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Kinetics and specificity of L-alanine transport across the basolateral cell surface in isolated oxyntic glands

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The time course, kinetics, specificity and sodium-dependence of alanine uptake by isolated oxyntic glands were studied. The uptake of alanine by the hydrolyzed cells was measured directly, after incubation of the glands with L-[^{3}H]alanine. L-Alanine total influx was saturable and apparently mediated by a single entry system (K_t = 7.93 mM and V_{max} = 8.0 μmol · mg^{-1} · 30 s^{-1}). The K_t was comparable to previously reported values for L-alanine transport in other epithelial cells. Kinetic studies performed in the presence and absence of Na^+ suggest that L-alanine uptake is mainly mediated by a Na^+-dependent carrier system, but in addition, a minor diffusional component has been detected. Cross inhibition experiments performed over a wide range of concentrations (1 to 100 mM) suggest that the Na^+-dependent transport system for alanine resembles system A and displays higher affinity for L-serine (K_i = 1.81 mM) than for L-alanine (K_t' = 4.86 mM); a lower affinity was found for L-cysteine (K_i = 16.30 mM). Results obtained with MeAIB support the hypothesis that system A is present at the basolateral membrane of the gland cells.

Introduction

Known transport systems which are ubiquitous in cells of higher animals [1–3], account for the influx of amino acids from the blood to tissue cells in the small intestine [4–6] and accessory digestive organs [7–9].

There is currently little direct evidence about the transport pathways for amino acids in the stomach. In previous reports [10,11] we showed that intraarterially injected L-amino acids are taken up by tissue cells of the stomach at rates which are greater for acidic than for neutral or basic amino acids. Uptake values increased when gastric secretion was stimulated with histamine. In a recent study [12] by using L-leucine and L-alanine at models of neutral amino acids, we demonstrated that characteristic transport systems, similar to 'L' and 'A/ASC' types, are operative at the blood/tissue interface of the stomach. The kinetic parameters obtained for alanine were comparable with those measured in other organs, whereas the same was not valid for leucine. However, these results were obtained by means of the paired-tracer dilution technique [9] in the vascularly perfused stomach, with which it is almost impossible to discriminate between the different gastric layers and cell types involved in amino acid transport.

In the present study, by measuring the kinetic constants and the specificity of L-alanine transport through the basolateral cell surface in isolated oxyntic glands, we have characterized a Na^+-dependent transport system which is shared by short-chain neutral amino acids. Part of this work has been published in abstract form [13].

Materials and Methods

Isolation of the oxyntic glands

Rabbits (1–1.5 kg) were used for the experiments. By following the method described by Berglindh and Öbrink [14] the gastric glands were isolated from the corpus of the non-stimulated stomach in the anaesthetized (30 mg/kg nembutal) rabbit. After subdiaphragmatic ligatures of the aorta and of the mesenteric vessels, the stomach was perfused with a saline solution (SS: 154 mM NaCl, 10 mM KCl) under high pressure through an aortic cannula introduced in a retrograde direction. When the stomach appeared totally exsanguinated it was rapidly removed, cut open the lesser curvature and rinsed in saline solution. The mucosa of the corpus was stripped off, minced into small pieces

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and transferred to a 200 ml flask containing 25 ml collagenase solution. This solution consisted of 1 mg collagenase Type IV dissolved in 1 ml incubation medium (IM), with the following composition (mM); NaCl, 132.4; KCl, 10; $CaCl_2$, 1; $MgCl_2$, 0.8; Na_2HPO_4, 5; NaH_2PO_4, 1.2; pyruvic acid, 1; Glucose, 11.1 and bovine serum albumin, 1 mg/ml (pH 7.4). The flask was gassed with 100% oxygen, sealed, incubated at 37°C and continuously stirred with a magnet during 40 min. The glandular suspension was filtered through a nylon cloth (pore size approx. 230 μm) into 15-ml test tubes with conical bottoms. Thus the glands were separated from isolated cells by sedimentation and repeated washing. The yield of gastric glands from this material amounted to approx. 7 mg wet weight, corresponding to a dry weight of 1 mg.

The viability of the isolated oxyntic glands was determined by the dye exclusion technique. Equal volumes of trypan blue (400 mg/100 ml IM) and gland suspension were mixed and immediately examined by light microscopy. About 96% of the glands were able to exclude the dye. In each tinged gland only 1–2 cells were unable to exclude the trypan blue. The dependence of L-alanine transport upon extracellular sodium was studied by measuring the uptake of the labelled amino acids by the isolated glands incubated with the IM in which Na^+ was iso-osmotically replaced with choline (as choline chloride). Inhibition experiments were performed by measuring the uptake of ^{3}H-labelled alanine in the presence and absence of unlabelled amino acids at concentrations which ranged between 2 and 100 mM.

Measurement of amino acid uptake

A mixture reaction, previously gassed with 100% oxygen, was incubated at 37°C in 1.5 ml Ependorf tubes. In each tube the mixture reaction contained: the isolated glands (equivalent to 1 mg dry weight) suspended in 1 ml of the incubation medium and L-[^{3}H]alanine (at a final concentration of 26.7 μM) At indicated time intervals (15 to 600 s), the mixtures were removed and immediately centrifuged at $11\,630 \times g$ (Microfuge, Heraus) during 15 s. The obtained pellets were dried (overnight, 75°C) and hydrolyzed with 20% HNO_3 at 75°C during 60 min. After cooling, 1 ml hydrolyzate containing 1 mg dry weight glands was mixed with 10 ml liquid fluor and 4 ml absolute ethanol. Standards of the mixture reaction and the hydrolysate were counted concurrently in a Beckman model LS-100 C automatic scintillation spectrometer as described previously [12]. The cellular uptake (U%) of the labelled amino acids was calculated as (cpm[^{3}H] · ml^{-1} (sample)/cpm [^{3}H] · ml^{-1} (standard)) · 100. From the U values and the specific activity of the ^{3}H-labelled amino acid (75 Ci/mmol for alanine) the cellular uptake was expressed as (nmol · mg^{-1}). When unlabelled alanine was added to the test tube, the total influx of alanine (μmol · mg^{-1} · 30 s^{-1}) was calculated as (U(%)/100) · [Ala], in which [Ala] corresponds to total alanine concentration in μmol · ml^{-1} and the influx time was 30 s. A similar procedure was followed to measure the [^{14}C]methylaminoisobutyric acid (MeAIB) uptake.

In order to investigate the possibility that a considerable fraction of [^{3}H]alanine could be distributed in an extracellular compartment, oxyntic glands were incubated with [^{14}C]sucrose during 30 to 600 s and processed like above described for tritium counting. Only 2% of the extracellular marker remained in the hydrolyzate of the dried glands.

In the kinetic studies of alanine uptake and when other amino acids were used as competitors, control measurements of [^{3}H]alanine uptake were performed in the presence of sucrose at equivalent concentrations to those of unlabelled alanine or competitors. Sucrose showed no appreciable effect on alanine uptake in previous experiments. Statistical significance was assessed using Student's t-test, with differences at the 0.001 level considered significant.

Chemicals

The radioactive molecules L-[^{3}H]alanine (75 Ci/mmol), [^{14}C]methylaminoisobutyric acid (48.4 mCi/mmol) and [^{14}C]sucrose (645.4 Ci/mmol) and the liquid scintillation fluors were purchased from New England Nuclear (U.S.A.). All other chemical used were obtained from Merck (F.R.G.) and Sigma Chemical Co (U.S.A.).

Results

The uptake of L-alanine by isolated oxyntic glands of the rabbit stomach was investigated by using uptake-time course, kinetic and inhibitor experiments. Sodium-dependent and sodium-independent uptakes were also measured.

Uptake time courses

Fig. 1 shows the time-dependent uptake of ^{3}H-labelled alanine by the isolated oxyntic glands measured in the absence (control conditions) of unlabelled alanine both in a normal sodium-containing medium and in a sodium-free medium. It is apparent that in the normal medium, the cellular content of the amino acid allows a steady state from 60 to 300 s. A similar uptake-time course was observed in one experiment in which the uptakes were measured in the presence of unlabelled 1.0 mM alanine (results not shown). Fig. 1 also illustrates that alanine uptake was roughly linear up to 30 s. Therefore, in order to compare results obtained under different experimental conditions 30 s incubation was allowed. At this time there was a substantial (74% of maximum) influx of the amino acid when its efflux might not be significant.

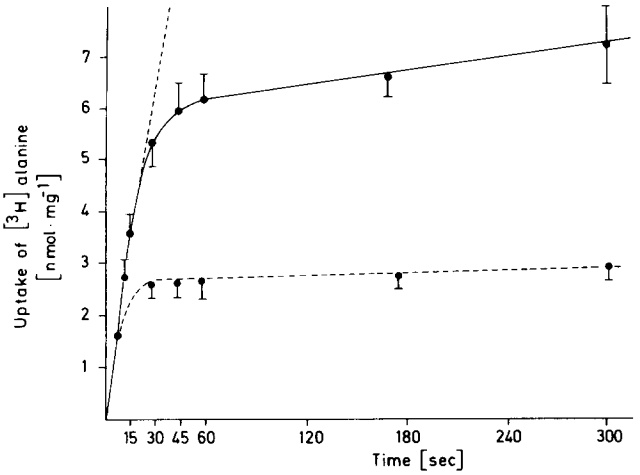

Fig. 1. Time course of alanine uptake in isolated oxyntic glands. Glands were incubated at 37°C with 26.7 μM L-[^{3}H]alanine in both a normal Na^+-containing (●———●) and a Na^+-free medium (choline, ● — — — ●). The amino acid uptake was measured at indicated times as described in Material and Methods. Each point denotes the mean ± S.D. of seven measurements. All values obtained in the absence of Na^+ are statiscally different from those observed in the normal medium ($P < 0.001$).

Kinetic of L-alanine influx

In order to evaluate whether the influx of L-alanine was saturable, the cellular uptake of the ^{3}H-labelled alanine was measured in the isolated oxyntic glands in the presence of different unlabelled alanine concentrations (0.25 to 15 mM). Because of variability in uptake measured in different preparations, the values obtained at various concentrations of the unlabelled amino acid were normalized in relation to control uptake values. From these normalized values the total influx of alanine was calculated as described in Material and Methods. Fig. 2 shows that the total influx of L-alanine varied with its extracellular concentration in a manner resembling saturation. A Michaelis-Menten kinetics analysis (Fig. 2, inset) suggested one component uptake pattern, with an apparent $K_t = 7.93 \pm 0.4$ mM and $V_{max} = 8.0 \pm 0.2$ μmol · mg^{-1} · 30 s^{-1}). A Hofstee transformation supports the hypothesis that one saturable component prevails in alanine transport (results not shown).

Effect of sodium-free medium on L-alanine uptake

In order to investigate the sodium-dependence of L-alanine uptake the oxyntic glands were incubated during different lengths of time (15 to 600 s) with 26.7 μmol L-[^{3}H]alanine under conditions similar to those above described for measurements in the normal medium, except that Na^+ was replaced by isoosmolar concentrations of choline (Fig. 1). Uptake values obtained in absence of sodium were significantly inhibited. From the ratio of the integral of the uptake-time curves ($\int_0^t U(t) \cdot dt$) in the normal medium with that obtained in the sodium-free medium it can be inferred that the uptake of alanine in the absence of Na^+ was reduced by 55%, i.e., about 45% of total alanine uptake appears to occur via a pathway which is sodium independent.

To assess the concentration dependence of alanine uptake and whether a component of alanine transport

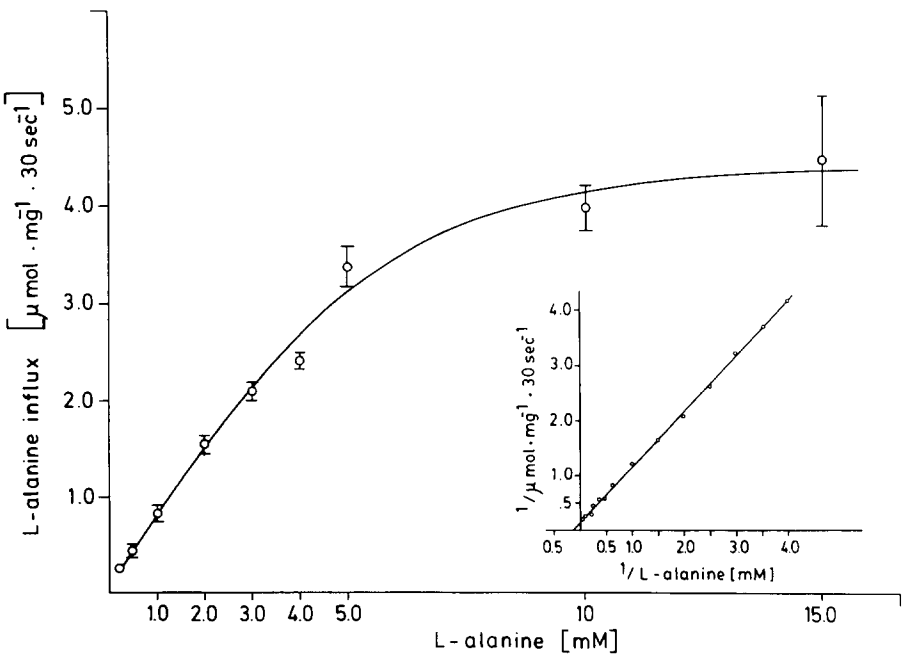

Fig. 2. Kinetic of unidirectional influx of L-alanine. Total influx of alanine was measured as a function of its concentrations (0.25 to 15 mM) according to procedures described in Materials and Methods.The influx time was 30 s. The continuous curve is a rectangular hyperbola obtained by a direct fit to the mean influx values. Each point denotes the mean ± S.D. of 11 to 20 measurements. The inset illustrates a Lineweaver-Burk plot of the data: kinetic constants obtained were (mean ± S.E.): $K_t = 7.93 + 0.4$ mM and $V_{max} = 8.0 \pm 0.2$ μmol · mg^{-1} · 30 s^{-1}.

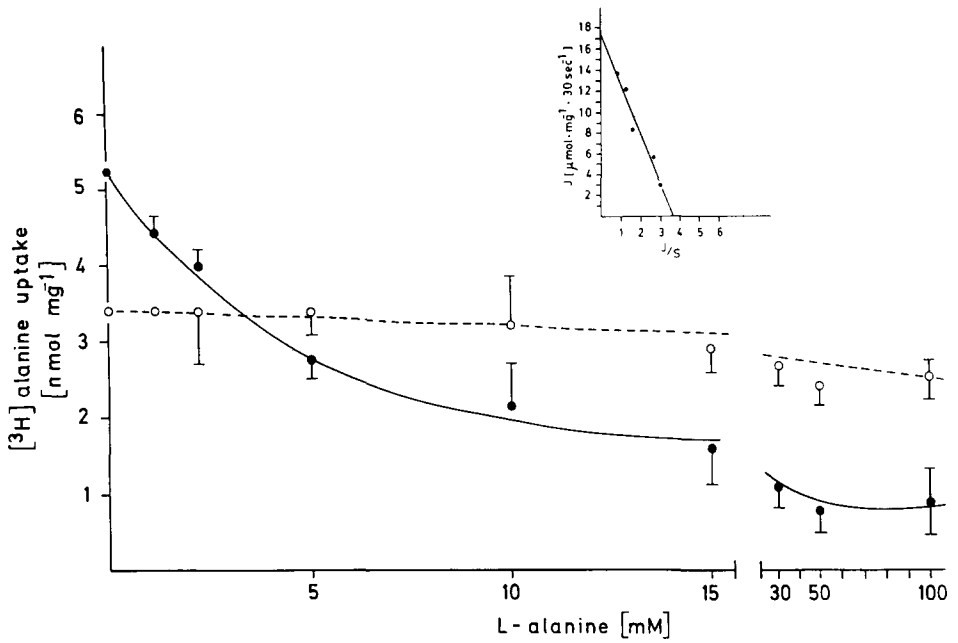

Fig. 3. Kinetic of L-alanine uptake. The uptake of 26.7 μM L-[^{3}H]alanine was measured in presence of 2 to 100 mM unlabelled alanine in a normal Na^+-containing medium (●———●) and in a Na^+-free medium (●— — —●). Values obtained at 100 mM unlabelled alanine have been considered as due to simple diffusion [14]. Each point refers to the mean ± S.D. of at least six measurements. Values were expressed as % of the respective initial uptake, i.e., that measured at 0 mM unlabelled amino acid. All percentages obtained in absence of sodium were statistically different from those observed in the normal medium ($P < 0.001$). The inset illustrates a Hofstee plot (J vs. J/S) of the Na^+-dependent carrier mediated component of alanine influx (J) calculated from values displayed in Fig. 3. Abcissa units are (μmol·mg^{-1}·30 s^{-1}/mM). The influx was calculated by: $J = [Ala]\cdot(U_{Na} - U_{ch})$, in which U_{Na} is the total uptake of [^{3}H]alanine measured in presence of Na^+ minus the diffusional uptake obtained in similar conditions. U_{ch} involves analogous measurements of [^{3}H]alanine uptake but in the absence of sodium (choline). Kinetic constants obtained were (mean ± S.E.): $K_t' = 4.86$ mM and $V_{max} = 17.7\ \mu$mol·mg^{-1}·30 s^{-1}.

across the basolateral plasma membrane of the oxyntic glands is carrier-mediated or just simple diffusion, the kinetics of the process in the presence and absence of Na^+ was studied (Fig. 3). In the presence of Na^+ the uptake of L-[^{3}H]alanine was asymptotically inhibited as the concentration of alanine was increased, the diffusional component being responsible for about 17% of total uptake. No significant inhibition of the uptake was observed even at 100 mM alanine when the glands were incubated in the sodium free medium. Therefore passive diffusion appears to be the primary pathway for alanine influx in the absence of Na^+.

The Na^+-dependent carrier mediated influx of alanine was calculated by considering uptake of L-[^{3}H]alanine measured in the absence and presence of unlabelled alanine (1 to 15 mM, Fig. 3) in both a sodium-containing and a sodium-free medium. Values obtained were analysed by Hofstee plot (Fig. 3, inset) which shows that the graph was resolvable into a single straight line. The kinetic constants were: $V_{max} = 17.70$ (μmol · mg^{-1} · 30 s^{-1}) and $K_t' = 4.86$ mM.

Inhibition of L-alanine uptake by neutral amino acids

Unlabelled neutral amino acid were tested for their effectiveness to inhibit the uptake of alanine by the gastric oxyntic glands. Fig. 4 depicts the results obtained when the uptake of 26.7 μmol [^{3}H]alanine was measured after the addition of the unlabelled amino acid (1 to 100 mM), in the presence (Fig. 4A) and then in the absence (Fig. 4B) of sodium. In the presence of sodium both short-chain neutral amino acids significantly inhibited the transport of L-alanine; the inhibition produced by low or intermediate concentrations of L-serine, which ranged between 28.6 (at 1 mM) and 51% (at 10 mM), was greater. In the absence of sodium (Fig. 4B) the effect of serine was significantly but not totally inhibited whilst cysteine showed no effect on alanine transport. Two long-chain neutral amino acids, L-leucine and L-isoleucine, did not inhibit the uptake of L-alanine either in the presence or absence of sodium. These overall results suggest that the uptake of alanine is primarily mediated by a Na^+-dependent system which is shared by other short-chain neutral amino acids.

The apparent inhibitor constants (K_i) for Na^+-dependent alanine transport were calculated for the two inhibitor amino acids [16,17] by assuming a single Na^+-dependent carrier for alanine. The K_i was calculated using:

$$K_i = \left[\frac{U_i}{U_{Na^+} - U_i}\right]\left[\frac{K_t\cdot[I]}{K_t + [Ala]}\right]$$

Where U_{Na^+} = uninhibited Na^+-dependent carrier mediated uptake of 26.7 μmol [^{3}H]alanine by the oxyntic

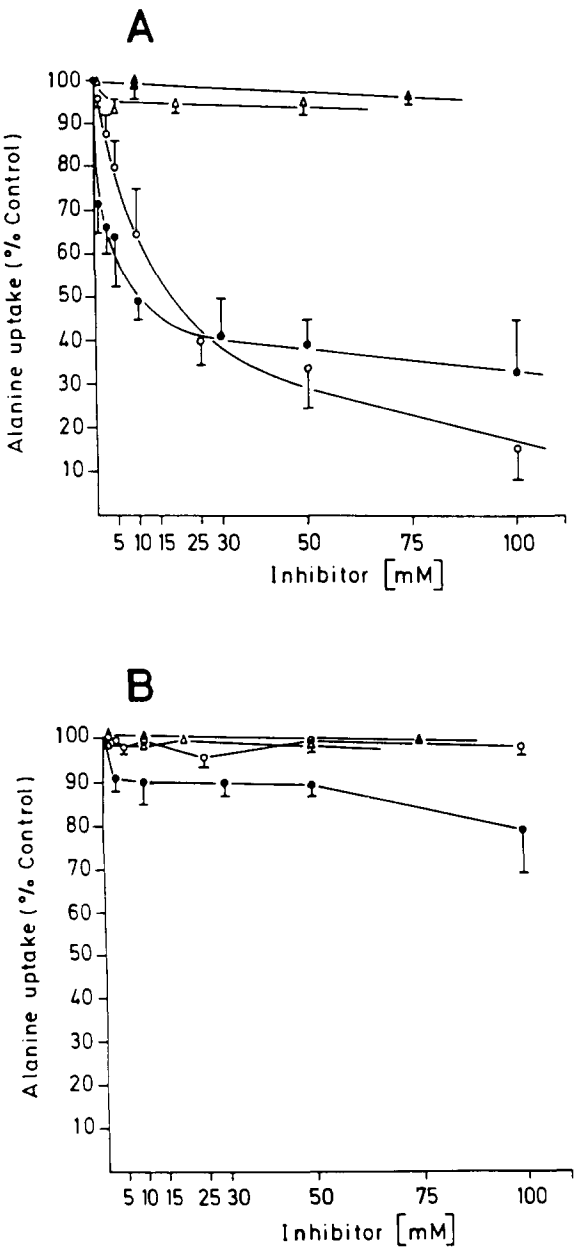

Fig. 4. Inhibition of 26.7 μM L- [^{3}H]alanine uptake by unlabelled L-neutral amino acids, either in a sodium-containing (A) and a sodium-free (B) medium. The values were determined by: % control = (uptake in presence of unlabelled amino acid/uptake in absence of unlabelled amino acid)·100. Each point denotes the mean ± S.D. of 4–8 measurements. The symbols are: serine (●), cysteine (○), leucine (△) and isoleucine (▲). Only serine and cysteine significantly inhibited the L-alanine uptake in the Na^+-containing medium. In the absence of sodium, no amino acid significantly inhibited the alanine uptake.

glands, which was obtained as: (total uptake in presence of Na^+ uptake by diffusion in presence of Na^+) minus (total uptake in absence of Na^+ uptake by diffusion in absence of Na^+); U_i involves analogous uptake measurements in the presence of the inhibitor. The concentrations of inhibitor (I) used were 1 mM serine and 10 mM cysteine. The apparent K_i for Na^+-dependent carrier-mediated transport of alanine were 1.81 mM serine and 16.30 mM cysteine.

Fig. 5 shows the effect of various amino acids assayed as inhibitors of methylaminoisobutyric acid (MeAIB). Both alanine and serine significantly inhibited the uptake of MeAIB, whereas cysteine only produced slight inhibition (Fig. 5A). When similar uptake measurements were performed in a sodium-free medium (Fig. 5B) no appreciable effect was observed, except that serine produced incomplete inhibition of MeAIB uptake. The long-chain neutral amino acid, leucine, did not significantly inhibit MeAIB uptake either in the presence or absence of sodium. By following a similar procedure yet described for alanine uptake [17,18] the apparent K_i of 5 mM serine and 5 mM alanine for the Na^+-dependent carrier-mediated transport of MeAIB were calculated. The respectives values were 1.23 mM serine and 8.90 mM alanine.

Discussion

As was shown by Berglindh and colleagues [14,18] gastric glands isolated from the corpus of the rabbit stomach are appropriate for studying the function of gastric epithelial cells in a state closely resembling the in vivo conditions. This model was selected to study the characteristics of the pathways involved in the basolateral transport of alanine because, in contrast with complete cell separation, isolated oxyntic glands maintain the polarity of the basolateral and luminal sides. Furthermore, the basolateral surface of the glands is more accessible than the lumen [19]. The present report provides evidence that the isolated gastric glands are viable (dye exclusion) and functional (high capacity of carrier-mediated transport). Because parietal cells amount to about 50% of the total cell volume of the isolated gland [14] and are peripherically located [19], the data obtained in this investigation would correspond principally to amino acid transport through the basolateral membrane of the parietal cells. Our findings have demostrated the presence of a saturable Na^+-dependent transport system for L-alanine at the basolateral side of isolated oxyntic glands in rabbit stomach. Although this system appears to provide the major route, simple diffusion seems to be another component involved in alanine influx which is evident in a sodium free medium (Fig. 3).

When the substrate specificity of the L-alanine transport system was investigated it was demonstrated to exhibit preference for short-chain neutral amino acids (Fig. 4A) L-serine being the most effective inhibitor of alanine uptake. Other neutral amino acids assayed as possible inhibitors of alanine failed to cause a significant effect on its transport. The results presented in Fig. 1 suggested that in the absence of sodium, uptake of alanine was considerably but not completely inhibited

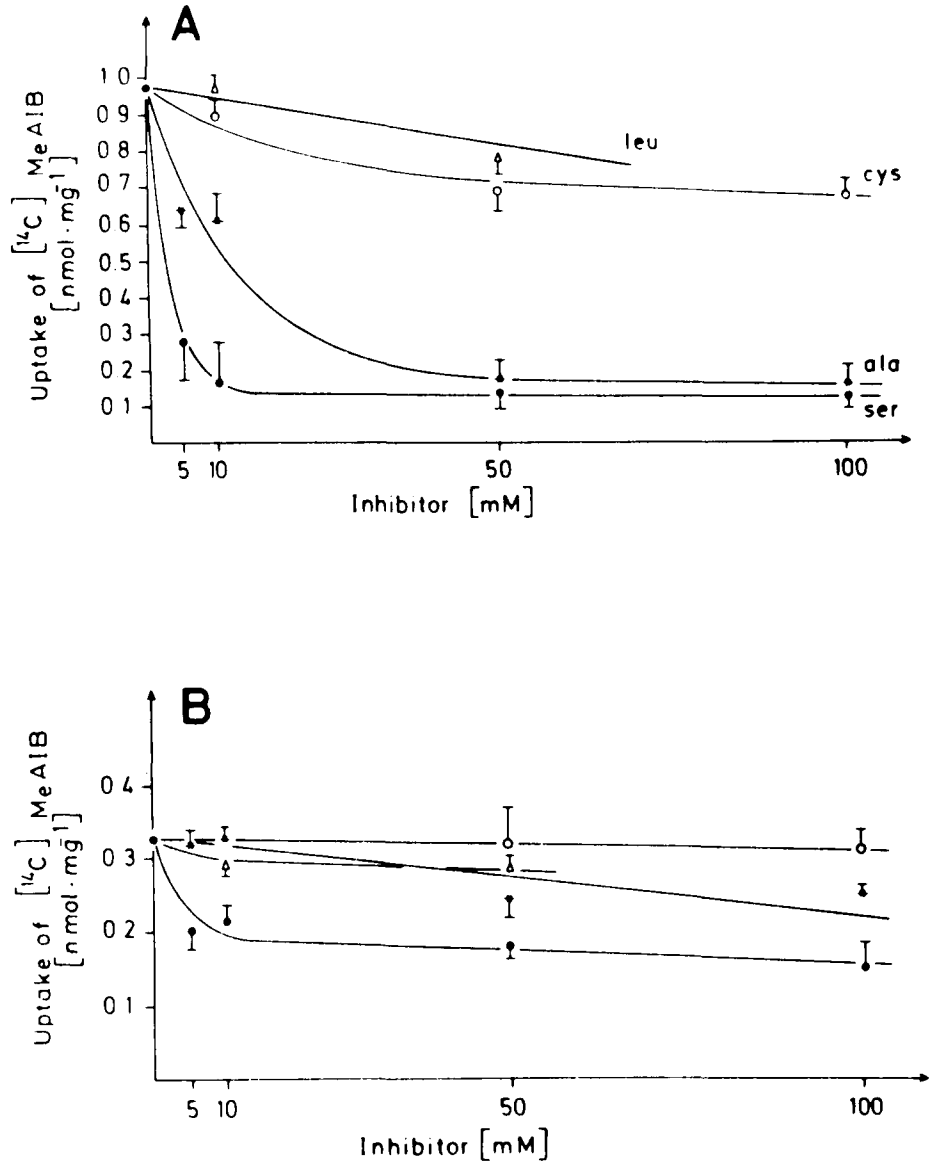

Fig. 5. Effect of unlabelled neutral amino acids: alanine (★), cysteine (○), serine (●) and leucine (△), on the uptake of [^{14}C]methylaminoisobutyric acid, either in a sodium-containing (A) or in a sodium-free (B) medium. Each point denotes the mean ± S.D. of 4–8 measurements. In the normal medium all the unlabelled short-chain neutral amino acids, except 10 mM cysteine, significantly inhibited the MeAIB uptake ($P < 0.001$). With exception of serine, no other amino acid significantly inhibited the uptake of MeAIB when measurements were performed in the sodium-free medium.

(approx. 55%). The question may be raised whether efflux of Na^+ from cellular sources could have contributed to amino acid-sodium cotransport even though the glands were exposed to a sodium free medium up to 5 min. However, a Na^+-containing medium was essential to maintain the inhibitory action of unlabelled alanine (Fig. 3) and cysteine (Fig. 4A) on L-[^{3}H]alanine uptake, whilst serine was shown to be a very weak inhibitor in the absence of the sodium gradient (Fig. 4B).

Furthermore, the uptake of the non-metabolizable selective substrate of the system A, MeAIB [2], was markedly inhibited by short-chain neutral amino acids only in the presence but not in the absence of Na^+. No appreciable effect was produced by larger neutral amino acids (Fig. 5). In addition the K_i of the Na^+-dependent transport of MeAIB for serine (1.23 mM) was closely similar to that obtained for alanine (1.81 mM). These overall results strongly suggest that a sodium-dependent transport system resembling system A is operative at the basolateral cell membrane in oxyntic glands, and support previously reported results [12] which suggested the presence of system A or ASC at the blood-tissue interface of the gastric wall. Mircheff et al. [5] showed that an A-like system, parallel to an ASC-like and an L-like amino acid transport system, exists in jejunal basolateral membrane vesicles.

As analyzed in Results (Fig. 3) the sodium-independent component of alanine transport appears mainly to

correspond to a non-carrier mediated process, i.e. simple diffusion, which is also appreciated by the negligible effect that other amino acids show in absence of Na^+ (Fig. 4B). Thus, apparent inhibitor constants (K_i) for only the sodium-dependent carrier-mediated transport of alanine for both short-chain neutral amino acids were calculated and compared with the apparent K_t of the Na^+-dependent carrier-mediated transport of alanine (K_t', Fig. 3). Results suggest that the Na^+-dependent transport system is shared by short-chain neutral amino acids with different affinities: serine (K_i = 1.81 mM), alanine (K_t' = 4.86 mM) and cysteine (K_i = 16.30 mM).

The kinetics of total L-alanine influx across the basolateral side of oxintic glands revealed a saturable process with an apparent K_t = 7.93 mM (Fig. 2). This value is similar to that reported for alanine transport in basolateral membrane vesicles of renal epithelial cells (8.60 mM) [20], isolated enterocytes (4.50 mM) [21], and both in the fetal (8.4 mM) and maternal (10.3 mM) side of the perfused guinea-pig placenta [22]. In all the cases described alanine transport was Na^+-dependent. Higher affinities for alanine influx have been obtained at the basolateral side of other epithelial cells, e.g. intestine (0.73 mM) [5] and salivary gland (0.83 mM) [8]. Interestingly, the K_t here measured for total alanine influx in isolated oxyntic glands is comparable to that previously obtained for alanine transport at the blood/tissue interface in the perfused dog stomach [12], which might indicate the predominance of the epithelium on other tissues of the gastric wall in the uptake process.

Acknowledgements

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