Absorption of homocitrulline from the gastrointestinal tract

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(*Received 9 July 1982 – Accepted 21 July 1982*)

1. Transport of L-homocitrulline, an amino acid which occurs in milk products, was studied with rat small intestine in vitro and from the human mouth in vivo. Absorption was partially dependent, in both systems, on the presence of sodium ions.

2. Metabolic inhibitors decreased L-homocitrulline uptake across the small intestine. Transport across the intestine did not occur against the concentration gradient but did show saturation kinetics.

3. The barbiturate, amytal, did not inhibit buccal absorption. Saturation kinetics were demonstrated.

4. Experiments were conducted with L-citrulline, or other amino acids, as possible inhibitors of L-homocitrulline transport. Results were compatible with Na^+-dependent carrier-mediated uptake across the buccal mucosa. Active transport could be involved with the small intestine assuming that L-homocitrulline has a low affinity for the carrier system.

L-Homocitrulline is a structural analogue of L-citrulline with an extra CH_2 group. Homocitrulline was identified in the urine of human infants by ion-exchange chromatography while older children or adults only excreted very small amounts (Gerritsen *et al.* 1961, 1962). Subsequent studies revealed that urinary homocitrulline was dietary in origin. It arose during the processing of cow's milk by evaporation and canning (Gerritsen *et al.* 1963). A large proportion of orally-fed homocitrulline was recovered from the urine of two babies (Gerritsen *et al.* 1962). Hence it was concluded indirectly that homocitrulline from processed milk was well absorbed from the gastrointestinal tract, was metabolically inert and poorly reabsorbed in the renal tubules.

The aim of the present study was first, to compare the intestinal absorption of L-homocitrulline with that of L-citrulline and second, to compare L-homocitrulline absorption from mammalian small intestine with that from the human buccal cavity. Many nutrients show similarities in their absorption characteristics with these two very different locations in the gastrointestinal tract.

MATERIALS AND METHODS

L-Homocitrulline (ICN Life Sciences Group, Cleveland, Ohio) was assayed by a spectrophotometric method I (Prescott & Jones, 1969) which also reacted with citrulline. Purity of the homocitrulline was checked by thin-layer chromatography (TLC) on laboratory-prepared cellulose layers or commercial aluminium plates coated with silica gel F_{254} (E. Merck, Darmstadt, West Germany). The solvent systems for one-dimensional TLC were: *n*-butanol–acetone–acetic acid–water (70:70:20:40, by vol.) (Smith & Seakins, 1976) and isopropanol–formic acid–water (75:12·5:12·5, by vol.) (White, 1968). Amino acids were detected by spraying with ninhydrin (2·5 g/l aqueous alcohol (950 ml/l)). Homocitrulline and citrulline were detected by a second spray, Ehrlich's reagent (20 g *p*-dimethylaminobenzaldehyde/l aqueous hydrochloric acid (50 ml/l)). Homocitrulline was determined, in the presence of citrulline, using an automatic amino acid analyser (Locarte Scientific Co. Ltd,

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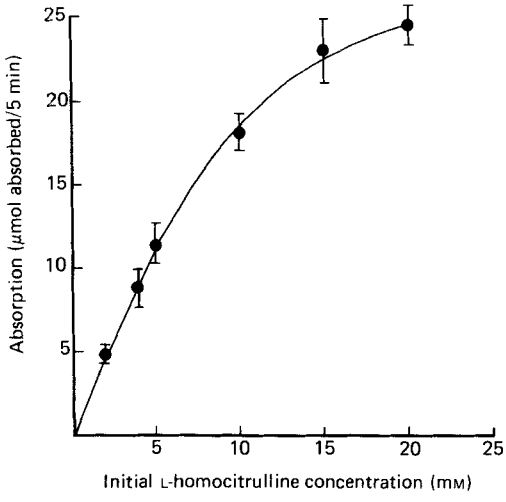

Fig. 1. The effect of the initial concentration (mM) of L-homocitrulline on absorption (μmol transported/5 min) across buccal mucosa in the human. Points are mean values, with their standard errors represented by vertical bars. Numbers of experiments at each concentration in parentheses. For details of procedures, see pp. 35–37.

Table 1. *Inter-subject variation in the buccal absorption (μmol/5 min) of L-homocitrulline in apparently healthy subjects*

(Mean values with their standard errors; no. of determinations in parentheses)

	Male						
Subject no ...	1		2		Female		
Age (years) ...	26*		36		24		Statistical significance of difference
L-Homocitrulline concentration (mM)	Mean	SE	Mean	SE	Mean	SE	
2	4·90	0·60 (6)	5·64	0·72 (4)	5·47	0·54 (4)	NS
5	11·6	1·13 (6)	12·5	0·98 (4)	10·8	1·75 (4)	NS
15	23·0	1·85 (8)	23·2	1·33 (4)	24·7	1·92 (4)	NS

NS, not significant.
* Main subject.

London). Amino acids were eluted by sodium citrate buffer gradient recommended by the manufacturer with DL-norleucine added as an internal standard.

Everted sacs of rat small intestine were prepared and used as previously described (Evered & Sadoogh-Abasian, 1979). Alternate sacs were taken down the length of the small intestine, with and without inhibitor respectively, to randomize the results. Two everted sacs each containing 0·4 ml sample were placed in 15 ml Krebs–Henseleit phosphate buffer, pH 7·4. After gassing with 95% O_2/5% CO_2 the flasks were sealed and incubated for 30 min at 37° while shaking. Buccal absorption was measured as previously described (Sadoogh-Abasian & Evered, 1979) but with 1·9 mM-calcium chloride added to the modified Krebs–Ringer buffer, pH 6, and 1·8 mM-citric acid replacing sodium citrate. Test samples (25 ml) or 'blanks' at 37° were circulated in the mouth for 5 min followed by 10 ml of fresh buffer at 37° for 5 s as a wash. Samples and washings were pooled, diluted and centrifuged before

Table 2. *Effect of replacing sodium ions by potassium ions on the buccal absorption (μmol/5 min) of 5 mM-L-homocitrulline*

(Mean values with their standard errors; nos. of determinations in parentheses)

Subject		Control* (136 mM-Na^+ + 15–45 mM-Na^+)		Test* (15–45 mM-Na^+)		Percentage inhibition	Significance of difference between treatments: P
Sex	No.	Mean	SE	Mean	SE		
♂	1	11·8	0·78 (6)	7·50	0·82 (6)	36	< 0·005
♂	2	10·2	0·61 (5)	6·67	0·75 (5)	35	< 0·005

* Indicates probable salivary contribution of Na^+ (Eastoe, 1961).

analysis of the supernatant. To investigate the effect of sodium ions on buccal absorption the sodium salts in the buffer were replaced by potassium salts in equimolar concentrations. When testing the possible effects of inhibitors it was necessary to presaturate the buccal mucosa with the chosen inhibitor. This was performed by including the inhibitor not only with the L-homocitrulline test solution but also with the buccal 'blank' solution preceding the test. Experiments were in random order, i.e. control then test or vice versa. Contamination of successive buccal samples with inhibitor was avoided by a pause of appropriate duration between experiments, usually more than 30 min. The effectiveness of this step was shown by a return to the usual value of the control experiments.

Possible metabolic loss was monitored by precirculating buffer solution in the buccal cavity, ejecting it into a homocitrulline solution and then measuring the homocitrulline content of centrifuged samples after incubation for 5 min at 37°.

RESULTS

Commercial L-homocitrulline contained a small amount of impurity which was thought to be lysine by the R_f values on TLC in two different solvents with two different absorbents. Quantitative amino acid analysis on an automatic analyser confirmed the identification of lysine and gave a value of 4% of the impurity on a molar basis.

Absorption of L-homocitrulline from the buccal cavity

Metabolic loss of L-homocitrulline during a 5 min incubation time, as used in buccal absorption experiments, was 0·44% from 25 ml 5 mM-L-homocitrulline. This is a negligible loss in relation to the rate of absorption except at low initial concentrations.

The rate of buccal absorption as a function of initial concentration of L-homocitrulline in the range from 2 to 20 mM was curvilinear (Fig. 1). A reciprocal plot (Lineweaver & Burk, 1934) gave a linear plot (not shown here).

There was only small inter-subject variation in the buccal absorption of L-homocitrulline with three Caucasian individuals none of whom wore dentures (Table 1).

Replacing Na^+ with K^+ at equimolar concentrations diminished significantly the uptake of L-homocitrulline across the buccal mucoasa (Table 2). The barbiturate, amytal, did not inhibit uptake but ethacrynic acid did inhibit absorption (Table 3). Of the amino acids tested only L-leucine, L-methionine and L-lysine significantly inhibited L-homocitrulline absorption (Table 3). The D-isomers of leucine and methionine, and L-glutamic acid, did not act as inhibitors under the experimental conditions when the 'inhibitor' concentration exceeded the L-homocitrulline concentration fivefold on a molar basis. L-Citrulline under these

Table 3. *Effect of possible inhibitors on the buccal absorption of* L-*homocitrulline in male subject no. 1*

(Mean values with their standard errors; number of determinations in parentheses)

L-Homocitrulline concentration (mM)	Possible inhibitor	μmol Absorbed/5 min		Percentage inhibition	Significance of difference between control and test values (P)
		Mean	SE		
5	None (control)	11·08	0·47 (13)	—	—
	Amytal (2 mM)	11·7	0·64 (5)	—	NS
	Ethacrynic acid (2 mM)	7·85	0·44 (4)	29	< 0·005
	L-Citrulline (25 mM)	7·65	0·92 (4)	31	< 0·005
2	None (control)	5·03	0·26 (26)	—	—
	L-Leucine (10 mM)	2·90	0·40 (6)	42	< 0·005
	D-Leucine (10 mM)	4·94	0·66 (4)	—	NS
	L-Methionine (10 mM)	3·55	0·48 (4)	29	< 0·01
	D-Methionine (10 mM)	4·95	0·55 (4)	—	NS
	L-Lysine (10 mM)	2·68	0·42 (4)	47	< 0·005
	L-Glutamic acid (10 mM)	4·25	0·54 (4)	16	NS

NS, not significant.

conditions slightly inhibited L-homocitrulline transport. With these amino acids alone, at concentrations over the range 2–10 mM, L-citrulline absorption exceeded that of L-homocitrulline by approximately 35%.

One-sided Student's t tests were used throughout since the alternative hypothesis was, in all cases, that the possible inhibitor was indeed an inhibitor.

Absorption of L-*homocitrulline from the small intestine*

At an initial concentration of 1 mM the final serosal:mucosal concentration was measured along the length of the small intestine with everted sacs. The value did not rise above 1·0, i.e. transport was not against the concentration gradient (Fig. 2*a*). Analytical recovery of 1 mM-L-homocitrulline (serosal + mucosal) was 97·8 ± 1·1% (n 12). The remaining 2–3% presumably remained in the tissues of the everted sac which was not analysed and this small loss was neglected in our calculations. Serosal and mucosal samples examined by TLC and an amino acid automatic analyser did not reveal any likely metabolites of L-homocitrulline. The high analytical recovery was also compatible with a negligible metabolic loss.

Transmural absorption was measured with L-homocitrulline outside the sac (mucosal) and only buffer solution within the everted sac. Rat small intestine did not show an optimal site for L-homocitrulline transport (Fig. 2*b*).

Downhill transfer of L-homocitrulline was measured over the concentration range of 1–10 mM outside the everted sacs with only buffer solution within. To correct for Na^+-independent transfer, rates of transfer in the absence of Na^+ replaced by K^+ were subtracted from rates of transfer in the presence of Na^+ (Fig. 3).

Inhibition of L-homocitrulline was significant with ouabain and the metabolic inhibitors sodium cyanide and 2,4-dinitrophenol (Table 4). The presence of some other amino acids was also inhibitory, e.g. the L-isomers of lysine, leucine and methionine (Table 4). L-Glutamic acid in fivefold excess on a molar basis was not inhibitory (Table 4).

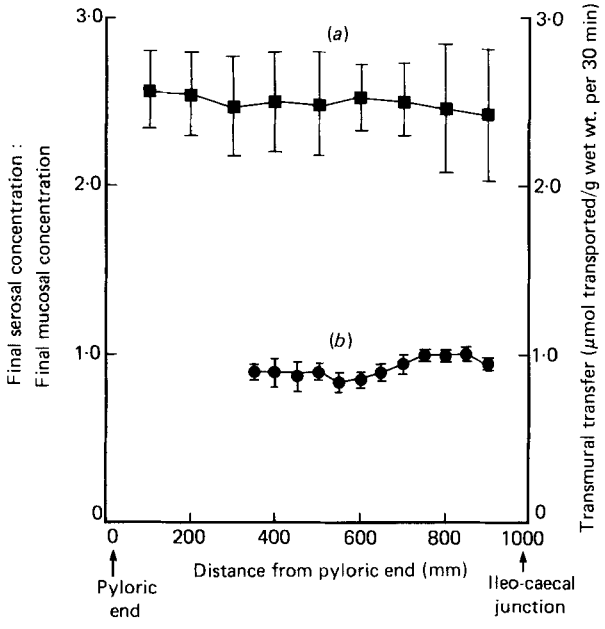

Fig. 2. The transport of L-homocitrulline along the rat small intestine using everted sacs. (*a*) (●) Mean concentration ratios with their standard errors represented by vertical bars for four experiments. Initial concentration of L-homocitrulline was 1 mM inside and outside the sac. (*b*) (■) Transmural transfer of L-homocitrulline initially at 5 mM concentration outside each everted sac. Points are mean values with their standard errors represented by vertical bars for six experiments. Each distance (mm) is measured from the pyloric sphincter. For details of procedures, see pp. 35–37.

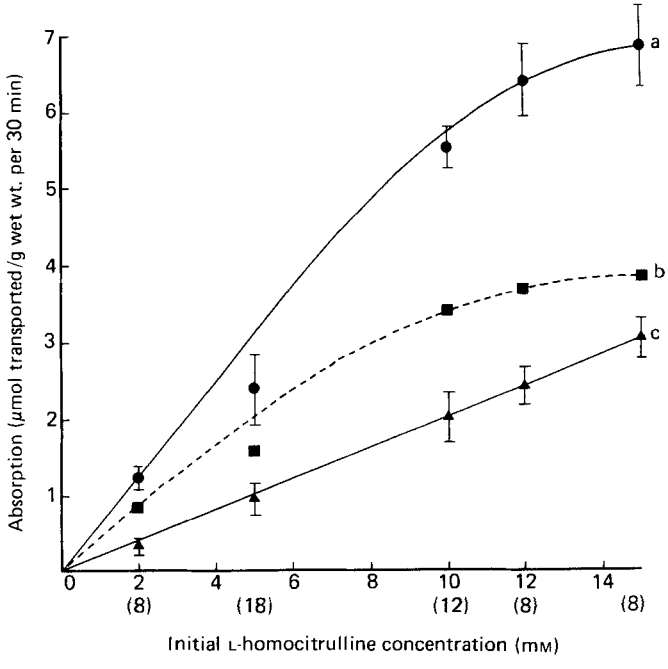

Fig. 3. The effect of the initial concentration (mM) of L-homocitrulline on the absorption (μmol transported/g wet wt tissue per 30 min) across everted sacs of rat small intestine. (●), The uptake before correction for diffusion; (■), the uptake after correction; (▲), the uptake in the absence of sodium ions. Points are mean values with their standard errors represented by vertical bars. Numbers of experiments at each concentration in parentheses. For details of procedures, see pp. 35–37.

Table 4. *Inhibition of 5 mM-L-homocitrulline transport (μmol/g wet tissue per 30 min) across everted sacs of rat small intestine*

(Mean values with their standard errors; no. of experiments in parentheses)

Inhibitor	Inhibitor absent		Inhibitor present		Percentage inhibition	Significance of difference between treatments: *P*
	Mean	SE	Mean	SE		
Ouabain (1 mM)	2·54	0·39 (13)	1·65	0·28 (13)	35	< 0·05
Sodium cyanide (2 mM)	2·26	0·14 (6)	1·77	0·21 (6)	22	< 0·05
2,4-Dinitrophenal (0·2 mM)	2·43	0·26 (6)	1·22	0·30 (6)	49·8	< 0·05
L-Lysine (0·2 mM)	3·15	0·16 (6)	2·86	0·21 (6)	9·2	NS
L-Lysine (25 mM)	3·15	0·16 (6)	0·92	0·10 (6)	71	< 0·005
L-Leucine (25 mM)	2·63	0·25 (6)	0·93	0·38 (6)	64·5	< 0·005
L-Methionine (25 mM)	2·29	0·33 (6)	1·03	0·30 (6)	55	< 0·01
L-Cysteine (25 mM)	3·23	0·27 (6)	2·18	0·29 (6)	32·5	< 0·025
L-Glutamic acid (25 mM)	2·98	0·28 (4)	2·52	0·30 (4)	15·4	NS
L-Citrulline (25 mM)	3·03	0·22 (6)	2·10	0·28 (6)	31	< 0·025

NS, not significant.

DISCUSSION

Loss of L-homocitrulline from the buccal cavity was assumed to be a measure of mucosal absorption. Negligible metabolic loss indicated by recovery experiments strengthened the validity of this assumption. A search for possible metabolites, with negative results for both the buccal cavity and small intestine, also indicated that metabolic losses were negligible.

The downhill transport rate in both systems was non-linear, showing saturation kinetics with an increase in the initial concentration of L-homocitrulline, suggesting carrier-mediated transport in both locations. In the intestine a significant proportion of L-homocitrulline uptake in vitro was Na^+ independent, thus uptake may be by passive absorption. It was difficult to seek a similar situation during buccal absorption since the salivary Na^+ constantly contaminated a Na^+-free buffer solution in the buccal cavity during the test period of 5 min.

Reabsorption mechanisms in the kidney often reflect absorption or malabsorption from the intestine (Asatoor *et al.* 1962). Therefore, poor absorption of L-homocitrulline from the intestine would be consistent with the high urinary excretion of L-homocitrulline given by mouth or arising metabolically (Gerritsen *et al.* 1962, 1963; Shih *et al.*, 1969).

For transport into everted sacs of rat small intestine the final value for serosal:mucosal concentrations did not exceed unity even at the lowest initial concentration (1 mM). Hence, either the small intestine lacks an active transport process for L-homocitrulline or such a postulated carrier has a very low affinity for this substrate. The high K transport (K_t) value obtained in the present study is consistent with the latter view. Transport against the concentration gradient could not be investigated with buccal mucosa since L-homocitrulline is effectively absent from the blood plasma of healthy humans (Gerritsen *et al.* 1962). This aspect could be studied by raising the plasma level above that in the buccal cavity by intravenous infusion of L-homocitrulline solutions. Such a procedure is beyond the scope of the present investigation and would also raise ethical problems with healthy subjects.

Buccal absorption of L-homocitrulline was not inhibited by the barbiturate, amytal, a known inhibitor of the electron transport chain (Jalling *et al.* 1955). This evidence, taken in isolation, would suggest facilitated diffusion not requiring an expenditure of energy. There

was partial inhibition of the intestinal absorption by an uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, and an inhibitor of the respiratory chain, sodium cyanide. This suggested the presence of an energy-dependent, carrier-mediated process for intestinal absorption of L-homocitrulline.

Omission of Na^+ from the buffer solution revealed that buccal absorption was Na^+-dependent. This was confirmed by the inhibition of L-homocitrulline absorption by ethacrynic acid, an agent known to inhibit the active extrusion of Na^+ from kidney cells (Whittembury, 1968). Intestinal absorption was also partly inhibited (64%) by the omission of Na^+ from the buffer solution. A smaller inhibition was produced by ouabain (35%) which inhibits the membrane pump for Na (Crane, 1968). This evidence suggests the presence of an additional Na-exchange system for L-homocitrulline which is not sensitive to ouabain.

Inhibition studies, using other amino acids, were chosen to represent specific transport pathways. It was first established that L-lysine, at the concentration present as a contaminant in the L-homocitrulline, did not inhibit transport of L-homocitrulline across the wall of rat small intestine. L-Lysine at a much higher concentration than that of the L-homocitrulline (five times) was inhibitory, suggesting that a carrier common to L-lysine may be preferred. This is not surprising as the two compounds share structural similarities. L-Homocitrulline also appears to have some affinity for the methionine-carrier system (Newey & Smyth, 1964) and the leucine site (Lerner, 1978). In spite of their structural similarity it seems likely, from the inhibition experiments, that L-homocitrulline and L-citrulline use different transport systems.

Buccal absorption of L-homocitrulline was inhibited by lysine, leucine and methionine. This inhibition was stereospecific for the L-isomers. Inhibition by L-citrulline was statistically significant.

In summary, it seems likely that dietary L-homocitrulline is transferred across the mucosa of the human buccal cavity and rat small intestine by a Na^+-dependent carrier-mediated process. Intestinal absorption was partially energy dependent.

These findings are surprising in view of the different embryological origins of these mucosae and also their different epithelial histology. This parallel has been described for another amino acid, the cyclic antibiotic cycloserine (Wass & Evered, 1971; Sprake & Evered, 1979). This phenomenon was also shown for some nutrients, e.g. monosaccharides and disaccharides (Manning & Evered, 1976; Evered & Sadoogh-Abasian, 1979), vitamin C (Sadoogh-Abasian & Evered, 1979), nicotinic acid and nicotinamide (Sadoogh-Abasian & Evered, 1980; Evered *et al.* 1981). Buccal absorption is small, nutritionally speaking, but could be a useful model for simulating intestinal absorption.

The authors acknowledge with thanks the financial support of the Lord Dowding Trust for Humane Research for the experiments on buccal absorption. Also they thank their human subjects for their co-operation and for their informed consent to the conduct of the buccal experiments. Mr E. C. Crutcher gave skilled technical assistance in preparing the everted sacs. Mr P. J. Milligan (Department of Mathematics) gave invaluable advice on the statistical treatment of data.

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Printed in Great Britain