na present	Methionine absent	Methionine present	
1.5	13.8	20.1	109.0	116.6	1.2 ± 0.5
3.0	15.1	25.1	70.3	91.7	2.0 ± 0.3
6.0	37.2	50.1	111.2	141.8	2.5 ± 0.3
12.5	51.5	56.4	86.5	109.9	4.6 ± 0.6

acid varied from 62.8 to 93.3 nmol · cm^{-2} · min^{-1}, a range similar to that found using alanine at 25 mM Na (Table II). Amino acid influx in the absence of Na was lower for the hydrophilic amino acids serine and threonine (and alanine, Table II) than for the hydrophobic amino acids, isoleucine, leucine and methionine. The coupling ratio varied from 0.9 for serine to 4.7 for methionine.

The variation observed in the coupling ratio for different amino acids may be explained by assuming that the stoichiometry of coupling is the same for all amino acids tested, but that some of these amino acids also cross the brush border membrane on the Na-dependent carrier (system 1) in the absence of Na. J_A in the absence of Na would then include some system 1 influx and ΔJ_A would thereby be decreased without any decrease in ΔJ_{Na}. If this explanation is correct, one would predict that the coupling ratio should vary with the concentration of amino acid used. This was tested using methionine over a 10-fold range of concentration. The results are shown in Table IV.

The ability of Na to increase methionine flux was always present but the absolute stimulation became very small using a methionine concentration of 12.5 mM. The ability of methionine to increase Na influx was also present for all concentrations, the effect being smallest using a methionine concentration of 1.5 mM. The coupling ratio showed a steady decline from 4.6 to 1.2 on lowering the methionine concentration from 12.5 to 1.5 mM. This value of 1.2 was not significantly different from unity.

Description of a model and quantification of delta analysis

Any model chosen to describe the results obtained for methionine should apply equally well to alanine, since it has been shown previously that both these amino acids share the same Na-dependent entry system in rabbit ileum [3]. The reaction order in which substrates bind to the Na-dependent carrier is assumed to be the amino acid first followed by Na. The model used to describe coupling of Na to amino acid influx in rabbit distal ileum is as follows:

$$\begin{array}{ccccc} E & \overset{K_A}{\rightleftharpoons} & EA & \overset{K_{Na}}{\rightleftharpoons} & EANa \\ \uparrow & & \downarrow k_1 & & \downarrow k_2 \end{array} \qquad (1)$$

where E is the carrier, A the substrate, K_A and K_{Na} dissociation constants and k_1 and k_2 translocation rate constants for the complexes EA and EANa respectively. At least two other simple alternative models can be considered, one in which Na binds before the amino acid and another in which a random order of binding is assumed. The first is readily discarded as it implies that coupling ratios will never exceed unity. The second could explain our results but in order to do this additional assumptions have first to be made concerning the magnitude of the equilibrium constants and rate constants for translocation.

Defining J_2 as the Na influx in the absence of substrate and J_1 as the Na influx when substrate is present, $J_1 = k_2\,[EANa] + J_2$, and

$$\Delta J_{Na} = J_1 - J_2 = k_2[EANa] \qquad (2)$$

$$= \frac{k_2[E_t]}{1 + \frac{K_{Na}}{[Na]} + \frac{K_A K_{Na}}{[A][Na]}} \qquad (3)$$

where $[E_t]$ is the total concentration of carrier sites exposed to the external medium. In the absence of Na:

$$J_4 = \frac{k_1[E_t]}{1 + \frac{K_A}{[A]}} + J_{ind} \tag{4}$$

where J_4 is the total influx of substrate measured in the absence of Na and J_{ind} is the amount of influx of substrate taking place on the Na-independent carrier.

$$J_3 = k_1[EA] + k_2[EANa] + J_{ind} \tag{5}$$

$$= \frac{[E_t]\left(k_1 \frac{K_{Na}}{[Na]} + k_2\right)}{1 + \frac{K_{Na}}{[Na]} + \frac{K_A K_{Na}}{[A][Na]}} + J_{ind} \tag{6}$$

where J_3 is the substrate influx measured in the presence of Na. The change due to Na, ΔJ_A, is:

$$J_3 - J_4 = \frac{[E_t]\left(k_2\left(1 + \frac{K_A}{[A]}\right) - k_1\right)}{\left(1 + \frac{K_{Na}}{[Na]} + \frac{K_A K_{Na}}{[A][Na]}\right)\left(1 + \frac{K_A}{[A]}\right)} \tag{7}$$

The coupling ratio, R, defined as $\Delta J_{Na}/\Delta J_A$, then becomes:

$$R = \frac{J_1 - J_2}{J_3 - J_4} = \frac{k_2\left(1 + \frac{K_A}{[A]}\right)}{k_2\left(1 + \frac{K_A}{[A]}\right) - k_1} \tag{8}$$

It can be seen that this expression eliminates $[E_t]$, i.e., it is independent of between-animal variation in J_{max} values. Eqn. 8 was rearranged in the form:

$$(R - 1)\,K_A - (R[A])\frac{k_1}{k_2} = (1 - R)[A] \tag{9}$$

to allow the calculation of the two parameters K_A and k_1/k_2 for methionine using the results summarized in Table IV. This equation, solved by least-squares analysis using matrices for four values of the coupling ratio, gave a K_{Met} value of 3.6 ± 1.1 mM and a k_1/k_2 ratio of 1.01 ± 0.07. The correspondence between the theoretical curve generated using these constants and the experimental results obtained for coupling ratio is shown in Fig. 2.

The apparent equality of k_1 and k_2 makes the function rectilinear with an intercept of 1 and a slope of $1/K_{Met}$. The Na-dependent carrier appears, by this analysis, to possess a reasonably high affinity for methionine in the absence of Na, though this is still an order of magnitude less than that found using methionine in the presence of 140 mM Na [2]. It has been known for some time that the transport of hydrophobic amino acids is less dependent upon the presence of Na than that of hydophilic amino acids. The present results suggest

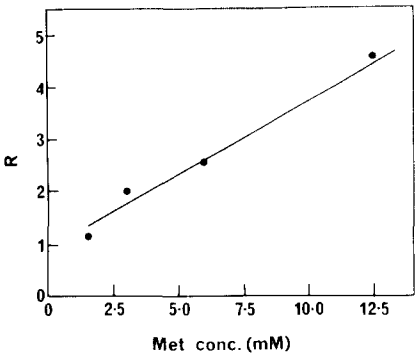

Fig. 2. Delta analysis of methionine influx in rabbit distal ileum. The experimental values of the coupling ratio (R), taken from Table IV, are plotted as a function of the concentration of amino acid used. The line is the best fit to Eqn. 9 in the text using a value of 3.6 ± 1.1 mM for K_{Met} and a value of 1.01 ± 0.07 for the ratio k_1/k_2.

that part of this discrepancy can be accounted for by the ability of hydrophobic amino acids to use the Na-dependent carrier in the absence of Na. At 30 mM, for instance, it is predicted that the influx of methionine on the Na-dependent carrier in the absence of Na will be 90% of that measured at 140 mM Na (98% of total influx taking into account the additional entry of methionine on the Na-independent system). The absolute dependence of alanine influx on the presence of Na can be explained by assuming that alanine does not bind to the carrier in the absence of Na or else that the permeability coefficient for the EA complex is zero.

Coupling ratios greater than unity have also been reported for pigeon red blood cells, their origin being attributed to 'futile cycling' of amino acids (Fig. 3 in Ref. 13). This work, which was carried out using hydrophilic amino acids, showed no dependency of the coupling ratio on amino acid concentration. This system appears, therefore, to be very different from that reported for the rabbit ileum. Transconcentration effects on amino acid influx, seen to take place in other cells [14], appear to be absent from the brush border membrane of the mammalian enterocyte [15,16]. Changes in the electrochemical potential have also been held responsible for leading to anomalous measurements of the coupling ratio in intestinal cells [17]. Again, this appears not to complicate the present results, where a 10-fold change in external Na concentration has been shown to have no measurable effect on the stoichiometry of alanine coupling to Na influx.

The present analysis of coupling ratios, which we call delta analysis for want of a better term, could prove of general use when studying the mechanisms available for amino acid translocation across membranes, particularly where straightforward kinetic analysis proves ineffective. It could also prove valuable in assessing the structural requirements which determine the binding and translocation constants for different neutral amino acids.

References

1 Sepúlveda, F.V. and Smith, M.W. (1978) J. Physiol. 282, 73—90
2 Paterson, J.Y.F., Sepúlveda, F.V. and Smith, M.W. (1979) J. Physiol. 292, 339—350

3 Paterson, J.Y.F., Sepúlveda, F.V. and Smith, M.W. (1980) J. Physiol. 298, 333—346
4 Curran, P.F., Schultz, S.G., Chez, R.A. and Fuisz, R.E. (1967) J. Gen. Physiol. 50, 1261—1286
5 Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1970) Biochim. Biophys. Acta 515, 329—366
6 Christensen, H.N. (1979) Adv. Enzymol. 49, 41—101
7 Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33—66
8 Paterson, J.Y.F., Sepúlveda, F.V. and Smith, M.W. (1980) J. Physiol. 303, 78p
9 Paterson, J.Y.F., Smith, M.W. and Sepúlveda, F.V. (1980) Biochim. Biophys. Acta 598, 184—188
10 Smith, M.W. and Sepúlveda, F.V. (1979) Biochim. Biophys. Acta 555, 374—378
11 Alvarez, O., Goldner, A.M. and Curran, P.F. (1969) Am. J. Physiol. 217, 946—950
12 Sepúlveda, F.V. and Robinson, J.W.L. (1978) J. Physiol. (Paris) 74, 575—583
13 Koser, B.H. and Christensen, H.N. (1971) Biochim. Biophys. Acta 241, 9—19
14 Christensen, H.N. (1975) Curr. Top. Membranes Transp. 6, 227—258
15 Schultz, S.G., Curran, P.F., Chez, R.A. and Fuisz, R.E. (1967) J. Gen. Physiol. 50, 1241—1260
16 Robinson, J.W.L. (1974) Biochim. Biophys. Acta 367, 88—101
17 Kimmich, G.A. and Randles, J. (1980) Biochim. Biophys. Acta 596, 439—444