Biochimica et Biophysica Acta, 689 (1982) 55–62
Elsevier Biomedical Press

BBA 71220

THE TRANSPORT OF ALANINE AND GLUTAMINE INTO ISOLATED RAT INTESTINAL EPITHELIAL CELLS

NORAH M. BRADFORD and JOHN D. McGIVAN

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD (U.K.)

(Received November 23rd, 1981)

Key words: Amino acid transport; Alanine; Glutamine; Amino acid exchange; (Rat intestinal epithelial cell)

(1) The transport of alanine and glutamine into isolated rat intestinal epithelial cells, prepared as described previously (Watford, M., Lund, P. and Krebs, H.A. (1979) Biochem. J. 178, 589–596), was studied. (2) Cells isolated by this method accumulated alanine 7-fold from an external concentration of 0.5 mM, and by this criterion appear more suitable for transport studies than do previous rat intestinal cell preparations. (3) In these cells, it was shown using several different approaches that the major part of the transport of alanine and glutamine is mediated by a common carrier which is Na^+ dependent and is sensitive to inhibition by 2-methylaminoisobutyric acid. (4) These results are in contrast to the situation in isolated hepatocytes where glutamine is transported by a carrier system (System N) distinct from that that which mediates the transport of alanine. (5) It is suggested that a major metabolic function of this transport system in intestinal cells is the exchange of extracellular glutamine for intracellular alanine, which is a major product of glutamine metabolism in the gut.

Introduction

The major nitrogen-containing products of amino acid metabolism in skeletal muscle are alanine and glutamine. Alanine is mainly utilized by the liver, where it is a major substrate for the synthesis of glucose and urea. Although glutamine can be metabolized to glucose in perfused liver or in isolated hepatocytes it is not a major substrate for liver metabolism. The glutamine produced by the skeletal muscle is metabolized mainly in the intestine, where it is a major fuel of respiration, and in the kidney where glutamine metabolism is involved in the regulation of acid/base balance (for a recent review, see Ref. 1).

Previous work in our laboratory led to the postulate that glutamine and alanine are transported across the hepatocyte plasma membrane by separate transporting systems [2]. A transport system in isolated hepatocytes specific for glutamine, asparagine and histidine was identified and characterized by Kilberg et al. [3] and given the designation 'System N'. The system could be distinguished from System A (which catalyzes the major part of alanine transport in hepatocytes) by its lack of inhibition of 2-methylaminoisobutyrate. It is not at present known whether System N occurs uniquely in the liver cell plasma membrane, or whether it exists also in the plasma membranes of other mammalian cells. It was therefore of interest to study the characteristics of alanine and glutamine transport in tissues where the metabolic roles of these amino acids are different from their functions in the liver. A good example of such a tissue is the intestinal epithelium where glutamine is an important substrate while alanine is an end-product of glutamine metabolism. Accordingly, it

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

0005-2736/82/0000–0000/$02.75

was decided to study the transport of alanine and glutamine in cells isolated from rat intestinal epithelium.

Amino acid transport in the intestinal epithelium has been studied mainly in various preparations of the intact tissue. Although several techniques for the isolation of intestinal epithelial cells have been described (see Refs. 4 and 5 for references) relatively few studies of active transport in such cells have been performed. Cells isolated from chicken intestine by treatment with hyaluronidase have been used extensively for the study of Na^+-dependent transport by Kimmich [4]; however, the same method is reported to produce cells from rat intestine which have minimal ability for Na^+-dependent transport. Amino acid transport in isolated rat intestinal epithelial cells was studied in some detail by Reiser and Christiansen [6] but their cell preparation accumulated L-leucine only 2-fold.

The recent report by Watford et al. [5] of an improved procedure for the isolation of rat intestinal epithelial cells suggested to us that this preparation might be appropriate for the study of amino acid transport. In this paper, it is shown that, under appropriate conditions, alanine and glutamine are accumulated by these cells. Evidence is presented that, contrary to the situation in the liver, alanine and glutamine are transported into intestinal epithelial cells by a common transporting system.

Methods

Preparation of cells

Isolated intestinal epithelial cells were prepared from normally fed female Wistar rats (250–300 g weight) exactly as described by Watford et al. [5]. The cells were suspended in Krebs-Henseleit bicarbonate-buffered saline (pH 7.5) [7], containing 2.5% (w/v) bovine albumin (fraction V) under an atmosphere of 95% O_2 + 5% CO_2 at a concentration of 5–10 mg cell protein/ml. The albumin had been dialyzed but not defatted. The use of glass apparatus was avoided during the preparation, since the cells tended to clump on contact with glass.

Measurement of transport

For the measurement of the transport of alanine and glutamine, an aliquot of the cell suspension was added to Krebs-Henseleit medium containing [^{3}H]inulin plus 10 μg/ml unlabelled inulin to act as a marker of the extracellular space together with [U-^{14}C]alanine or [U-^{14}C]glutamine to give the required final concentration. All incubations also contained a final concentration of 1 mM aminooxyacetate, a specific inhibitor of transaminase enzymes. The cell suspension was shaken in an orbital shaker. The final cell concentration was 2–4 mg protein/ml. The transport reaction was terminated by centrifuging the cells through silicone oil into perchloric acid. Tubes for the Beckman Model 152 centrifuge were prepared as follows: 50 μl of 1.83 M $HClO_4$ were placed at the bottom of the tube and 100 μl of a 1:1 (v/v) mixture of dinonylphthalate and silicone fluid (Dow Corning 550) (Hopking & Williams, Essex, U.K.) were layered above it. To stop the transport reaction, aliquots of 0.25 ml of the incubation mixture were pipetted on top of the oil layer and centrifuged for 40 s. A sample of the supernatant layer was counted for radioactivity and the tubes were then frozen and cut through the silicone oil layer. The entire contents of the perchloric acid layer were shaken well in scintillation fluid (Unisolve E; Koch Light Laboratories, U.K.) and assayed for ^{3}H and ^{14}C by dual-channel liquid scintillation counting. The intracellular content of alanine or glutamine was taken as the ^{14}C counts in the total pellet corrected for the extracellular water as measured by the [^{3}H]inulin space. This method has been used routinely for the measurement of transport in isolated hepatocytes [3,8,9]. A similar method was previously used to measure pyruvate transport in enterocytes by Lamers and Hulsmann [10]. The method offers superior time resolution, more rapid quenching of metabolic reactions and more accurate determination of extracellular water than methods in which transport is terminated by dilution with ice-cold medium followed by washing of the pellet.

Aminooxyacetate completely inhibits the metabolism of alanine in hepatocytes and has been utilized to allow the assay of alanine transport by isotope uptake in this system [2,11]. Similar results (not shown) were obtained in epithelial cells in the

present investigation. However, these cells metabolize glutamine relatively rapidly. Since it is not possible to inhibit glutamine metabolism, the uptake of ^{14}C label from [^{14}C]glutamine does not necessarily accurately represent net glutamine uptake where glutamine metabolism is extensive. It was found on assaying glutamine metabolism by the measurement of NH_3 production that when the cells were incubated with 0.5 mM glutamine under the conditions used for transport studies, between 5 and 10% of this glutamine was metabolized during the first 2 min, and this increased to 30% after 10 min. Initial rates of [^{14}C]glutamine uptake measured over the first 2 min should therefore represent the uptake of glutamine fairly accurately under the conditions used here.

Assays

For the measurement of metabolites, an aliquot of the cell suspension as prepared was incubated with the appropriate substrate and protein depleted by the addition of $HClO_4$, 0.61 M final concentration. After centrifugation, the supernatant was neutralized with a small volume of 3 M KOH. Lactate [12], NH_3 [13] and alanine [14] were assayed enzymically. ATP was measured by the luciferase method [15]. Cell protein was determined by a biuret method [16]. This is convenient for measuring cell concentration, since it can be performed rapidly and cell suspensions can be adjusted to the appropriate concentration at the start of an experiment. In order to compare these results with those of others who measure cell concentration on a dry weight basis, it was found that the ratio of dry weight to protein was 1.58 ± 0.12 (mean $\pm$ S.E. for eight preparations).

Properties of isolated rat epithelial cells in relation to transport studies

Cell viability was determined by measuring intracellular ATP and lactate production from 10 mM glucose. The values obtained were comparable to those reported by Watford et al. [5]. In accordance with their observations, it was found that the cell ATP level and the rate of lactate production decreased markedly with time. Cells were routinely used for transport experiments immediately after preparation. The cells could be kept on ice for up to 30 min, during which time after warming to 37°C the initial ATP level and initial rate of alanine transport were unchanged; the cells deteriorated if kept on ice for longer than this period. Loss of cell viability in general was accompanied by clumping of the cells, which then failed to pass through the silicone oil layer on centrifugation. The internal volume of the cells as measured by the difference in the volume available to [^{14}C]inulin and ^3H_2O was 5.03 ± 0.30 μl/mg protein, and the volume of extracellular water carried down with the cells was 10.52 ± 0.39 μl/mg protein (mean $\pm$ S.E. of 14 determinations).

Results

Isolated rat epithelial cells as an experimental system for the study of intestinal transport

The time course of the transport of alanine (0.5 mM) into isolated epithelial cells at three different temperatures is shown in Fig. 1. The maximum accumulation ratio for alanine at 37°C was greater than 7. This is comparable with the ratios obtained for the transport of alanine into hepatocytes. At 37°C, the cell content of alanine was maximal after 10 min and declined sharply thereafter. That

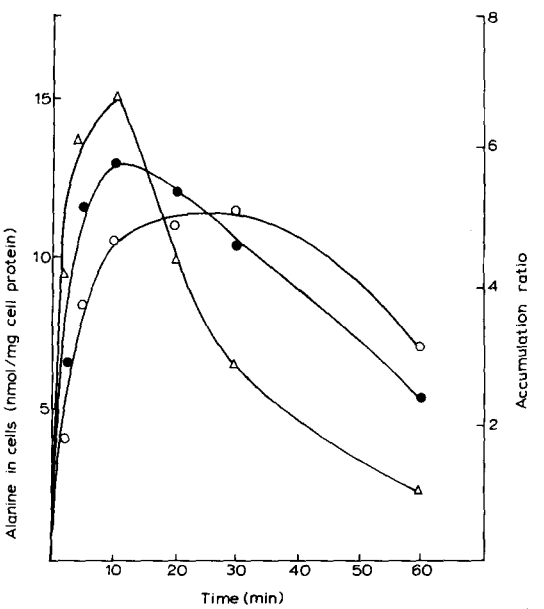

Fig. 1. Time course of alanine uptake into rat intestinal cells. Cells were incubated with 0.5 mM alanine at three different temperatures. The experiments were performed concurrently. (○——○) 22°C, (●——●) 30°C, (△——△) 37°C.

the decrease in alanine content was associated with loss of cell viability is indicated by the observation that after 10 min, a decreased proportion of the cells penetrated the silicone layer on centrifugation. In other experiments (not shown), it was found that the addition of glucose (10 mM) affected neither the initial rate of transport of alanine nor the cell ATP level. The loss of alanine from the cells after 10 min was also unaffected by the presence of glucose. It was concluded that the loss of cell viability was an inherent property of this cell preparation rather than being due to lack of provision of intracellular ATP. At lower temperatures, the decrease in cell viability occurred only after a longer period of time. At 22°C, the cell content of alanine remained constant between 10 and 30 min. The rate of alanine transport was shown to be linear with time over the first 2 minutes; initial rates of transport for 0.5 mM alanine in the experiment shown in Fig. 1 were 2.00, 3.25 and 4.82 nmol/min per mg cell protein at 22, 30 and 37°C, respectively.

Fig. 2 shows the relative rates of transport of alanine and glutamine (added at 0.5 mM) at 23°C.

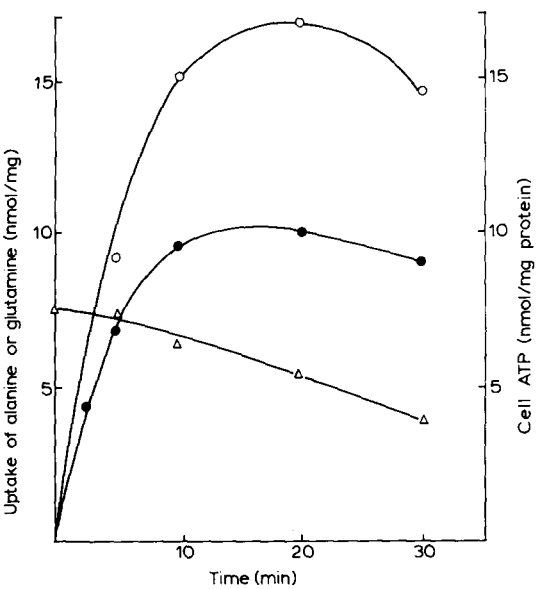

Fig. 2. Time course of the uptake of alanine and glutamine at 23°C. Cells were incubated with 0.5 mM glutamine or 0.5 mM alanine. Samples were withdrawn at the times shown and spun through silicone oil to terminate transport; parallel samples were protein depleted and assayed for ATP. (●——●) alanine, (○——○) glutamine, (△——△) ATP.

The initial rate of transport of glutamine was somewhat faster than that of alanine. The ATP content of the cells remained steady for the first 5 min but declined steadily thereafter, in accordance with previous findings [5].

The uptake of alanine and glutamine would be expected to be Na^+ dependent by analogy with amino acid transport in other cell systems and with valine transport in chicken enterocytes in particular [4]. Table I shows the inhibition of alanine transport by ouabain (an inhibitor of the $(Na^+ + K^+)$-ATPase) and FCCP (an uncoupler of oxidative phosphorylation). At saturating concentrations of each of these inhibitors, alanine merely equilibrated across the cell membrane, and the energy-dependent uptake was abolished. The Na^+ dependence of transport is shown in Fig. 3. In the absence of Na^+, the initial rates of transport of both alanine and glutamine were less than 20% of the maximum rate in the presence of Na^+. Na^+ had a half-maximum effect on the rate of transport when present at 15 mM for each amino acid. The kinetics of transport were determined under optimal conditions at two different tempera-

TABLE I

INHIBITION OF ALANINE ACCUMULATION BY OUABAIN AND FCCP IN ISOLATED RAT ENTEROCYTES

Cells were incubated at 18°C with 0.5 mM alanine and various concentrations of inhibitors. Alanine uptake was measured after 15 min, by which time the alanine distribution ratio had reached a steady value. The results shown are the mean ± S.E. for four separate determinations using one cell preparation.

Additions	Alanine in cells (nmol/mg)	Alanine accumulation ratio
None	11.8 ± 0.27	4.90
0.05 mM ouabain	9.95 ± 0.6	4.15
0.1 mM ouabain	9.39 ± 0.72	3.91
0.5 mM ouabain	5.26 ± 0.33	2.19
1 mM ouabain	3.57 ± 0.13	1.50
2 mM ouabain	2.80 ± 0.10	1.16
2.5 μM FCCP	9.06 ± 0.25	3.78
5.0 μM FCCP	7.10 ± 0.31	2.96
7.5 μM FCCP	3.34 ± 0.11	1.39
10 μM FCCP	4.04 ± 0.12	1.68

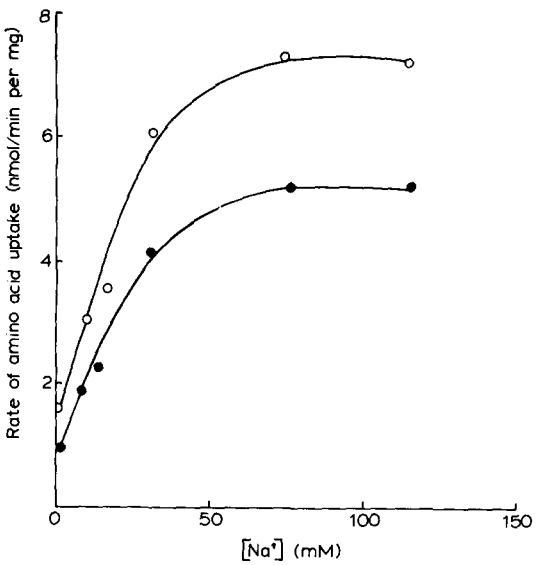

Fig. 3. Na^+ dependence of the transport of alanine and glutamine. Cells were incubated with 0.5 mM alanine or 0.5 mM glutamine at 37°C in a series of media containing modifications of Krebs-Henseleit solution where various proportions of the Na^+ content were replaced with the corresponding choline salts. Transport was measured after 2 min. (●———●) alanine, (○———○) glutamine.

tures (Fig. 4). The transport of both amino acids gave linear double-reciprocal plots, indicating simple saturation kinetics. Half-maximal rates of transport were obtained at 4.5 mM alanine or at 2.1 mM glutamine.

The above results are taken to indicate that the isolated rat enterocyte preparation of Watford et al. [5] is a suitable experimental system for the quantitative study of amino acid transport. The major experimental limitation of this system is the relatively short period of cell viability at 37°C. However, initial rates of transport can be measured satisfactorily at this temperature. Other parameters are determined more satisfactorily at lower temperatures. The degree of accumulation of amino acids, Na^+ dependence and energy dependence of this process are comparable with similar parameters obtained for several other isolated cell preparations from different tissues.

Interaction of the transport of alanine and glutamine in intestinal cells

As stated in the Introduction, alanine and glutamine are transported by separate systems in hepatocytes, and it was of interest to determine whether the same situation would be obtained in intestinal epithelial cells. Glutamine was found to inhibit alanine transport. Fig. 5 shows a typical experiment where the results are presented as a Dixon plot. The lines intersect above the x-axis, and this is characteristic of competitive inhibition. The K_i value for glutamine was approx. 2 mM, which is similar to the K_m value for glutamine transport.

The transport of alanine and glutamine was inhibited by various other amino acids. In Table II it is shown that asparagine, leucine and serine inhibited the transport of alanine and glutamine in parallel. This is in contrast to the previous findings

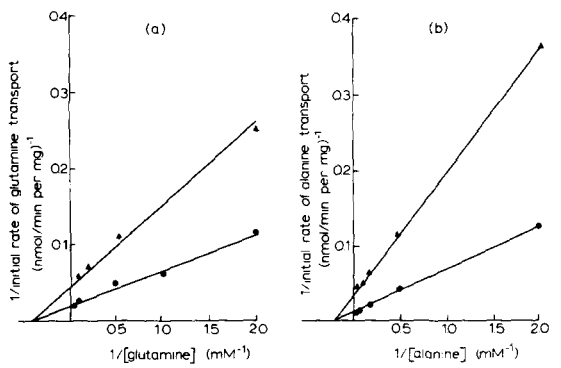

Fig. 4. Concentration dependence of alanine and glutamine transport into rat intestinal cells. Cells were incubated in Krebs-Henseleit bicarbonate medium. The initial rate of alanine and glutamine transport was measured after 2 min. The results are presented as a double-reciprocal plot. (a) Glutamine, (b) alanine. (▲) 25°C, (●) 37°C.

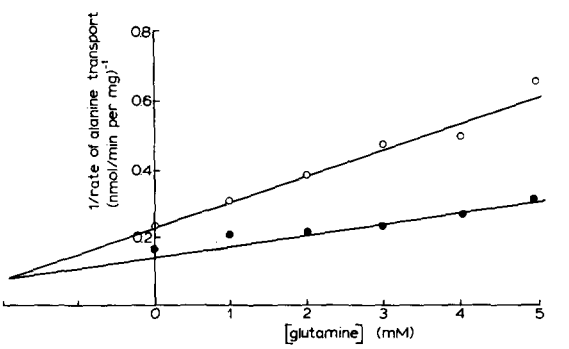

Fig. 5. Inhibition of alanine transport by glutamine. Cells were incubated with alanine plus glutamine at the concentrations shown at 37°C. Transport was measured after 2 min. (○———○) 0.25 mM alanine, (●———●) 0.5 mM alanine.

TABLE II

INHIBITION OF ALANINE AND GLUTAMINE TRANSPORT BY VARIOUS AMINO ACIDS

Cells were incubated at 37°C with 0.25 mM alanine or 0.25 mM glutamine together with amino acids as indicated added at 5 mM final concentration. The results shown are the mean ± S.E. of six determinations. Mean control rates were: alanine 2.96 ± 0.11 nmol/min per mg; glutamine 4.53 ± 0.15 nmol/min per mg.

Substrate	% inhibition of initial rate of transport		
	L-Asparagine	L-Leucine	L-Serine
Alanine	74.7 ± 1.0	86.1 ± 1.0	67.7 ± 3.0
Glutamine	73.1 ± 1.1	91.9 ± 0.7	64.8 ± 1.7

in hepatocytes [2] where asparagine and serine inhibit alanine transport far more than that of glutamine while leucine inhibits the transport of glutamine in preference to that of alanine. Fig. 6 shows the inhibition of the initial rates of transport of both alanine and glutamine by the non-metabolized amino acid analogue 2-methylaminoisobutyrate which is considered to be a specific inhibitor of System A for amino acid transport. The transport of alanine was inhibited by 2-methylaminoisobutyrate to approximately the same extent as that of glutamine. In hepatocytes, the transport of alanine, but not that of glutamine, is inhibited by this compound. That none of the above inhibitory effects is due to electrical interaction of independent systems is indicated by the finding that glucose, which is transported on an Na^+-linked system in enterocytes, did not affect the initial rate of transport of either alanine or glutamine.

The above results strongly suggest that in rat enterocytes, the transport of alanine and that of glutamine are mediated by the same transporting system. If this is the case, it should be possible to demonstrate the exchange of intracellular alanine for extracellular glutamine. Fig. 7 shows an experiment in which cells were first exposed to ^{14}C-labelled alanine (in the presence of aminooxyacetate to inhibit alanine metabolism). After accumulation of the alanine had occurred, the addition of glutamine to the system caused a rapid release of alanine. The addition of glucose did not cause alanine efflux in this system and it was noted previously that glucose does not affect the initial rate of alanine transport. In similar experiments,

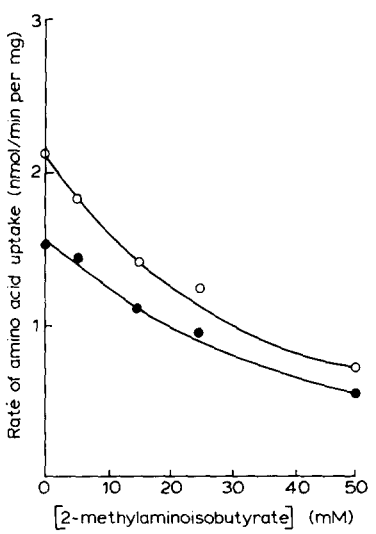

Fig. 6. Inhibition of alanine and glutamine transport by 2-methylaminoisobutyrate. Cells were incubated with 0.1 mM alanine or 0.1 mM glutamine together with 2-methylaminoisobutyrate at the concentrations shown. Transport was measured after 2 min. The temperature was 37°C. (●——●) alanine; (○——○) glutamine.

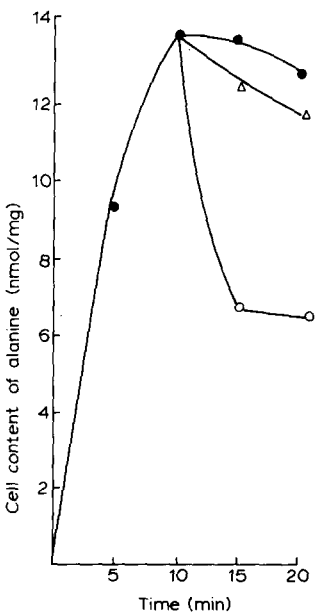

Fig. 7. Exchange of alanine for glutamine. Cells were incubated with 0.5 mM alanine at 30°C. After 10 min, glutamine (5 mM) or glucose (5 mM) was added to aliquots of the suspension. (●——●) control, (○——○) glutamine after 10 min, (△——△) glucose after 10 min.

citrulline and proline, which are end-products of glutamine metabolism in the gut, also did not exchange with alanine. The reverse experiment was not attempted due to uncertainty about the identity of the ^{14}C-labelled compounds when cells were incubated with [^{14}C]glutamine for prolonged intervals.

Discussion

Isolated rat intestinal epithelial cells prepared by the method of Watford et al. [5] appear to be a suitable system for the study of some aspects of intestinal transport. The cells accumulated alanine 7-fold from an external concentration of 0.5 mM. This accumulation ratio is similar to, or greater than, those obtained for the transport of 1 mM galactose and 1 mM valine in chicken epithelial cells [4], and may be compared with the 2-fold accumulation of 1 mM L-leucine observed in rat intestinal cells prepared by a previous method [6].

Kimmich [4] has argued that the uptake of sugars by isolated chicken enterocytes occurs mainly across the brush border membrane on the grounds that this process is strongly inhibited by phloridin which has been reported to be effective at the mucosal but not at the serosal boundary of the intact intestine [17]. In the case of amino acid transport, such a criterion is not available, and it does not seem possible to discriminate between transport across the serosal membrane and that across the brush border membrane in the present study.

The results presented in this paper show that glutamine and alanine transport into isolated rat enterocytes is mediated mainly by a common carrier for these amino acids which is Na^+ dependent and inhibited by 2-methylaminoisobutyrate, properties which are characteristic of System A in other tissues. The existence of a common carrier is deduced from the following observations: (a) alanine transport is inhibited competitively by glutamine, and the K_i for inhibition approximates to the K_m for glutamine transport; (b) the inhibitory effects of serine, asparagine, leucine and 2-methylaminoisobutyrate on the transport of alanine are parallel with those on glutamine transport; (c) alanine which has been accumulated by the cells is released by addition of glutamine but not by the addition of glucose which is transported on a separate Na^+-dependent system. These results contrast strongly with those obtained in isolated hepatocytes where glutamine transport is inhibited only slightly by serine [2] and not at all by 2-methylaminoisobutyrate [3], and the inhibition of alanine transport by glutamine is non-competitive [2]. It follows that System N, the major transport system for glutamine in liver cells [3], does not make a major contribution to glutamine transport in rat enterocytes.

In the intact intestine, glutamine is used as a major respiratory substrate and the major end-products of glutamine metabolism are NH_3, alanine, citrulline and proline [18]. However, in the isolated cell preparation used here, glutamine is metabolized to alanine plus ammonia, with little or no formation of citrulline or proline [5]. Alanine formed by the intestine is rapidly metabolized by the liver. Watford et al. [5] have suggested that an important function of the conversion of glutamate to alanine in the gut may be the avoidance of accumulation of glutamate in the portal blood. Glutamate is not readily metabolized by the liver. It is possible that a major metabolic function of the transport system for alanine and glutamine identified in the present study is the inward transport of glutamine across the serosal membrane in exchange for the outward transport of its metabolic product, alanine.

Acknowledgements

We thank Miss S. Small for assistance with cell preparation. We also thank The Medical Research Council for financial support.

References

1 Lund, P. (1979) FEBS Lett. 117, K86–K92
2 Joseph, S.K., Bradford, M.M. and McGivan, J.D. (1978) Biochem. J. 176, 827–836
3 Kilberg, M.S., Handlogten, M.E. and Christensen, H.N. (1980) J. Biol. Chem. 255, 4011–4019
4 Kimmich, G.A. (1975) Methods Membrane Biol. 5, 51–115
5 Watford, M., Lund, P. and Krebs, H.A. (1979) Biochem. J. 178, 589–596
6 Reiser, S. and Christiansen, P.A. (1971) Biochim. Biophys. Acta 255, 123–129
7 Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66

8 McGivan, J.D., Bradford, N.M. and Mendes-Mourao, J. (1977) FEBS Lett. 80, 380–384
9 McGivan, J.D., Ramsell, J.C. and Lacey, J.H. (1981) Biochim. Biophys. Acta 644, 295–304
10 Lamers, J.M.J. and Hulsmann, W.C. (1975) Biochim. Biophys. Acta 394, 31–45
11 Edmondson, J.W., Lumeng, L. and Ti, T.-K. (1977) Biophys. Biochem. Res. Commun. 76, 751–757
12 Hohorst, H.J. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.), pp. 266–270, Academic Press, New York
13 Kirsten, E., Gerez, C. and Kirsten, R. (1963) Biochem. J. 237, 312–319
14 Williamson, D.H., Lopes-Vereira, O. and Walker, B. (1967) Biochem. J. 104, 497–502
15 Stanley, P.E. and Williams, S.G. (1969) Anal. Biochem. 29, 381–392
16 Gornall, H.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–756
17 Newey, H., Parsons, B.J. and Smyth, D.H. (1959) J. Physiol. 148, 83–89
18 Windmueller, H.C. and Spaeth, A.E. (1974) J. Biol. Chem. 249, 5070–5079