INTESTINAL ABSORPTION OF β-ALANINE, ANSERINE AND CARNOSINE IN RATS

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Summary Absorption β-alanine, anserine or carnosine from rat intestine was studied in vivo by a force feeding method and in vitro using an everted sac method. Possibility of anserine and carnosine hydrolysis prior to intestinal absorption was also investigated using a glycylleucine dipeptidase-containing fraction prepared from rat intestine. The following results were obtained.

1) Anserine and carnosine were absorbed as they were from rat small intestine.
2) Both anserine and carnosine were partially hydrolyzed in vitro by the glycylleucine dipeptidase-containing fraction. Carnosine was hydrolyzed faster than anserine.

The above rather conflicting results suggest that physiological amounts of anserine and carnosine might be absorbed from rat small intestine in dipeptide forms.

Anserine and carnosine are found in considerable amounts in the skeletal muscle of animals. In spite of extensive studies, their physiological role and metabolic fate have not been clarified yet. The authors reported previously that these dipeptides were biosynthesized from β-alanine formed in the metabolic pathway of uracil (1). These dipeptides are usually found in food ingested by carnivorous animals. Anserine and carnosine in food (meat) were stable under extreme conditions (i.e. stirring at 100°C for 60 min), and 95% of them was recovered in free form afterwards (2).

NATHANS et al. (3) and LIN et al. (4) studied intestinal absorption of amino acids extensively using everted sacs and found the β-amino acids were not actively transported across the intestinal wall. It is generally accepted, with a few exceptions (5), that peptides do not cross the intestinal epithelium as they are but

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L-1-Methylhistidine in this paper is L-N²-methylhistidine.
reach the serosal side in the form of free amino acids (6–8). It is suggested that peptides or amino acid residues of small peptides are translocated across the intestinal brush border by a mechanism different from that for free amino acids, for example, hydrolysis after tissue accumulation (9–11). Enzymatic hydrolysis of small peptides in significant amounts does not take place in the intestinal lumen but does in the mucosal cells (12).

In the present study, the authors investigated the influx of β-alanine, anserine and carnosine across the intestinal mucosal border of albino rat in in vivo and in vitro experiments using a force feeding method and an everted sac method (13), respectively. Enzymatic hydrolysis of these dipeptides was also investigated.

**EXPERIMENTAL**

**Animals.** Wistar strain male rats, weighing approximately 100 g, were used in the present experiments. They were kept in individual cages at room temperature (23±1°C) and fed with commercially available pellet foods (Clea Japan, Inc.). These animals were divided into 3 experimental groups according to body weight to ensure uniform weight distribution. After each treatment, animals were killed by decapitation. Samples of blood, liver and gastrocnemius muscle were removed from each rat as described previously in detail (1) for analysis.

**Everted sac experiments.** An everted sac prepared from the ileum according to CRANE and WILSON (13) and a modified apparatus (4) were used in in vitro dipeptide transfer experiments. The abdomen was opened by a midline incision and the entire small intestine was flushed out with a Tyrode’s solution. The small intestine was then removed after cutting the upper end of the duodenum and the lower end of the ileum and manually stripping the mesentery from the intestine. The everted intestine, 10 cm in length, was tied at one end. The amount of Tyrode’s solution was 50 ml outside and 1 ml inside. The apparatus was set in a water bath kept at 37°C and clean air was passed through the apparatus. After 30 min incubation dipeptide concentration was determined in both inside and outside of the sac (14).

**In vivo intestinal absorption.** Aliquots of β-alanine, anserine or carnosine solution were given to rats through a stomach tube. Animals were killed at regular intervals and samples for analysis were removed in the manner described previously (1).

**Hydrolysis of anserine and carnosine by dipeptide hydrolase in rat small intestine.** Two rats, starved for 24 hr, were decapitated. Dipeptide hydrolase was prepared according to the method used for glycyllleucine dipeptidase (EC 3.4.3.2) by SMITH (15). The small intestines were quickly removed and washed out with ice-cold saline. The ileum was chopped up with scissors and homogenized in
0.3 M phosphate buffer (pH 7.8) for 1 min with a Potter Elvehm homogenizer. The homogenate (10% ) was preincubated at 40°C for 2 hr without adding Mn++ and Co++, and centrifuged at 800×g for 15 min. The supernatant solution was filtered through two layers of gauze and used as an enzyme solution.

The reaction mixture consisted of 1.0 ml of substrate (1.5 μmole of Gly-Leu, anserine or carnosine), 1.0 ml of 0.3 M phosphate buffer (pH 7.8) and 0.5 ml of the enzyme solution. After incubation the reaction was stopped by adding 7.5 ml of absolute ethanol. After centrifugation, the supernatant and washings were combined and condensed to dry below 40°C. The residue was used as an analytical sample. β-Alanine, anserine and carnosine in the analytical smaples were determined with an amino acid analyzer as described previously (1).

RESULTS AND DISCUSSION

In vitro intestinal absorption

Figure 1 shows the ratio of internal and external concentrations of the test compound and its constitutive amino acids 30 min after adding β-alanine, histidine, anserine or carnosine after 30 min incubation. Initial concentration of test compound was the same on both sides (0.3 mM). According to CRANE and WILSON (13), the everted sac from rat ileum (9 cm long) was incubated at 37°C in air saturated Tyrode's solution containing test compounds. Volume of external solution was 50 ml and that of internal solution was 1 ml. Concentration ratio of component amino acids of test compound was also investigated.

Fig. 1. The relative ratio of internal and external concentration of β-alanine, histidine, anserine or carnosine after 30 min incubation. Initial concentration of test compound was the same on both sides (0.3 mM). According to CRANE and WILSON (13), the everted sac from rat ileum (9 cm long) was incubated at 37°C in air saturated Tyrode's solution containing test compounds. Volume of external solution was 50 ml and that of internal solution was 1 ml. Concentration ratio of component amino acids of test compound was also investigated.
anserine or carnosine to both sides of an everted sac. The active transport of L-histidine was reconfirmed by the fact that the concentration of L-histidine on the serosal side (inside) was more than 4 times of that of the mucosal side (outside). But no difference was observed in the concentration ratio of β-alanine. After incubating the intestinal sac with anserine or carnosine, concentration of β-alanine and 1-methylhistidine or histidine in the serosal side increased clearly. The concentration of anserine or carnosine, however, remained unchanged or slightly decreased. Increase in β-alanine concentration on the serosal side was not due to the hydrolysis of dipeptides before passing through the intestinal membrane, because β-alanine itself was not actively transported as seen in Fig. 1. These dipeptides, therefore, had to be hydrolyzed during or after permeation through the membrane.

The result reveals that both anserine and carnosine pass through the intestinal membrane, but anserine is transported at a higher rate than carnosine. The finding that two component amino acids of anserine increased markedly in the serosal side solution was interesting when compared with the results obtained in the cell-free system as shown in Figs. 8, 9 and 10.

In vivo intestinal absorption

The β-alanine solution was given daily for a week in a dose of 5 g per kg of body weight. As shown in Fig. 2, β-alanine accumulated in both liver and gastrocnemius muscle. Anserine and carnosine were not detected in the liver, while the concentration of these dipeptides increased in the muscle after β-alanine ad-

![Graph showing content of β-alanine, anserine and carnosine in rat force-fed with β-alanine for a week. Open bar: control group without β-alanine administration. Hatched bar: test group administered with 5 g/kg of β-alanine daily. Each group contained 3 animals.](image-url)
Absorption of Anserine and Carnosine in Rat

Fig. 3. Changes of β-alanine level in rat organs after force feeding with β-alanine (5 g/kg of body weight). Each value shows a mean of 3 animals. Blood ———, gastrocnemius ———, liver ———.

ministration. Concentration of anserine in gastrocnemius muscle of the test group was 50% higher than that of the control group, and carnosine level in muscle of the test animals was 6 times of that of the control animals. This result indicates that the absorbed β-alanine is used for biosynthesis of β-alanyl dipeptides.

Figure 3 shows the time course of changes in β-alanine concentration in the blood, liver and gastrocnemius muscle of rats after oral administration of β-alanine (5 g/kg). β-Alanine is not usually detected in blood by chemical methods, however, β-alanine level increased strikingly 15 min after its administration. β-Alanine concentration reached its peak 6 hr after its administration and then decreased during the subsequent 12 hr.

To see what proportion of the administered β-alanine was absorbed, β-alanine level in organs was analyzed 2 hr after several dosage levels of β-alanine were given to rats orally. As could be seen in Fig. 3, organs were not saturated with β-alanine 2 hr after oral administration of β-alanine. The results are shown in Fig. 4. The administered dosage was represented logarithmically and each dosage was threefold greater than the previous one (Initial dosage was based on the ordinary amount contained in the diet consume daily). β-Alanine levels in the blood, liver and gastrocnemius muscle increased linearly in proportion to the dosages above 18.7 mg. β-Alanine levels in blood showed a sigmoid curve above 6.2 mg. The maximum concentration could not be obtained in this experiment because of a technical problem. β-Alaninesolution of higher concentration was too thick to give to animals through force-feeding.

Anserine and carnosine concentrations in the blood, liver and gastrocnemius
Fig. 4. Changes of β-alanine level in rat organs 2 hr after oral administration of several dosages of β-alanine. Each value shows a mean of 3 animals. The body weight of each rat was approximately 100 g. Blood — — , gastrocnemius — — , liver — — .

Fig. 5. β-Alanine, anserine and carnosine contents in rat organs 2 hr after oral administration of anserine at 3 dose levels. L stands for 16.7 mg/rat of anserine, M for 150.21 mg/rat and H for 450.36 mg/rat, respectively. Hereafter L, M and H are used as abbreviations for each treatment. The results obtained in the M and H treatments of anserine showed the highest anserine level which was not usually encountered in the blood (Fig. 5). A small amount of β-alanine was found in the liver, which was not converted into
carnosine. The amount of anserine in the liver increased linearly with the increase in administered dosages. β-Alanine also increased markedly in the liver (Fig. 5). This indicates that the amount of accumulated anserine becomes far greater than the amount of hydrolyzed anserine at a certain point between L and M treatments. The gastrocnemius muscle usually contains about 0.2 mg of anserine and 0.2 to 1.1 mg of carnosine per g of wet tissue, but not the detectable amount of β-alanine. The content of anserine in the gastrocnemius muscle doubled in L treatment and that of carnosine increased to some extends in L and M treatments. The contents of anserine and carnosine in the muscle, however, decreased markedly in H treatment but β-alanine was not detected. It is difficult to explain these results. There might be some physiological relation between the muscle function and the biosynthesis or accumulation of the dipeptides.

Figure 6 shows the concentrations of carnosine, anserine and β-alanine 2 hr after administration of carnosine at 3 dose levels. Carnosine was detected in the blood in treatment H, but anserine and β-alanine were not. ASATOOR et al. (16) studied intestinal absorption of carnosine in humans and found that carnosine was taken in the intestinal cells as it was but delivery of carnosine to the portal blood was slower than the free constituent amino acids. Our experimental result demonstrated that carnosine was absorbed from the intestine as it was. When M and H dosages of carnosine were administered, carnosine was detected in the liver and the amount of β-alanine in the liver was higher than that of carnosine. These observations agree with previous report (17) showing that carnosine was hydrolyzed to β-alanine and histidine, and was not converted into anserine.

![Graph showing concentrations of carnosine, anserine, and β-alanine in rat organs](image)

**Fig. 6.** β-Alanine, anserine and carnosine contents in rat organs 2 hr after oral administration of carnosine at 3 dose levels. L stands for 15.74 mg/rat of carnosine, M for 141.44 mg/rat and H for 424.07 mg/rat, respectively.
The content of carnosine in the gastrocnemius muscle did not increase after administration of L and M dosages of carnosine. The concentration of carnosine in muscle after administration of H dose of carnosine decreased to 1/2 of that of control, and anserine also decreased to the nondetectable level, which was quite similar to the situation encountered in H dose of anserine administration. The phenomenon of anserine and carnosine decreasing in the muscle after administration of H dose of either dipeptide cannot be well explained by the mechanism for intestinal absorption. The increase of anserine in the muscle after administration of the M dose of carnosine agrees with previous observations (17) that the administered carnosine is hydrolyzed in the liver, the formed β-alanine is transported to the muscle and used for anserine biosynthesis.

Hydrolysis of anserine and carnosine by intestinal dipeptidase

The above results reveal that anserine and carnosine pass through the intestinal membrane in both in vitro and in vivo experiments. As these dipeptides were β-aminopeptidases, they might be immune to the action of dipeptidase. In the present studies, the glycylleucine dipeptidase (EC 3.4.3.2)-containing fraction from rat intestine was used for the hydrolysis of these dipeptides. SMITH showed that glycylglycine dipeptidase (EC 3.4.3.1) did not act on β-alanyldipeptides (18). Glycyl-L-leucine from Osaka University Peptide Center was employed as reference for an enzyme reaction.

Figure 7 represents the substrate diminution and formation of reaction products after 30 min incubation. The optimum pH for the hydrolysis of anserine

Fig. 7. Effect of pH on hydrolysis of anserine, carnosine and glycylleucine by the dipeptidase-containing fraction from rat intestine homogenate. Reaction mixture; 1.5 μmole of substrate (*) and 0.5 ml enzyme solution, in 2.5 ml of 0.3 M phosphate buffer (pH 7.8), incubated for 30 min at 37°C. The dotted line shows hydrolized component.
Fig. 8. Time-course of alteration by hydrolysis of anserine, carnosine and glycylleucine. Enzyme was dipeptidase containing fraction from rat intestine. Substrate is indicated by an asterisk (*). The dotted line shows the free constituent amino acids formed after hydrolysis of substrate.

Fig. 9. Chemical equilibrium of anserine hydrolysis. 1.5 μmole of anserine was added as substrate in each tube. (A) Amount of 1-methylhistidine formed. (B) Amount of β-alanine formed.

and carnosine was between 7.6 and 7.8, and the reaction rate of carnosine was higher than that of anserine. Though anserine and carnosine passed through the intestine as they were, some portions of these dipeptides were supposed to be hydrolyzed by the enzyme. The fact that β-alanine level in blood didn’t increase even after administering large dose of anserine or carnosine (cf. Figs. 5
Fig. 10. Michaelis constant of carnosine (A) and anserine (B), hydrolyzed by the enzyme from rat intestine. The dotted line shows the effect of adding 10 μmole/tube of β-alanine. (A) based on the formed histidine (μmole) for 30 min reaction. (B) based on the formed 1-methylhistidine (μmole) for 30 min reaction. S: μmole in 2.5 ml.

and 6) might suggest that there were some differences between in vivo and in vitro conditions or that metabolic rate of β-alanine in blood was quite fast. It is well known that the injected 14C-β-alanine disappears quickly from circulating blood(19).

Figure 8 shows the reaction time and equilibrium in this system. The diminution of substrate agrees with the formation of products. Glycylleucine and carnosine were hydrolyzed completely after 30 min and 1 hr respectively, but only 86% of anserine was hydrolyzed after 24 hr. No interconversion between the two dipeptides was observed in this system. When freshly prepared enzyme solution was added again to the anserine hydrolyzing system after 24 hr incubation, the reaction did not proceed any further.

Figure 9 shows the linear relationship between reciprocals of reaction time and concentration of formed product. It was apparent that the enzyme was not inactivated during the 24 hr incubation. The critical equilibrium point of anserine hydrolysis was 94% according to the circumscribing method on the figure.

Figure 10 shows the relationship between the substrate concentration and reaction velocity. When carnosine was used as a substrate, addition of 10 μmole of β-alanine did not show any effect. The following data were obtained by the graphical method of Lineweaver and Burke. The $K_m$ value of carnosine was $7.48 \times 10^{-4}$ M, which was similar to that of glycylleucine. As for anserine, $K_m$ value was $8.58 \times 10^{-5}$ M and $V_{max}$ was 0.292 (μmole/30 min). In this case addition of β-alanine accelerated the reaction velocity and the effect was remarkable in the higher concentration of anserine. The above findings are very interesting but it is quite difficult to explain the difference between added β-alanine and produced β-alanine. Further studies, including enzyme purification, are required to elucidate this point.

These rather conflicting facts between the intact cell and the cell free system
were also shown in the data obtained from human carnosineaemia (16,20,21).

REFERENCES