

## INTESTINAL ABSORPTION OF THE INTACT PEPTIDE CARNOSINE IN MAN, AND COMPARISON WITH INTESTINAL PERMEABILITY TO LACTULOSE

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### SUMMARY

1. Healthy humans ingested the dipeptide carnosine (L- $\beta$ -alanyl-L-histidine). Their plasma levels and urinary outputs of carnosine and  $\beta$ -alanine were monitored over the following 5 h.

2. Large amounts of intact carnosine (up to 14% of the ingested dose) were recovered in the urine over the 5 h after ingestion. However, carnosine was undetectable in the plasma unless precautions were taken to inhibit blood carnosinase activity *ex vivo* during and after blood collection.

3. The amount of carnosine recovered in urine varied substantially between subjects. It correlated negatively with carnosinase enzymic activity in the plasma. Highest carnosinase activities were observed in those subjects who regularly underwent physical training.

4. Urinary recovery of the disaccharide lactulose also varied considerably between subjects, but was substantially lower than that of carnosine. There was no significant correlation between the recoveries of carnosine and lactulose.

5. When lactulose was ingested with a hypertonic solution, the urinary recovery of lactulose was generally increased. When carnosine was ingested with a hypertonic solution, the urinary recovery of carnosine was reduced: hence the paracellular route probably is not dominant for absorption of intact carnosine.

6. Intact carnosine must have crossed the intestine to an extent much greater than hitherto recognized. Rapid post-absorptive hydrolysis is a severe obstacle to quantification of intact peptide absorption.

### INTRODUCTION

Peptide transport systems in the small intestinal brush border are well documented and are thought to play a major role in absorption of the digestion products of dietary protein (e.g. Matthews, 1975, 1991). However, it is commonly assumed that peptides absorbed via these mechanisms are hydrolysed in the epithelial cytosol so that only free amino acids enter the circulation. Much evidence suggests that this

view is incorrect (Gardner, 1984; Gardner & Wood, 1989), but it has hitherto been impossible to obtain reliable estimates of the total amounts of intact peptides that enter the circulation during assimilation of protein. This assumes special significance since (i) biologically active peptides can be produced experimentally by peptic digestion of dietary proteins (e.g. Zioudrou, Streaty & Klee, 1979), and (ii) such peptides might arise under physiological conditions in the intestinal lumen. If such peptides were absorbed, and a number of factors are now recognized to enhance intestinal 'permeability' to small and medium-sized molecules (e.g. van Hoogdalem, de Boer & Breimer, 1989), they might exert activity in peripheral tissues (e.g. Gardner, 1985). An understanding of routes and mechanisms for absorption of intact peptides is also potentially relevant for the design of strategies for oral delivery of peptide and peptide-like drugs.

In order to investigate the extent of intact peptide absorption and possible transport mechanisms, we have studied the absorption of a dipeptide, carnosine (L- $\beta$ -alanyl-L-histidine; mol. wt 226), that is already believed to be absorbed to some extent in intact form (e.g. Perry, Hansen, Tischler, Bunting & Berry, 1967; Matthews, Addison & Burston, 1974). Absorption has been assessed by oral tolerance tests: plasma levels and, particularly, urinary outputs have been measured over 5 h after ingestion of a dose of carnosine by healthy humans. Since hypertonic solutions enhance permeability to various molecules including lactulose (e.g. Laker & Menzies, 1977; Wheeler, Menzies & Creamer, 1978), supposedly absorbed via the paracellular route, we have investigated the effect of hypertonic solutions on the urinary recovery of carnosine. Most of our test meals included also the monosaccharide rhamnose (mol. wt 164) and the disaccharide lactulose (4-*O*- $\beta$ -D-galactopyranosyl-D-fructose; mol. wt 342) since these are now widely used as probes of intestinal 'permeability' (e.g. Hamilton, 1986).

After our early experiments suggested that hydrolysis of carnosine in blood might be a determinant of urinary recovery, we measured hydrolytic activity against carnosine ('carnosinase') in plasma. Since early data suggested that the exercise status of the subjects might be relevant, we classified the subjects as 'exercisers' or 'non-exercisers'.

Preliminary communications have described part of this work (Gardner & Wood, 1987, 1989).

## METHODS

### *Subjects*

Nine healthy subjects, male and female, aged between 23 and 50 years participated. They gave informed consent, and the study was approved by a University of Bradford ethics committee. Eight subjects had no history of gastrointestinal disease; one had undergone 11 years previously a truncal vagotomy and pyloroplasty for gastric stress ulceration following a hypotensive crisis. Five subjects ('exercisers') regularly undertook formal exercise; the other four ('non-exercisers') did not.

### *Experimental procedure*

Subjects fasted overnight from 23.00 h before an experiment. At the start of the experiment, between 08.30 and 09.30 h the next day, subjects emptied their bladders and ingested a test meal which was designated (a) 'blank', (b) 'isotonic' (nominally) or (c) 'hypertonic'. All subjects

consumed at least one of each test meal, and at least 1 week elapsed before anyone underwent another experiment. The 'blank' meal comprised 92.5 ml hot water, 7.5 ml Duphulac syrup (Duphar Laboratories Ltd, Basingstoke, Hants, UK) and 1 g rhamnose (Sigma Chemical Co.). The Duphulac contained 5 g lactulose and traces of galactose and lactose in aqueous solution. The 'isotonic' test meal contained the same ingredients with the addition of 4 g carnosine (Sigma Chemical Co.). (In some experiments on one subject, smaller doses of carnosine were ingested.) The osmotic pressure of this solution was 344 mosmol/kg measured cryoscopically. The 'hypertonic' test meal was the same as the 'isotonic' one, but with the addition of 35 g lactose and 35 g sucrose (both from Sigma Chemical Co.); it had an osmotic pressure of approximately 2300 mosmol/kg. (In an additional experiment on one subject, an approximately isotonic test meal containing 2 g  $\beta$ -alanine plus 2 g histidine instead of carnosine was taken. In another experiment, the test meal contained 0.4 g of glycyl-sarcosine supplied by Sigma Chemical Co. in place of carnosine.)

Subjects were allowed free intake of water or orange juice (recommended to be 200–500 ml per hour) during the experiment. Urine was collected either every hour for five hours or over the 5 h period into bottles containing 10% w/v thiomersal (0.2 or 1.0 ml, respectively).

In some experiments, a cannula was inserted in a superficial vein in the forearm before the start of experiment, and blood samples (10 ml) were taken at the following times after the test meal: 0, 30, 60, 90, 120, 150, 180 and 360 min. Blood was transferred to lithium heparin tubes, and plasma was separated rapidly. In one experiment, the syringes, anticoagulant tubes, and the centrifuge were pre-chilled to 4 °C. All plasma samples were stored at –20 °C pending assay.

#### Analytical methods

*Carnosine,  $\beta$ -alanine and glycyl-sarcosine.* These were determined on an ion-exchange amino acid analyser with ninhydrin detection ('Alpha-Plus', L.K.B. Instruments Ltd). Plasma or urine (1 ml) was deproteinized with sulphosalicylic acid (0.1 ml; 30 mg/ml) containing norleucine (100 nmol/ml) as internal standard. Eighty microlitres of the supernatant were loaded onto the analyser, and a standard 'physiological fluid' programme with lithium citrate buffers was used. Carnosine emerged as a single peak at approximately 116 min, and analyses of 'blank' urine specimens indicated that it did not co-elute with any other ninhydrin-positive constituent in the urine.  $\beta$ -Alanine eluted at approximately 78 min, and glycyl-sarcosine at 60 min. Peak areas were integrated and were related to both external and internal standards.

*Lactulose and rhamnose.* Urine (50  $\mu$ l) with inositol as internal standard was analysed by gas-liquid chromatography as follows. Oxime derivatives of the sugars were formed by incubation with 100  $\mu$ l 2.5% ethoxylamine in pyridine at 60 °C for 30 min followed by trimethylsilylation with 20  $\mu$ l hexamethyldisilazane and 20  $\mu$ l trimethylchlorosilane at 60 °C for 30 min. Samples were dried on a sand bath at 60 °C under a stream of N<sub>2</sub> and taken up in 1 ml heptane. One microlitre was injected onto an SE-54 capillary column (30 m  $\times$  0.25 mm i.d. splitless mode) at an injection temperature of 250 °C and flame-ionization detector temperature of 350 °C. The initial separation temperature, 80 °C, was maintained for 2 min followed by a stepwise rise to 290 °C at 16 °C/min at a column head pressure of 0.8 bar. The recovery ( $\pm$  s.d.) of lactulose added to urine (10 samples) was 97.2  $\pm$  6.2%; the between-batch coefficient of variation was 4.0%.

*Plasma carnosinase activity.* This was assayed by the method of Bando, Shimotsuji, Toyoshima, Hayashi & Miyai (1984) as modified by Bando, Ichihara, Shimotsuji, Toyoshima, Koda, Hayashi & Miyai (1986) in which the appearance of free histidine is measured fluorimetrically following incubation of the plasma with carnosine at 37 °C.

## RESULTS

### Urinary output of carnosine

Subjects not ingesting a carnosine test meal excreted 24.0  $\pm$  2.36  $\mu$ mol carnosine in 5 h ( $n = 9$ ). No adverse effects were caused by carnosine ingestion although some subjects experienced mild and transient digital paraesthesia within the first hour, a similar effect having been reported by Asatoor, Bandoh, Lant, Milne & Navab (1970). Figure 1 shows the urinary content of carnosine over the first 5 h after

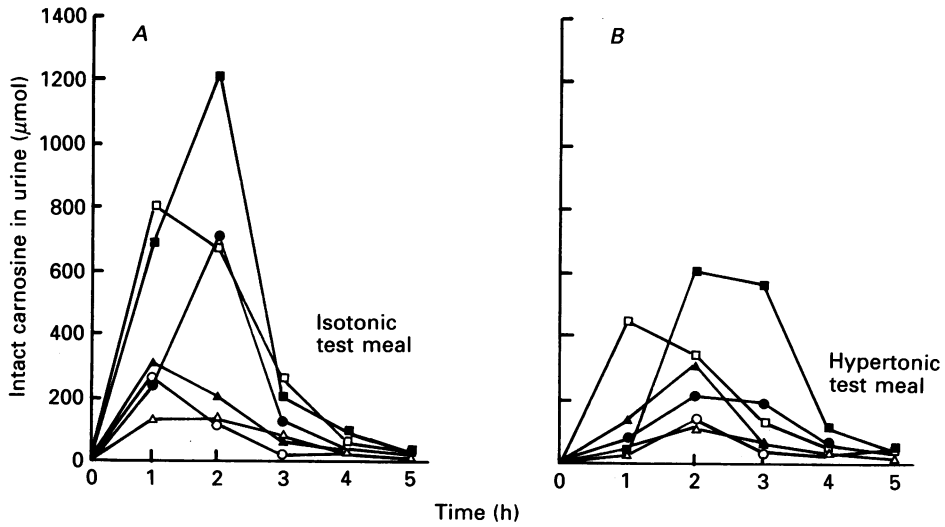


Fig. 1. Hourly urinary output of intact carnosine following ingestion of 4 g carnosine with an isotonic (A) or a hypertonic (B) test meal. Each symbol represents a different subject.

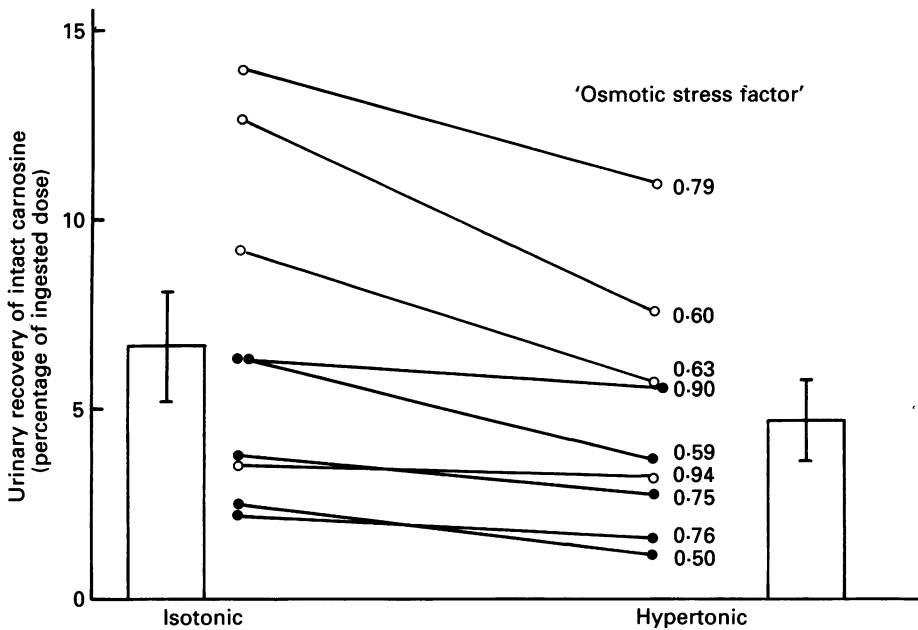


Fig. 2. Urinary recoveries of intact carnosine for 5 h after ingestion of 4 g carnosine with either an isotonic or a hypertonic test meal. Each pair of points represents a single subject. The filled symbols (●) represent 'exercisers' and the open symbols (○) represent 'non-exercisers'. The bars show the mean value  $\pm$  S.E.M. for the nine subjects.

ingestion of 4 g of carnosine by six subjects. It was maximal at 1–2 h and returned to basal levels by 5 h after both the isotonic (Fig. 1A) and hypertonic (Fig. 1B) test meals. The area under the curve varied substantially between subjects. The recovery

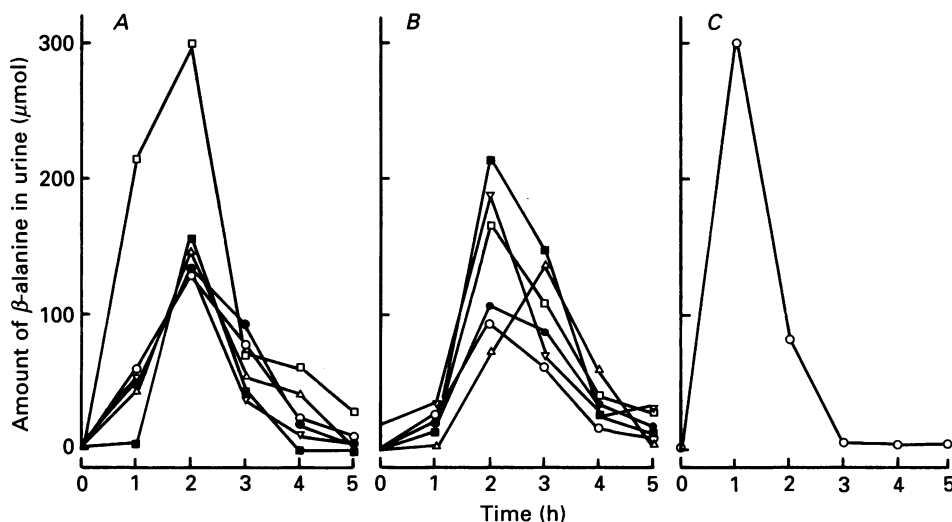


Fig. 3. Urinary output of  $\beta$ -alanine for 5 h after ingestion of 4 g carnosine in an 'isotonic' test meal (six subjects) (A), 4 g carnosine in a 'hypertonic' test meal (six subjects) (B) and 2 g L-histidine plus 2 g L- $\beta$ -alanine (one subject) (C). Each symbol represents a different subject. In some cases overlapping symbols have been omitted for clarity.

TABLE 1. Reproducibility of urinary carnosine recovery by four subjects undergoing replicate experiments

	Isotonic meal (%)	Hypertonic meal (%)
Subject 1	10.3; 9.4; 7.1; 6.7	5.5; 5.2; 6.5
Subject 2	3.6; 3.8	2.7; 2.8
Subject 3	6.4; 6.3	3.1; 4.4
Subject 4	1.5; 1.8	

(percentage of ingested dose) varied between subjects from 1.2 to 14.0% (197 to 2480  $\mu$ mol; see Fig. 2). Figure 2 shows also that the recovery of intact carnosine was less for each of the nine subjects when the test meal was made hypertonic (approx. 2300 mosmol/kg). The effect of tonicity has been expressed as an 'osmotic stress factor' (urinary recovery after hypertonic meal divided by recovery after isotonic meal) and, for carnosine, this ranged from 0.5 to 0.94 (Fig. 2). The correlation between the recoveries under isotonic and hypertonic conditions was highly significant ( $r = 0.958$ ;  $P < 0.001$ ). The amount of  $\beta$ -alanine (a product of carnosine hydrolysis) appearing in the urine over 5 h corresponded to approximately 2% of the ingested carnosine, and this was much higher than the basal excretion of  $\beta$ -alanine (Fig. 3A and B).

Four subjects performed replicate experiments. The limited data from these showed that the urinary recovery of carnosine was acceptably reproducible for each subject (Table 1). One subject also consumed test meals containing 1, 2 or 4 g of carnosine. Urinary recoveries were 1.3, 2.3 or 3.4% respectively of the consumed doses. In a single experiment to investigate Block, Hubbard & Steele's (1965)

suggestion that the urinary carnosine might have arisen from resynthesis of carnosine after gastrointestinal hydrolysis, one subject ingested a test meal containing histidine (2 g) and  $\beta$ -alanine (2 g). Only traces (approximately basal amounts) of carnosine were detected in the urine; substantial amounts of  $\beta$ -alanine

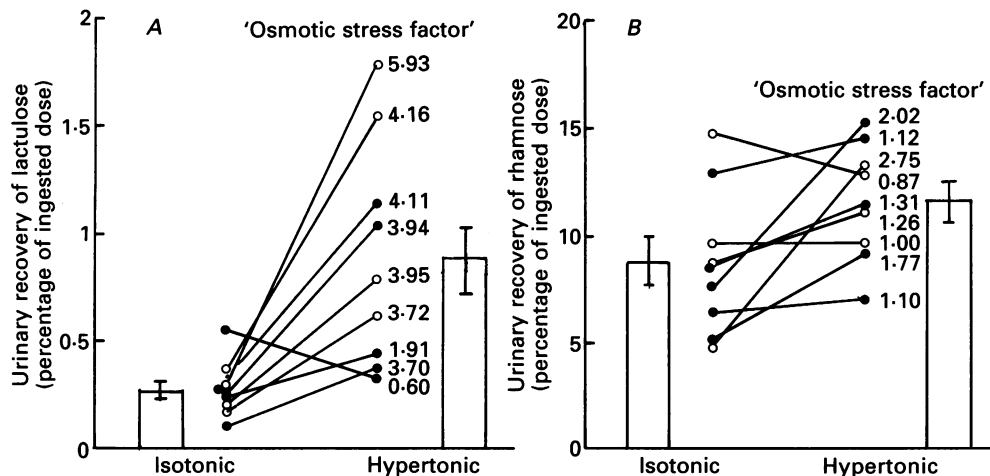


Fig. 4. Urinary recoveries of lactulose (A) and rhamnose (B) for 5 h after ingestion of an isotonic or a hypertonic test meal. Each pair of points represents a single subject. The filled symbols (●) represent 'exercisers' and the open symbols (○) represent 'non-exercisers'. The bars show the mean value  $\pm$  S.E.M. for the nine subjects.

appeared in the urine (corresponding to approximately 2% of the ingested dose over 5 h; see Fig. 3C).

#### Urinary output of lactulose and rhamnose

Figure 4 shows the urinary recoveries of lactulose and rhamnose for each subject. Lactulose recoveries ranged from 0.1 to 0.55% of the ingested dose when the test meal was isotonic and from 0.33 to 1.78% when it was hypertonic. In eight of the nine subjects the hypertonic meal caused an increase in lactulose recovery, their 'osmotic stress factors' ranging from 1.91 to 5.93 (Fig. 4A). One subject ingested six isotonic and six hypertonic test meals over a 12 week period, and his lactulose recoveries were always higher after the hypertonic test meal than after the isotonic one ( $1.14 \pm 0.200$  vs.  $0.292 \pm 0.0306$  % respectively;  $P < 0.01$ ). Figure 4B shows that the rhamnose recoveries for all subjects ranged from 4.84 to 15.25%, and the effect of the hypertonic test meal was small and less consistent though with a trend towards an increase. A significant increase was, however, seen for the subject undertaking the replicate experiments:  $11.35 \pm 0.559$  % ( $n = 6$ ) after a hypertonic meal vs.  $8.74 \pm 0.552$  % ( $n = 6$ ) after an isotonic meal ( $P < 0.01$ ).

#### Correlations between recoveries of carnosine and lactulose or rhamnose

No significant correlations were observed between the urinary recovery of carnosine and that of either lactulose or rhamnose under either isotonic or hypertonic conditions.

*Urinary recovery of glycyl-sarcosine*

In a single experiment a subject ingested 0.4 g of glycyl-sarcosine (glycyl-*N*-methyl-glycine) in 100 ml of water. Hourly urine collections showed that 10.5% was recovered in 6 h, with the maximum amount (6% of the ingested dose) being

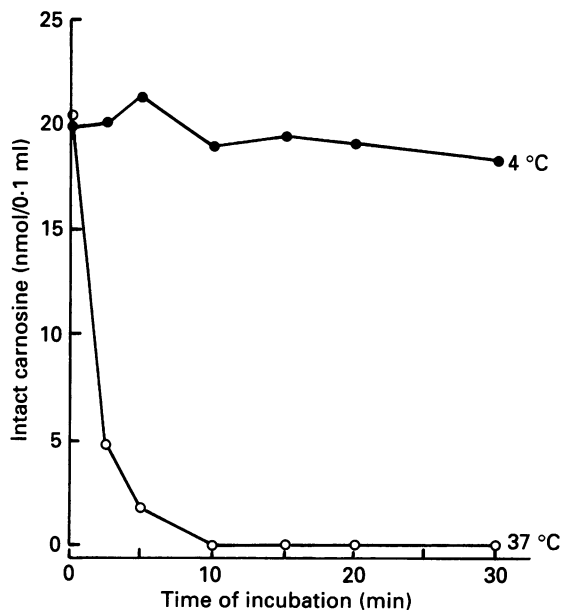


Fig. 5. Time course of disappearance of carnosine after addition to plasma *in vitro*. Samples were incubated either at 37 °C (○) or on ice (●).

excreted in the first hour. No sarcosine was detected on the amino acid chromatogram. Incubation of glycyl-sarcosine with blood or plasma for 40 min at 37 °C showed negligible hydrolysis.

*Plasma levels of carnosine and carnosinase activity*

In initial experiments, no carnosine was detected in plasma (limit of detection approx. 5 nmol/ml) after carnosine ingestion even though large amounts were found in corresponding urine specimens. The reason for this is apparent from Fig. 5 which shows the recovery of carnosine added *in vitro* to plasma and incubated at either 4 or 37 °C. This suggests that enzymic activity in plasma hydrolyses the added carnosine with a half-life of the order of 1 min. A further experiment on a subject who had ingested carnosine (4 g) showed that small amounts of carnosine (81.1, 44.4, 4.6 nmol/ml plasma) could be detected in the blood (at 0.5, 1.0, 1.5 h respectively) if the collection syringe, specimen bottle and centrifuge were chilled. Further attempts to measure plasma levels of carnosine were abandoned owing to the difficulties of halting peptide hydrolysis *ex vivo* during blood collection.

Carnosinase activity in plasma from each subject was measured, and this showed wide variability. Figure 6 shows that there is a highly significant negative correlation

between the plasma carnosinase activity and the urinary recovery of intact carnosine ( $r = -0.815$ ;  $P = 0.004$ ). These data are for the isotonic test meal, but a similar correlation was observed also for the hypertonic test meal ( $r = -0.787$ ;  $P = 0.006$ ).

#### DISCUSSION

Although it has previously been shown that intact carnosine appears in urine after ingestion of rabbit or chicken meat (e.g. Block *et al.* 1965; Perry *et al.* 1967), to our

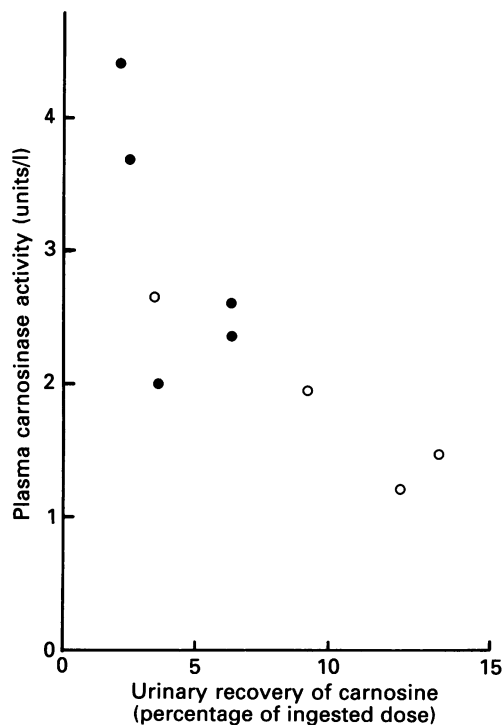


Fig. 6. Relationship between each subject's carnosinase activity in plasma and the urinary recovery of intact carnosine during the 5 h after ingestion of 4 g of carnosine with an isotonic test meal. The correlation is significant ( $r = -0.815$ ;  $P = 0.004$ ). A similar relationship was seen for the recovery of carnosine after the hypertonic test meal. Each point represents a different subject. Filled symbols (●) represent 'exercisers' and open symbols (○) represent 'non-exercisers'.

knowledge this is the first time that this process has been quantified. Mechanisms have been found for the active transport of carnosine in the brush border of rat and hamster small intestine, competition studies suggesting that the transporter is shared by some other peptides and by amino- $\beta$ -lactam antibiotics (e.g. Matthews *et al.* 1974; Tsuji, 1987). The brush-border transport of carnosine is said to be regulated by dietary levels of amino acids, peptides and/or proteins (Ferraris, Diamond & Kwan, 1988). A putative proton-linked peptide carrier has also recently been partially characterized in basolateral membranes, and carnosine inhibited the transport of glycyl-L-proline by it (Dyer, Beechey, Gorvel, Smith, Wootton &



Shirazi-Beechey, 1990). However, carnosinase activity has been identified in the small intestine of several species including humans (Parshin, 1946; Sadikali, Darwish & Watson, 1975) though Parshin (1946) found less activity in human mucosa than in other species, and Hanson & Smith (1949) reported that it was absent from pig intestine though present in pig kidney.

Our results show that up to 14% of ingested carnosine may be excreted in intact form and, thus, must have been absorbed across the gastrointestinal tract in intact form (Fig. 2). The experiment entailing ingestion of histidine plus  $\beta$ -alanine eliminates the possibility that the urinary carnosine had arisen from resynthesis after hydrolysis of the ingested peptide. In the experiment performed on glycyl-sarcosine, a hydrolysis-resistant dipeptide (Addison, Burston & Matthews, 1972), 10.5% of the intact peptide was recovered in urine. In view of the remarkably short half-life of carnosine in plasma (Fig. 5), it is very likely that the amounts crossing the gastrointestinal tract in intact form greatly exceed the amounts collected in the urine. (Indeed, it is possible that a majority of, or even all, the carnosine had been absorbed in intact form.) The high negative correlation between the urinary recovery of the peptide and the carnosinase activity in each subject's plasma (Fig. 6) strongly suggests that this enzymic activity has a dominating effect on the urinary recovery and that the wide variation in urinary recoveries between subjects (Fig. 2) is predominantly influenced by the subjects' carnosinase activities. The lack of correlation between carnosine recovery and recovery of lactulose or rhamnose supports the view that extra-intestinal factors primarily determine the urinary recovery of carnosine.

The presence of such effective carnosinase activity in plasma explains why Asatoor *et al.* (1970) and Sadikali *et al.* (1975) failed to detect carnosine in plasma after carnosine ingestion by humans. It also emphasizes the danger in using plasma measurements as an index of intestinal absorption of intact peptides. Further, systemic hydrolysis may therefore be as serious an obstacle to the efficacy of peptide-like drugs as is intraluminal hydrolysis or poor intestinal absorption of such drugs.

It is impossible to estimate the role of intestinal carnosinase in the hydrolysis of carnosine but it may well be minimal compared to hydrolysis in blood, liver, kidney and other tissues. Sadikali *et al.* (1975) concluded that intestinal hydrolysis of carnosine was the rate-limiting step in carnosine absorption. This conclusion was based mainly on the observation that a patient with a deficiency of intestinal carnosinase produced a relatively low serum histidine tolerance curve after ingestion of carnosine. However, an equally plausible explanation would be that the subject, who had sub-total villous atrophy associated with untreated coeliac disease, had a deficiency of the relevant peptide carrier(s). It thus appears that intestinal hydrolysis of carnosine may be irrelevant to the absorption of carnosine, and certainly it does not appear to be a rate-limiting step.

These results pose a question as to the fate of the absorbed carnosine before it is excreted. Figure 1 suggests that intestinal absorption is fairly rapid, as would be expected from such a test meal in fasted subjects. However, carnosine is still being excreted by the fourth hour after ingestion. In the light of the very short half-life in blood (Fig. 5), it seems that absorbed carnosine may be very rapidly cleared from the plasma and sequestered in some compartment before it is excreted by the kidneys.

The identity of such a compartment is unknown, but the evidence of Hama, Tamaki, Miyamoto, Kita & Tsunemori (1976), who fed high doses of carnosine to rats, would favour accumulation in liver rather than muscle even though skeletal muscle is the major repository of endogenous carnosine.

The hypertonic test meal caused a substantial increase (up to 6-fold) in the lactulose recovery in all subjects except one, and there was marked variation in the effect of hypertonicity (see Fig. 4A). This is in agreement with the observations of Menzies (1974, 1984), Wheeler *et al.* (1978) and Maxton, Bjarnason, Reynolds, Catt, Peters & Menzies (1986), and is probably due to enhancement of paracellular permeability via the 'tight' junctions. Temporary cellular shrinkage might be responsible. However, it is noteworthy that hypertonic solutions had the converse effect on recovery of carnosine (Fig. 2): in all nine subjects a modest reduction was observed. Hence, it is considered unlikely that the paracellular route is an important one for carnosine absorption; however, this could be a reflection of the relative efficiency of the brush-border transport mechanism for carnosine. It may be significant that, when Fuessl, Domin & Bloom (1987) demonstrated biological activity of an octapeptide mini-analogue of somatostatin (Sandoz SMS-201-995) after oral administration to humans, they administered it with a strongly hypertonic glucose solution (2100 mosmol/kg nominally). Hence, hypertonic solutions may enhance intestinal absorption of intact peptides, but this may be relevant only when the specificity of brush-border carriers and/or the size of the peptides denies the use of transcellular carrier-mediated mechanisms.

Rhamnose, now used routinely by some workers as a probe for investigation of intestinal permeability in humans, is presumed to be absorbed predominantly via the transcellular route (Menzies, 1984). Figure 4B shows that hypertonic solutions did not have a consistent effect on urinary recovery of this monosaccharide though a marked (nearly 3-fold) increase was produced in some subjects and a significant 30% increase ( $P < 0.01$ ) was observed in the one subject undertaking replicate experiments. It is useful to note that there was no correlation between the rhamnose and lactulose recoveries ( $r = -0.2346$  and  $0.1710$  for isotonic and hypertonic conditions respectively, with sixteen degrees of freedom), which suggests that the variability in these was not predominantly caused by factors common to both rhamnose and lactulose recoveries (e.g. gastric emptying, transit time, extracellular volume, renal function etc.; see Menzies, 1984). This is further supported by the fact that the 'between-subject' coefficient of variation for the (lactulose/rhamnose) recovery ratio (71%) is greater than that for either lactulose recovery (38%) or rhamnose recovery (48%) alone, even though clinical experience has consistently shown the lactulose/rhamnose ratio to have better power than lactulose or rhamnose recovery alone to discriminate between diseased and healthy subjects (e.g. Figs 45.6 and 45.7 of Menzies, 1984).

Although this work was primarily intended to quantify and elucidate mechanisms of peptide absorption, two incidental findings of interest are that variability in plasma carnosinase activity may be related to the subject's exercise status (Fig. 6) and that variability in permeability to lactulose may be related to lean body mass or to protein turnover (M. L. G. Gardner & D. Wood, unpublished observations). The former would accord with the views inherent in the work of Bando *et al.* (1984)

and Duane & Peters (1988) that carnosinase is involved in muscle turnover, and this could explain why excretion of absorbed carnosine tended to be lower in our 'exercisers' than in our 'non-exercisers' (Fig. 2). Both these issues merit fuller investigation.

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