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**Comparative physiology of dipeptide transport in lower  
vertebrates (fishes) and invertebrates (lobster)**

**Thomotharan, Manikkavasagar, Ph.D.**

**University of Hawaii, 1994**

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COMPARATIVE PHYSIOLOGY OF DIPEPTIDE TRANSPORT IN LOWER  
VERTEBRATES (FISHES) AND INVERTEBRATES (LOBSTER)

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF  
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## CHAPTER 1

### CURRENT STATUS OF INTESTINAL DIPEPTIDE TRANSPORT MECHANISMS AND PROPOSED WORK

#### PROTEIN DIGESTION

There are both luminal and cellular phases in the digestion of proteins in the gastrointestinal tract. Protein digestion begins in the stomach by the action of pepsin in the presence of hydrochloric acid. The main end products of gastric digestion is a mixture of very large polypeptides and a small percentage of amino acids. Under normal physiological conditions further degradation of polypeptides does not take place in the stomach and the digesta enters the small intestine. In the small intestine further hydrolysis of polypeptides continues by the proteolytic enzymes of the pancreas.

There are still insufficient data about the composition of contents, particularly the peptide fraction of the small intestine after a protein-containing meal. Studies conducted on dog, rat and man show that after a protein meal the small intestinal lumen contains a mixture of peptides and amino acids in which small peptides usually predominate (19). Further hydrolysis by the action of peptidases at the absorptive surfaces yields tripeptides, dipeptides and amino acids, which are absorbed by the intestinal cells.

## DISTINCTION BETWEEN AMINO ACID AND PEPTIDE TRANSPORTERS

It is important to recognize that much evidence exists which support the independence of intestinal transport of peptides and amino acids across the brush border membrane. These data fall under the following categories: (1) Transport defectssuch as Hartnup disease. In this instance, because of a defect in neutral amino acid transport, absorption of dipeptides is able to deliver appropriate amounts of neutral amino acids (24). (2) Competitive inhibition studies have shown that intestinal transport of dipeptides is inhibited by other dipeptides, but not by amino acids (14). (3) Additive phenomenon. During saturated, mediated uptake of a free amino acid, the rate of absorption of the same amino acid can be substantially increased by addition of a peptide containing it (10, 11). (4) Site of absorption. In rat and hamster the sites of maximal absorptive capacity for amino acids and peptides appear to be different (29). (5) Cotransport agent. Certain amino acids are cotransported with  $\text{Na}^+$ , unlike peptides are cotransported with protons (15, 33). (6) Effect of papain on Brush border membrane vesicles (BBMV). Treatment with papain causes a reduction in amino acid transport by BBMV, but not peptides (8).

## ABSORPTION OF PEPTIDES

Early evidence drawing attention to absorption of intact peptides was shown by Folin and Berglund (13). This was followed by a series of investigations by several researchers and the first demonstration of active transport of dipeptides was made with the hydrolysis-resistant dipeptide,



glycylsarcosine (Gly-Sar) using rings of everted hamster small intestine (1). In this study the experimenters were able to show that hamster jejunum has the ability to transport Gly-Sar against a concentration gradient. They were also able to abolish the ability to concentrate Gly-Sar by anoxia, cyanide, DNP (2:4 dinitrophenol) and Na replacement with K or Li. About this time intestinal transport of intact peptides was demonstrated in many other animals and in man (2, 23), but the actual mechanism by which they were transferred across the brush border membrane of the cell remained unclear.

#### MAXIMUM CHAIN LENGTH OF PEPTIDES ABSORBED BY INTESTINAL CELLS

Initially it was believed that only dipeptides could be absorbed intact by the intestinal cells (25), but later studies showed that the transport was limited to di- and tripeptides (3). Apart from the absorption of di- and tripeptides, large biologically active peptides of very long chain-length can be absorbed from the small intestine. These results are likely due to special mechanisms responsible for uptake of whole proteins. The extent of whole protein absorption is not nutritionally significant, but sufficient enough to trigger immunologic response.

## INFLUENCE OF MOLECULAR STRUCTURE ON TRANSPORT OF DIPEPTIDES

Knowledge of molecular structural requirements for transport of dipeptides across the brush border membrane of the intestine is still not clear. Even though no detailed systematic investigation has been conducted so far, some differences have become apparent in recent years. Peptide transport is stereochemically specific and prefers peptides containing L-amino acids or glycine (7). Peptides containing D-amino acids are slowly transported as well as hydrolyzed. In rats two D-amino acid-containing dipeptides were more slowly absorbed than those containing only one (6). If the D-amino acid is on the N-terminal end of the peptide, it may have more effect on the transport than one at the carboxyterminal end (9). Further methylation or other substitution on the amino or carboxy terminal group significantly reduces the affinity for transport.

## MECHANISMS OF PEPTIDE TRANSPORT

### Effect of pH on intestinal absorption of dipeptides

Initially investigations using BBMV suggested that peptides were transported by non-concentrative, Na<sup>+</sup>-independent facilitated diffusion (26). Later, it became well-established that intestinal transport of neutral dipetides was electrogenic. In these experiments the authors suggested that transport of the dipeptides must be associated with a flow of an ion other than Na<sup>+</sup> (9). Ganapathy and his co-workers (15, 16) showed a pH gradient across the BBMV stimulated dipeptide transport and suggested a mechanism involving

co-transport with protons. This was then further confirmed by other researchers in this field (20). Ganapathy and Leibach (17) first suggested that, although Na replacement did not affect dipeptide transport under vesicle conditions, it might inhibit peptide transport by the intact intestine through interference with the Na/H exchanger. Therefore, peptide transport may not be coupled directly to Na, but, may still be indirectly dependent on transport of this cation under physiological conditions.

#### Kinetics of peptide transport

Many discrepancies exist between experimental results for the kinetics of intestinal transport of peptides obtained with whole tissue preparations and those recently published with BBMV. Most of these discrepancies could be attributed to an effect of an unstirred water layer in whole tissue preparations. Hence, none of the earlier data available enable a reliable estimate of the kinetic parameters of the respective peptides studied. Early work on intestinal peptide transport showed that total peptide uptake was a saturable process (21, 22). These studies particularly pointed out that transport of glycine containing di- and tripeptides showed higher  $K_t$  and higher  $V_{max}$  than does the transport of free glycine, reinforcing the independence of transport of amino acids and peptides. While many investigations suggested the presence of possibly one peptide carrier in the small intestine of animals, a few studies suggested the occurrence of high and a low affinity carriers for Gly-Pro in the small intestine of rabbit (28). The demonstration of more than one mechanism for peptide transport was not accepted at that time, but data now support this finding (30, 12, 31). Even though progress has been achieved in the field of intestinal peptide

transport, this still remains a relatively under-studied area. One reason for this may be that the nutritional benefit from peptide transport still remains to be established. We still do not know whether all 400 possible dipeptides that can arise during a digested protein meal are transported by one carrier or whether there are different carriers as those described for amino acids. Although protein digestion to peptides occurs practically in all animals, so far the study of peptide transport has been mostly limited to mammals.

#### PEPTIDE ABSORPTION IN FISHES

Peptide absorption in fishes was first investigated by Reshkin and Ahearn (27). This study investigated the mechanism by which glycyl-L-phenylalanine (Gly-Phe) was absorbed by the herbivorous tilapia, *Oreochromis mossambicus*. They found that Gly-Phe was hydrolyzed during a carrier-mediated, cation-independent transport event. In another study, Verri and his co-workers (32) showed proton-gradient-energized glycyl-glycine transport in the carnivorous eel, *Anguilla anguilla*. These investigators used fluorescence quenching of a pH sensitive dye, acridine orange, to monitor proton accumulation in eel intestinal cell BBMV, coupled to the presence of the dipeptide glycyl-glycine. These two investigations showed two possible mechanisms that might be present in teleost gut for absorption of different peptides. However, because they used two different experimental approaches to study dipeptide uptake, it is difficult to conclude whether the differences observed were due (1) to the two different dipeptides studied, (2) to species differences, or (3) to the techniques used. These contradictory results stress the need for a repeated

investigation of dipeptide transport in teleost fish intestinal cells. Apart from these two studies, no other investigations have been reported on brush border or basolateral uptake mechanism(s) of dipeptides in fish or invertebrate gastrointestinal cells.

### RESEARCH OBJECTIVES

In my study I propose to undertake an investigation to characterize the brush border uptake and basolateral efflux mechanisms of a biologically stable dipeptide, glycylsarcosine in an herbivorous teleost (tilapia, *Oreochromis mossambicus*). This will be the first study to characterize dipeptide uptake and efflux processes of a single dipeptide in any animal.

In order to extend our understanding of such a unique system, I would like to compare the characteristics of brush border uptake in the herbivorous tilapia to those of a carnivorous teleost (rock fish, *Sebastes caurinus*) and an omnivorous invertebrate (lobster, *Homarus americanus*).

The lobster hepatopancreas is a diverticulum of the pyloric stomach. Over the past few years a number of studies have focused on the mechanism of sugar and amino acid transport by hepatopancreatic BBMV (4,5). These investigations showed that the hepatopancreas plays a major role in the absorption of nutrients in this animal. A novel feature of these diverticula is that the luminal pH at times of feeding may drop to as low as 4 (18). A number of studies have shown that a drop in external pH stimulates sugar and amino acid transport into hepatopancreatic BBMV. The observed stimulation was attributed either to protonation of

amino acids with the protonated form being the preferred substrate, or protonation of the carrier resulting in an increase in the binding affinity for the sugars.

The acidic nature of these diverticula at the absorptive site, markedly affecting nutrient transport, make this an ideal animal model since the solutes under investigation (dipeptides) are known to be coupled to protons in other types of animals. It will be of interest to investigate: (1) whether such a proton coupled dipeptide mechanism exists in the brush border membrane of lobster hepatopancreas, (2) If so, are the affinities and transport capacities of these dipeptide transporters any different than those described for mammals and fishes, (3) Does the binding affinity of this transporter show any variation at different pH values?, and (4) Is the specificity of this transporter any different from those exhibited by vertebrates?

#### SIGNIFICANCE OF RESEARCH

Results of these experiments would not only shed light on peptide transport mechanisms in animals other than mammals, but also would strengthen the understanding of protein absorptive physiology across phyla.

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## CHAPTER 2

### INTESTINAL DIPEPTIDE TRANSPORT IN HERBIVOROUS AND CARNIVOROUS TELEOST

#### INTRODUCTION

The study of peptide uptake in mammalian gut by Newly and Smith in 1959 (13) initially challenged the idea of amino acids being the fundamental currency of protein metabolism. This study opened up a fast growing field of investigation on the uptake mechanisms of different peptides in mammalian gut and renal tissue. More recent studies showed quantitatively that amino nitrogen could be absorbed more rapidly when presented as small peptides in the intestinal lumen than when presented as an equivalent mixture of free amino acids (1).

Ingested protein is first presented to the gut mucosa predominantly in the form of 2-4 amino-acid residue peptides and as free amino acids (2). A large part of the protein meal is presented to the blood stream as short chain peptides (4). Studies also suggested that dipeptide transport plays a greater significance than amino acid transport during early growth (5). These investigations demonstrated that peptide absorption plays an important role in amino nitrogen balance in many groups of vertebrates. However most scientific work has been focused on characterizing amino acid transport systems rather than those of peptides.

Recent experiments conducted on brush border membrane vesicles (BBMV) isolated from mammalian small intestine and kidney have demonstrated that di- and tripeptides can be transported intact by proton-gradient-energized transport systems (6). Further studies using mammalian kidney BBMV showed the existence of multiple carriers for dipeptide transport in renal tissue (3,16). Reports also suggested the existence of a non-cation-stimulated, facilitated diffusional transport mechanism for glycyl-L-proline in intestinal BBMV of mice, rabbits and humans (14) and a possible anion/peptide antiporter in intestinal BBMV of humans (10)

In teleost gut epithelia proton-gradient-energized glycyl-glycine transport was demonstrated in intestinal BBMV of the carnivorous eel *Anguilla anguilla* (18) and cation independent, facilitated transport of glycyl-L-phenylalanine, which was hydrolyzed to its component amino acids during transfer was demonstrated in intestinal BBMV of herbivorous tilapia, *Oreochromis mossambicus* (15). For this reason in the present study we used  $^{14}\text{C}$ -glycyl-sarcosine ( $^{14}\text{C}$ -Gly-Sar), which is generally very resistant to hydrolysis and appears intact in mammalian intestinal tissue following absorption (11), to determine the characteristics of gastrointestinal dipeptide transport in the carnivorous rock fish (*Sebastes caurinus*) and herbivorous teleost, tilapia (*Oreochromis mossambicus*).

## Materials and Methods

### Collection and maintenance of animals

African tilapia (*Oreochromis mossambicus*) were obtained from the Marine and Research Training Center (M.R.T.C.) on Oahu, Hawaii and maintained in large holding tanks in the Department of Zoology at the University of Hawaii. Rock fish (*Sebastes caurinus*) were collected from waters around the University of Washington Friday Harbor Laboratory, Washington state, and were held in tanks with flowing sea water at Friday Harbor Laboratory until needed. Experiments with rock fish were conducted at Friday Harbor while those with tilapia were performed in Hawaii.

### Preparation of brush-border membrane vesicles

Fish were killed by a blow to the head. Intestine and pyloric ceca were removed for preparation of brush-border membrane vesicles (BBMV). In tilapia the upper half and in rock fish the first two-thirds of the intestine were used for preparation of BBMV. Vesicles were prepared using a magnesium-precipitation technique (15). Briefly, the tissue was homogenized in a solution containing 60 mM mannitol having 12 mM (Tris) HCl, 10 mM EGTA and 0.1 mM PMSF (buffer 1). Magnesium chloride was added and mixed with the homogenate to a final concentration of 10 mM and the resulting solution was allowed to stand on ice for 15 minutes (step 1). The suspension was centrifuged at 3000 g for 15 minutes and the supernatant at 27,000 g for 30 minutes (step 2). The pellet from the high speed spin was resuspended in 35 ml of the above buffer solution using a Potter-Elvehjem homogenizer. Step 1 and step 2 were repeated on this

homogenate and the resulting pellet was resuspended with the Potter-Elvehjem homogenizer in 10 ml of ice-cold transport buffer of appropriate composition, and the final suspension was centrifuged at 27,000 g for 30 minutes. The purified membrane pellet was resuspended in sufficient transport buffer to provide 8-10 mg/ml. protein content. Protein content of the preparation was assessed with the Bio-Rad protein assay.

#### Transport measurements

Transport studies using intestinal BBMV were conducted at 23° C using the Millipore filtration technique (9). <sup>14</sup>C-Gly-Sar was provided by Dr. F. H. Leibach, Dept. of Cell and Molecular Biology, Medical College of Georgia. Long-term <sup>14</sup>C-Gly-Sar uptake experiments were initiated by mixing 20 ul membrane suspension with 180 ul radiolabeled incubation medium. Composition of the incubation medium varied with the nature of the experiments. Uptake of <sup>14</sup>C-Gly-Sar was terminated by injecting 20 ul of reaction mixture into 2 ml of ice cold stop solution (same composition as the incubation medium without radiolabelled solute). This was then filtered onto a Millipore filter (0.65um) and washed with another 5 ml of ice-cold stop solution. Filters containing the washed vesicles were placed in scintillation cocktail and counted in a Beckman LS-8100 scintillation spectrometer.

Short-term influx experiments were initiated by mixing 5 ul of vesicle sample with 45 ul of radiolabeled incubation medium containing variable concentrations of unlabeled solute. Solute influx was terminated by injecting 20 ul of reaction mixture into 2 ml of ice-cold stop solution. This was then filtered, washed, and counted as described earlier.

Carrier-mediated  $^{14}\text{C}$ -Gly-Sar influx kinetics were determined by a curve-fitting procedure by which the individual influx values for all the replicates were computer fitted, using an iterative, nonlinear method to the Michaelis-Menten kinetic equation plus a nonsaturable carrier mediated transport component:

$$J_{oi} = \left\{ \left( J_{\max} \cdot [S] \right) / \left( K_t + [S] \right) \right\} + [K_{ns}] \cdot [S] \quad (1)$$

In this equation  $J_{oi}$  was  $^{14}\text{C}$ -Gly-Sar influx in picomoles per milligram protein, per 10 sec,  $[S]$  was external  $^{14}\text{C}$ -Gly-Sar concentration in mM;  $J_{\max}$  was maximal  $^{14}\text{C}$ -Gly-Sar influx;  $K_t$  was the concentration of S that yielded one half  $J_{\max}$  and  $K_{ns}$  was the nonsaturable carrier mediated transport component. All isotope transport values were corrected for a "vesicle blank" obtained by adding the incubation medium and vesicles directly to the respective stop solution, filtering onto Millipore filters, and counting. Each experiment was repeated using membrane vesicles prepared from different fish to confirm consistent experimental findings. Within a given experiment, each point represented the mean of 3 replicates and their standard error values. Significant differences between values were determined by student's t test.

Specificity of the  $^{14}\text{C}$ -Gly-Sar carrier was assessed by measurement of the influx of 0.01 mM  $^{14}\text{C}$ -Gly-Sar (a) in the presence of several external unlabelled dipeptides (cis inhibition), and (b) during the efflux of preloaded intravesicular 0.1 mM test peptides (trans-stimulation). Both experiments employed an inwardly-directed hydrogen gradient.



Previously it was shown in rabbit intestinal BBMV that Gly-Sar was very resistant to hydrolysis, and that no detectable hydrolysis of Gly-Sar was seen within 30 minutes of incubation, and less than 5% in 3 hours (7). In tilapia and rock fish intestinal and pyloric cecal BBMV, one hour of incubation of  $^{14}\text{C}$ -Gly-Sar with BBMV revealed no hydrolysis of  $^{14}\text{C}$ -Gly-Sar, when analyzed by TLC (silicagel, butanol : acetic acid : water 4:2:2) and reverse-phase HPLC (unpublished data).

## RESULTS

Time course of  $^{14}\text{C}$ -Gly-Sar transport in herbivorous tilapia intestinal BBMV

Effect of a pH gradient

In order to assess whether the transport of  $^{14}\text{C}$ -Gly-Sar was specifically activated by a proton gradient, uptake of 0.01 mM  $^{14}\text{C}$ -Gly-Sar was measured with vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM HEPES/Tris at pH 7.5 and, 50  $\mu\text{M}$  valinomycin. Figure 1-A illustrates the effects of transmembrane hydrogen ion gradients on the time course of 0.01 mM  $^{14}\text{C}$ -Gly-Sar uptake by tilapia upper intestinal BBMV. Gly-Sar uptake was characterized by a slow, hyperbolic, time course and was higher when  $\text{pH}_i$  (7.5) >  $\text{pH}_o$  (5.5) than when  $\text{pH}_i = \text{pH}_o = 7.5$ . Further the uptake was also higher when  $\text{pH}_i$  (7.5) >  $\text{pH}_o$  (5.5) than when  $\text{pH}_i = \text{pH}_o = 5.5$  (data not shown). Abolishing the pH gradient  $\text{pH}_i$  (7.5) >  $\text{pH}_o$  (5.5) by incubating the vesicles in the protonophore, CCCP (carbonylcyanidemchlorophenyl hydrazone, 10  $\mu\text{M}$ ), reduced Gly-Sar transport by these vesicles. These data show that (1)

external pH had a marked effect on transport of this dipeptide and (2) maximal peptide transport was attained with an inwardly-directed hydrogen ion gradient.

#### Effect of a membrane potential

The effect of a  $K^+$ -generated diffusion potential (inside negative) on  $^{14}C$ -Gly-Sar transport was studied in the presence of an inwardly-directed hydrogen ion gradient. In this experiment vesicles were preloaded with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris at pH 7.5 and, 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 100 mM mannitol or (2) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Imposing a membrane potential under these conditions resulted in increased uptake of  $^{14}C$ -Gly-Sar in tilapia BBMV compared to the uptake in short-circuited conditions (Fig 1-B).

#### Effect of a sodium gradient

The effect of  $Na^+$  ions on the uptake of  $^{14}C$ -Gly-Sar in tilapia was studied in the presence of an inwardly-directed hydrogen ion gradient along with a  $K^+$  generated diffusion potential (inside negative) condition. In these experiments vesicles were preloaded with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris, at pH 7.5, and, 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 100 mM mannitol; (2) 100 mM choline chloride, 100 mM mannitol; or (3) 100 mM sodium chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Addition of a 100

mM inwardly-directed  $\text{Na}^+$  gradient along with an outwardly-directed  $\text{K}^+$  generated diffusion potential (inside negative) did not stimulate  $^{14}\text{C}$ -Gly-Sar uptake over that induced by a  $\text{K}^+$  generated diffusion potential (inside negative) alone. These results suggest that  $^{14}\text{C}$ -Gly-Sar uptake in tilapia BBMV is  $\text{Na}^+$  independent. (Fig 1-B).

Time course of  $^{14}\text{C}$ -Gly-Sar transport in carnivorous rock fish pyloric cecal and intestinal BBMV.

Pyloric ceca

Effect of a pH gradient

Figure 2-A illustrates the effects of transmembrane hydrogen ion gradients on the time course of 0.1 mM  $^{14}\text{C}$ -Gly-Sar uptake by rock fish pyloric cecal BBMV preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris pH 7.5 or 20 mM Mes/Tris at pH 5.5 and, 50  $\mu\text{M}$  valinomycin.  $^{14}\text{C}$ -Gly-Sar uptake was characterized by a slow, hyperbolic, time course and was higher when  $\text{pH}_i$  (7.5) >  $\text{pH}_o$  (5.5) than when  $\text{pH}_i = \text{pH}_o = 7.5$ . These data suggest that in the carnivorous rock fish pyloric ceca (1) external pH had a marked effect on the transport of this dipeptide, and (2) maximal transport of this peptide was attained when presented with an inwardly-directed hydrogen ion gradient.

Effect of a membrane potential

The effect of a  $\text{K}^+$ -generated diffusion potential (inside negative) on  $^{14}\text{C}$ -Gly-Sar transport was studied in pyloric cecal BBMV in the presence of an inwardly-directed hydrogen ion gradient. In this experiment vesicles were preloaded

with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris at pH 7.5, and 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 100 mM mannitol or (2) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Imposing a membrane potential under these conditions resulted in increased uptake of  $^{14}\text{C}$ -Gly-Sar in pyloric cecal BBMV, illustrating the electrogenic nature of dipeptide uptake in this tissue (Fig 2-A).

## Intestine

### Combined effects of membrane potential and sodium gradient

The effect of  $\text{Na}^+$  ions on the uptake of  $^{14}\text{C}$ -Gly-Sar in rock fish intestinal BBMV was studied in the presence of an inwardly-directed hydrogen ion gradient along with a  $\text{K}^+$  generated diffusion potential (inside negative). In these experiments uptake of 1 mM  $^{14}\text{C}$ -Gly-Sar was measured with vesicles that were preloaded with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris at pH 7.5, and 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 100 mM mannitol (2) 100 mM choline chloride, 100 mM mannitol or (3) 100 mM sodium chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Addition of a 100 mM inwardly-directed  $\text{Na}^+$  gradient along with an outwardly-directed  $\text{K}^+$ -generated, diffusion potential (inside negative) did not stimulate  $^{14}\text{C}$ -Gly-Sar uptake over that of a  $\text{K}^+$ -generated diffusion potential (inside negative) alone in rock fish intestinal BBMV. These results suggest that  $^{14}\text{C}$ -Gly-Sar uptake in rock fish intestinal BBMV was electrogenic and  $\text{Na}^+$  independent. (Fig 2-B)

### Influence of $\text{HCO}_3^-$ gradient and buffer capacity of vesicles on $^{14}\text{C}$ -Gly-Sar transport in tilapia BBMV

In order to test a possible involvement of an anion exchanger with proton-coupled peptide transport in fish, experiments were conducted using outwardly-directed bicarbonate gradients. In these experiments vesicles were preloaded with these conditions: a) 100 mM K-gluconate, 100 mM mannitol, 20 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin; b) 100 mM K-gluconate, 100 mM mannitol, 50 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin; and c) 100 mM  $\text{KHCO}_3$ , 100 mM mannitol, 50 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin. Transport was initiated by exposing 20  $\mu\text{l}$  of vesicle suspension to 180  $\mu\text{l}$  of transport medium having either (a) 100 mM K-gluconate, 100 mM mannitol, 20 mM Mes/Tris at pH 6.5, or (b) 100 mM K-gluconate, 100 mM mannitol, 50 mM Mes/Tris at pH 6.5 (Fig 3). Results show significant stimulation of peptide uptake when internal vesicle buffering capacity was changed from having 25 mM Hepes/Tris to 50 mM Hepes/Tris. An outwardly-directed bicarbonate gradient along with an inwardly-directed pH gradient did not stimulate peptide uptake over that of a pH gradient alone when vesicles were loaded with 50 mM Hepes/Tris instead of 25 mM Hepes/Tris. These results show that there was likely no involvement of an anion exchanger in peptide uptake in fish gut.

### Kinetics of $^{14}\text{C}$ -Gly-Sar influx in tilapia intestinal BBMV

Apparent influx of  $^{14}\text{C}$ -Gly-Sar into tilapia intestinal BBMV was measured over a concentration range of 0.001 mM to 1.0 mM in the presence of an inwardly-directed hydrogen

ion gradient for 10 sec at 23<sup>0</sup> C. Membrane vesicles were preloaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 7.5 and, 50 uM valinomycin. Concentration-dependent uptake of <sup>14</sup>C-Gly-Sar was measured with an inwardly-directed proton gradient (pH<sub>in</sub> 7.5/ pH<sub>out</sub> 5.5). Total influx for this concentration range shown by the top line in Fig. 4. Data for total dipeptide influx was used in equation (1) to generate transport kinetic constants for both high and low affinity Gly-Sar carrier systems. This analysis yielded the following Michelis-Menten values for a saturable, high affinity system:  $K_t = 0.56 \pm 0.08$  mM, (b)  $J_{max} = 1954 \pm 174.6$  pmol/ mg.protein/ 10 sec. The curve shown in Fig. 4 representing this saturable, high affinity system (lower line) was drawn through the data using these calculated kinetic constants. In addition, a linear non-saturable process was disclosed from this mathematical treatment generating a  $K_{NS}$  of  $4514.0 \pm 28.1$  pmol/ mg protein/ 10 sec/ mM.

At <sup>14</sup>C-Gly-Sar concentrations between 1 and 10 mM, influx was a linear function of external concentration (Fig.5, top line), exhibiting a slope of 4010 pmol/ mg protein/ 10 sec/ mM, which approximates the value of  $K_{NS}$  obtained from curve fitting total peptide influx over the low concentration range shown in Fig. 4. Influx in the absence of any driving forces (pH<sub>i</sub> = pH<sub>o</sub> = 7.5) in Fig. 5, was considered to be apparent diffusion and its magnitude was estimated at each Gly-Sar concentration using the slope in Fig. 5 (480 pmol/mg.pro/10 sec/mM). From this analysis, diffusion was shown to account for less than 10% of the total measured influx over the entire concentration range employed in these experiments ( 0.001 to 10 mM; Table. 1).

DEP (diethylpyrocarbonate) has been shown to inhibit carrier-dependent dipeptide transport in intestinal BBMV of

rabbit (12) and eel (18). In order to assess whether non-saturable  $^{14}\text{C}$ -Gly-Sar influx seen in tilapia intestinal BBMV (Fig. 5 top line) was carrier mediated, a 10 mM concentration of DEP was used in uptake studies. In this experiment membrane vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 7.5, 50  $\mu\text{M}$  valinomycin and incubated with 10 mM DEP for 10 minutes, washed and resuspended in inside buffer. Concentration-dependent uptake of Gly-Sar (1-10 mM) was measured with DEP treated vesicles either having an inwardly-directed proton gradient (pH 7.5 in / pH 5.5 out) or with equal pH ( $\text{pH}_i = \text{pH}_o = 7.5$ ). The presence of an inwardly directed proton gradient gave a four fold higher rate of influx. In the presence of 10 mM DEP, total pH gradient-dependent  $^{14}\text{C}$ -Gly-Sar influx was significantly reduced (Fig. 5). These observations and the fact that apparent diffusion only accounts for less than 10% of the total influx, suggests that non-saturable peptide transport observed between 1 and 10 mM was mediated by a carrier and not due to simple diffusion. These combined results show the possible presence of both a high capacity, low affinity carrier and a low capacity, high affinity carrier for  $^{14}\text{C}$ -Gly-Sar in tilapia intestine. The relative contributions of high and low affinity carrier-mediated transport components to the total peptide uptake is shown in Table 1. Total measured influx and calculated kinetic constant values (equation 1) were used to determine the percentage of total  $^{14}\text{C}$ -Gly-Sar influx attributed to each transport component at a variety of external peptide concentrations in tilapia BBMV. The values show that the low affinity, high capacity, non-saturable, carrier component predominated at peptide concentrations above 1.0 mM while the high affinity, low capacity, saturable, carrier mechanism accounted for 40% of total transport at peptide concentrations below 1.0 mM.

## Specificity of the $^{14}\text{C}$ -Gly-Sar carrier in tilapia gut

### Cis inhibition

In order to investigate whether other dipeptides could inhibit the influx of  $^{14}\text{C}$ -Gly-Sar, entry of the labelled substrate was measured in the presence of several unlabelled dipeptides. In these studies membrane vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM HEPES/Tris at pH 7.5 and, 50  $\mu\text{M}$  valinomycin. Influx of 0.01 mM  $^{14}\text{C}$ -Gly-Sar into tilapia BBMV was measured with an inwardly-directed hydrogen ion gradient for 10 sec in the presence of 0.1 mM test peptide concentration. Results showed a significant ( $p < 0.01$ ) inhibition of Gly-Sar influx only when challenged with Gly-Sar and Gly-Pro (Fig. 6). The other peptides used were ineffective inhibitors of  $^{14}\text{C}$ -Gly-Sar influx at the concentration used.

### Trans-stimulation

Trans-stimulation of  $^{14}\text{C}$ -Gly-Sar influx by the efflux of preloaded intravesicular 0.1 mM test peptides was investigated. In these studies membrane vesicles were pre-loaded with 0.1 mM test peptide and 100 mM mannitol, 100 mM KCl and 20 mM HEPES/Tris at pH 7.5 and, 50  $\mu\text{M}$  valinomycin. Influx of 0.01 mM  $^{14}\text{C}$ -Gly-Sar into tilapia BBMV preloaded with test peptide was measured with an inwardly-directed hydrogen ion gradient for 10 sec at  $23^{\circ}\text{C}$ . Results showed significant ( $p < 0.01$ ) trans-stimulation of 0.01 mM  $^{14}\text{C}$ -Gly-Sar influx only with vesicles preloaded with 0.1 mM Gly-Pro (Fig. 7); all other dipeptides were ineffective trans-stimulators at the concentration used.



## DISCUSSION

The results of this study provide new insight into the mechanism and driving forces for dipeptide transport in fish gastrointestinal BBMV. Downhill movement of protons along their electrochemical gradient, providing a driving force for electrogenic dipeptide uptake, was initially hypothesized and demonstrated in mammalian renal epithelia (7, 17). Our data showed that  $^{14}\text{C}$ -Gly-Sar transport in fish was electrogenic in the absence of sodium. In both fish species studied, imposition of an inward proton gradient stimulated  $^{14}\text{C}$ -Gly-Sar transport and this stimulation was reduced when the proton gradient was dissipated by the presence of a protonophore. Because Gly-Sar existed predominantly as a dipolar zwitterion with zero net charge in the pH range tested in this study, the electrogenicity observed was likely due to cotransport of a proton with the electroneutral dipeptide.

In tilapia  $^{14}\text{C}$ -Gly-Sar uptake by intestinal BBMV appeared to be mediated by two carrier systems. One carrier was characterized as a high affinity, low capacity type and the other as a low affinity, high capacity type. Previously, high and low affinity transport carriers for different peptides have been shown in mammals for glycyl-L-glutamine (3) and glycyl-L-proline (16). The high affinity carrier in tilapia intestine exhibited a  $K_t$  value that was close to the analogous values of high-affinity carriers of other peptide transport systems described in kidney tissue of mammals. (3,16).

We could not demonstrate saturation of the low affinity carrier system for Gly-Sar in tilapia intestinal BBMV. Previously there has been only one study that examined Gly-Sar transport in intestinal tissue using BBMV (7). This study

used rabbit intestinal BBMV and reported an asymptotic uptake of Gly-Sar by the peptide carrier having a  $K_t$  of 17 mM. The apparent discrepancy between these studies could be attributed to the imposition of short-circuiting conditions in the tilapia experiments by allowing  $K^+$  to exchange for entering protons, coupled to the peptide. In the absence of valinomycin, transported  $H^+$  into vesicles might produce an inside-positive potential, contributing to a reduction of electrogenic peptide uptake. This effect could be interpreted as a saturation of uptake as the peptide concentration was increased. The finding with tilapia that non-saturable low affinity transport (1-10 mM Gly-Sar) could be blocked by DEP and also could be affected by pH suggests that there may be a carrier involved over this concentration range for the total uptake of Gly-Sar rather than uptake due solely to simple diffusion.

The suggestion of two peptide carrier systems in fish intestine raises several questions: (1). Are both carriers present along the full length of the gut?, (2) Are they distributed differently?, (3) Why might the distribution vary?, (4) Are the two carriers really two different proteins, or rather different functional states of the same protein. In the present study with tilapia only the first half of the gut was used to prepare BBMV. It is possible that as proteins get hydrolyzed, the upper half of the gut becomes exposed to high concentrations of dipeptides. It may be advantageous for this part to have a low affinity, high capacity carrier, as this will enable the animal to absorb the maximum possible amino nitrogen in the form of di- and tripeptides. As digestion proceeds and the digesta moves down the length of the gut, the concentration of peptides will be reduced compared to that of amino acids. At this point it may be advantageous for

the system to have a high affinity, low capacity, carrier to efficiently absorb the remaining di- and tripeptides. On the other hand, considering the fact that proteins may have hundreds of possible different dipeptides in their structure, during a protein meal digestion, the quantity of total peptides in the gut could vary within the millimolar range, but an individual peptide concentration may only show up in the micromolar range. If this is true, one might wonder why an animal should have a low affinity, high capacity carrier at all. One suggestion for this apparent physiological anomaly is that the low affinity carrier might be a transporter for another type of molecule in the gut, which is able to transport dipeptides too, but with reduced affinity.

The possible involvement of an anion exchanger in the peptide transport system of the mammalian gut has been recently been suggested (10). Our data shows that the stimulatory effect of bicarbonate ions could be also achieved by increasing the buffer capacity of the vesicle from having 25 mM HEPES to 50 mM HEPES. For this reason we conclude that there is likely no direct involvement of anions in peptide transport in the fish gut.

Most studies reporting the substrate specificity of peptide carriers have been conducted with kidney tissue. Investigations that used labelled Gly-Sar or Gly-Pro indicated that both share the same transport system in rabbit intestine (7) and kidney (16). Results reported here show that fish gut exhibited significant inhibition of  $^{14}\text{C}$ -Gly-Sar influx by Gly-Pro (Fig 6). Trans-stimulation of Gly-Pro uptake was shown with rabbit intestinal vesicles loaded with Gly-Sar (7). Our findings also showed that Gly-Pro was able to trans-stimulate  $^{14}\text{C}$ -Gly-sar influx (Fig 7). These findings,

illustrating competitive inhibition and trans-stimulation of Gly-Sar by Gly-Pro, suggest that a shared peptide carrier was involved in the transport of both Gly-Sar and Gly-Pro in the fish gut, and support the findings in mammalian gut and renal tissues.

The results obtained in the present investigation with two teleost fishes indicate that gastrointestinal peptide transport in these animals displayed the following characteristics. Brush border membrane vesicles from both intestine and pyloric ceca are qualitatively similar in response in ionic requirements and electrogenicity. Both carnivorous and herbivorous teleosts display  $^{14}\text{C}$ -Gly-Sar transport that is  $\text{H}^+$ -gradient-dependent and sodium-independent. In tilapia  $^{14}\text{C}$ -Gly-Sar influx occurred by the combination of three transport processes: (1) A high affinity carrier system with the following kinetic constants (a).  $K_t = 0.6 \text{ mM}$ , (b)  $J_{\text{max}} = 1945 \text{ pmol/ mg. protein/ 10 sec}$ ; (2) A low affinity carrier system ( $K_{\text{NS}} = 4514 \text{ pmol/ mg protein/ 10 sec/ mM}$ ), which was not saturable over the substrate range between 1 and 10 mM Gly-Sar, but was inhibited by 10 mM DEP; and (3) Apparent diffusion.

In summary results of this study lead to a model for dipeptide transport in brush border membrane of teleost intestinal epithelium, which is described in Fig. 8. In teleosts absorption of dipeptides into the intestinal epithelial cells occurs by specialized transport systems. There are at least two major dipeptide transport systems present in teleost intestinal epithelial brush border: (1). A proton-dependent, high affinity, low capacity, saturable carrier system; and (2). A proton-dependent, low affinity, high capacity, non-saturable, carrier system. These two systems apparently

contribute to more than 90% of total dipeptide uptake in teleost gut. The third way in which dipeptides are able to enter intestinal epithelial cells is by simple diffusion. This only contributes to less than 10% of total dipeptide uptake. Absorbed dipeptides may be hydrolyzed by cytoplasmic hydrolase activity of the intestinal epithelial cells yielding individual amino acids, or may leave the cell intact. It has been reported in mammals that several different dipeptides are transported across the basolateral membrane to the blood (8). In Fig. 8, efflux of both amino acids and dipeptides from the gut epithelial cell in fish is hypothesized to follow the mammalian paradigm. Currently experiments are under way to characterize the basolateral efflux mechanism(s) for dipeptides in teleost gut. Preliminary experiments show that teleost gut does possess carrier processes for dipeptides in the basolateral membrane. In mammals and fishes, the nutritional importance of dipeptide uptake by the intestinal mucosa highlights the necessity for further work to investigate the possible diversity of transporters that might exist in both epithelial brush border and basolateral membranes for the hundreds of possible dipeptide combinations that may arise from protein digestion.

Figure 1. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into intestinal BBMVs of tilapia. (a) Effect of pH gradient. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5, or (2) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (3) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5 and 10  $\mu\text{M}$  CCCP. (b). Effect of membrane potential and  $\text{Na}^+$  gradient. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (2) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (3) 100 mM mannitol, 100 mM NaCl and 20 mM Mes/Tris, at pH 5.5.

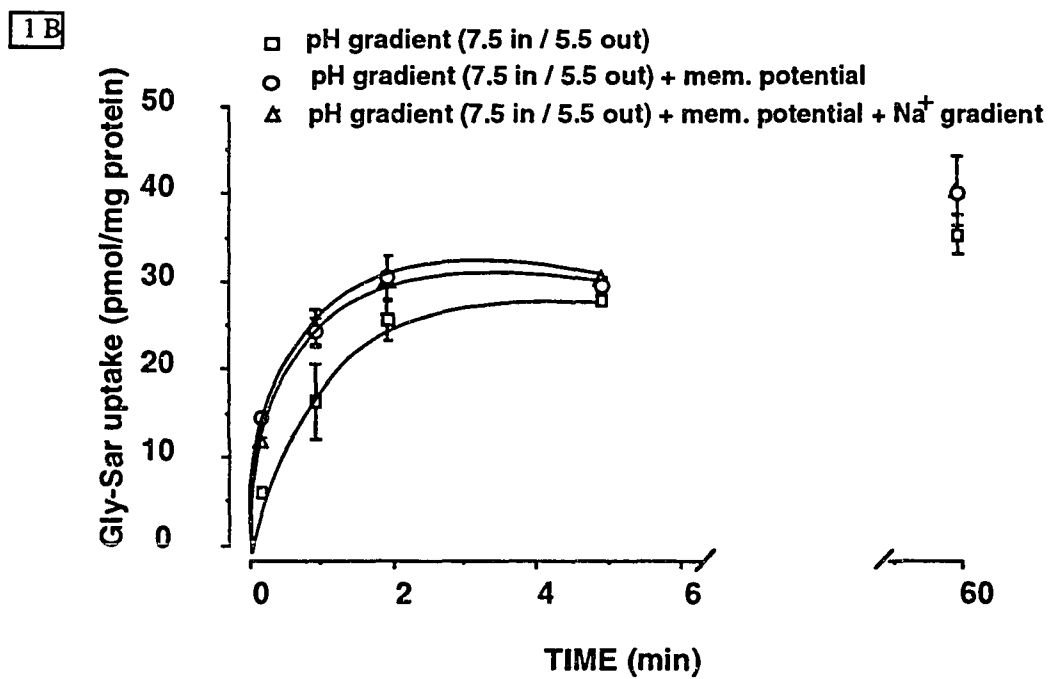
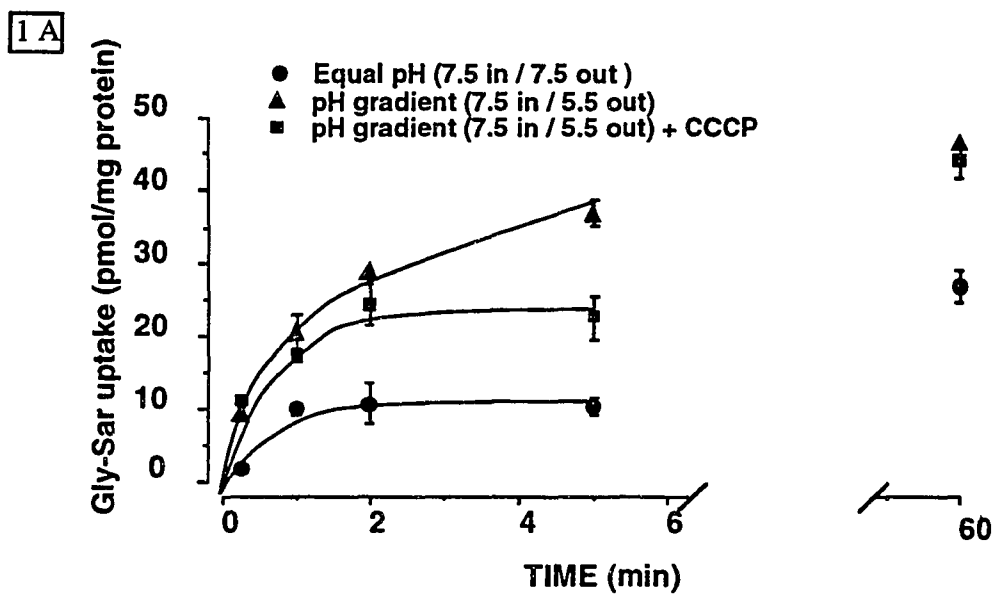
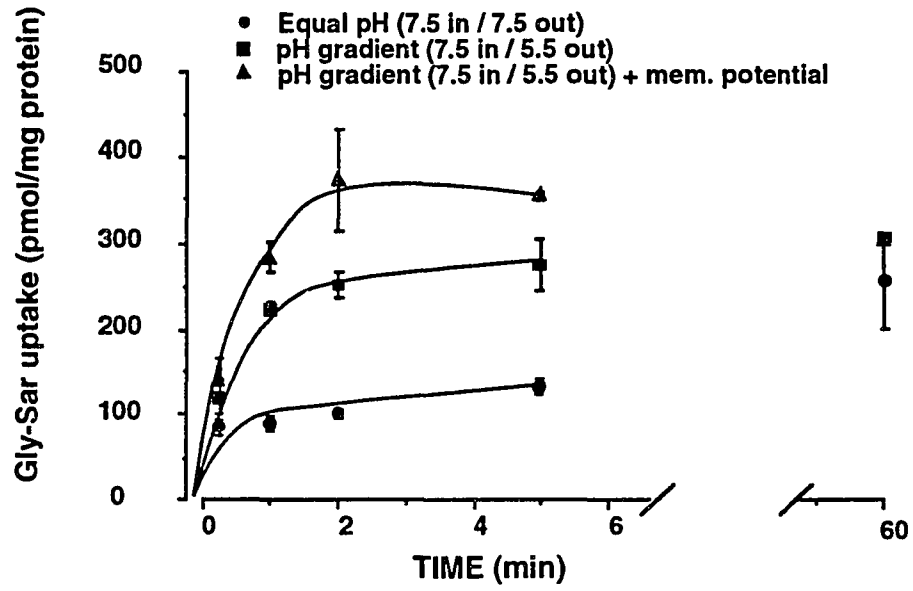


Figure 2. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into pyloric ceca and intestinal BBMV of rock fish. (a). Effect of pH gradient and membrane potential - pyloric ceca. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5, or (2) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (3) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5 (b). Effect of membrane potential and  $\text{Na}^+$  gradient - intestine. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5. Outside media were: (a) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (b) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (c) 100 mM mannitol, 100 mM NaCl and 20 mM Mes/Tris at pH 5.5 .



2A

## Pyloric Ceca



2B

## Intestine

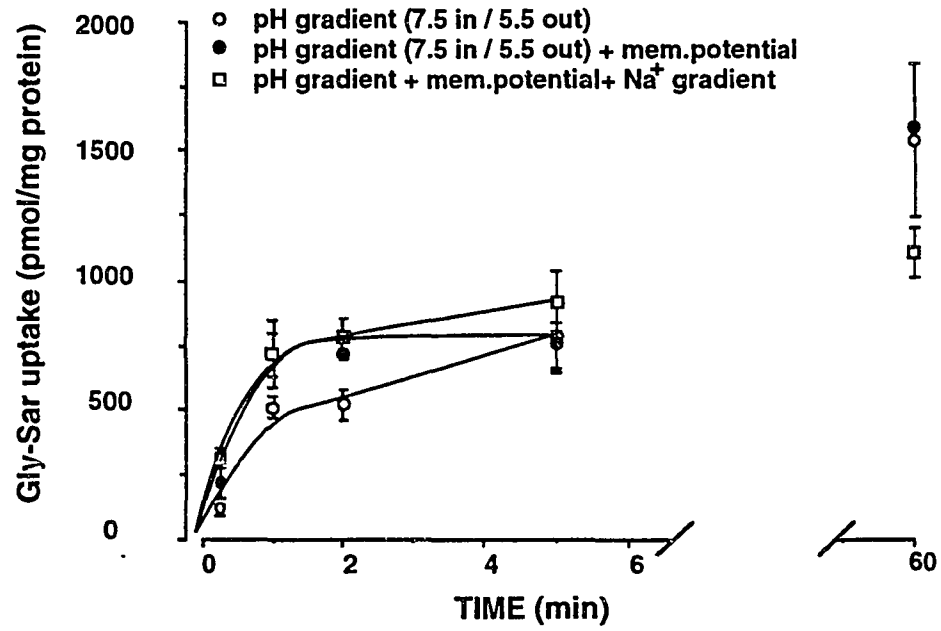


Figure 3. Influence of  $\text{HCO}_3^-$  gradient and buffer capacity of vesicles on the time course of  $^{14}\text{C}$ -Gly-Sar uptake into tilapia BBMV. vesicles were preloaded with either of the following: (a) 100 mM K-gluconate, 100 mM mannitol, 20 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin; (b) 100 mM K-gluconate, 100 mM mannitol, 50 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin; (c) 100 mM  $\text{KHCO}_3$ , 100 mM mannitol, 50 mM Hepes/Tris at pH 8.5, and 50  $\mu\text{M}$  valinomycin. Outside media were: (a) 100 mM K-gluconate, 100 mM mannitol, 20 mM Mes/Tris at pH 6.5, or (b) 100 mM K-gluconate, 100 mM mannitol, 50 mM Mes/Tris at pH 6.5

3

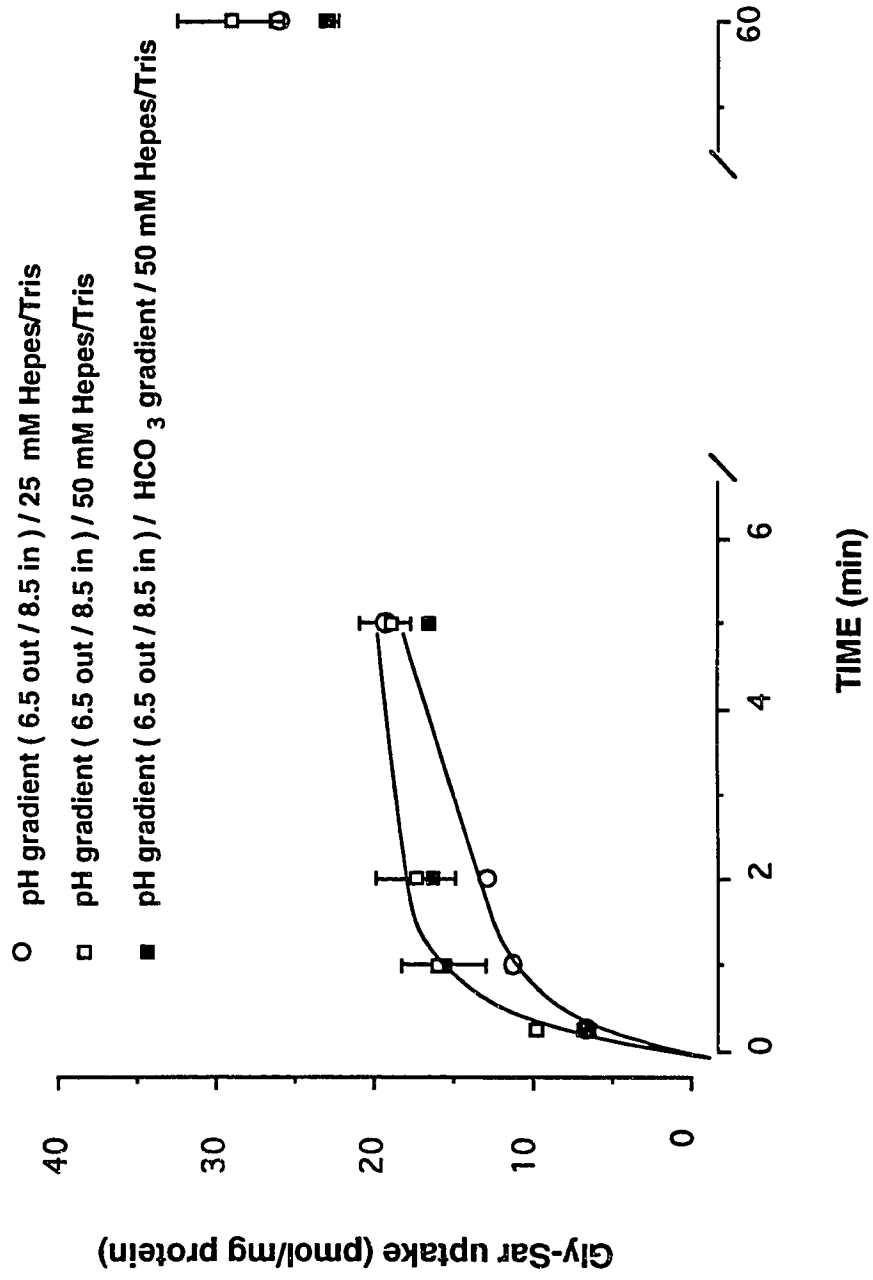


Figure 4. Uptake of  $^{14}\text{C}$ -Gly-Sar uptake into tilapia BBMV as a function of substrate concentration. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris, at pH 7.5 and 50  $\mu\text{M}$  valinomycin. External media either had 100 mM KCl and 20 mM Hepes / Tris, at pH 7.5, or 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris, at pH 5.5, with  $^{14}\text{C}$ -Gly-Sar concentration ranging from .001 to 1.0 mM. Data for total dipeptide influx was used in equation (1) to generate transport kinetic constants for both high and low affinity Gly-Sar carrier systems.

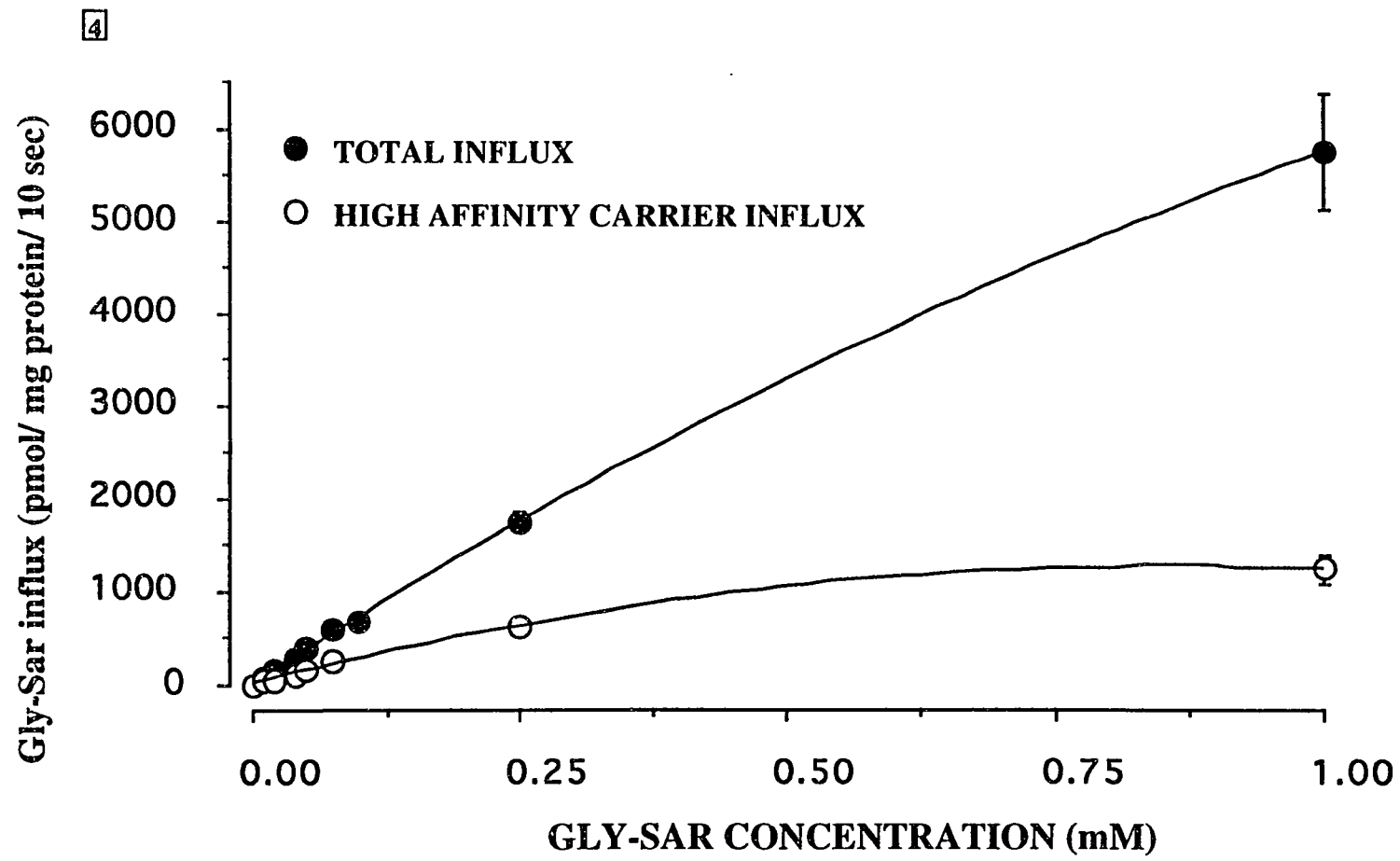


Figure 5. Effect of DEP (diethylpyrocarbonate) on  $^{14}\text{C}$ -Gly-Sar uptake into tilapia BBMV as a function of substrate concentration. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. External media either had 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 7.5, or 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris, at pH 5.5, with  $^{14}\text{C}$ -Gly-Sar concentration ranging from 1 to 10 mM. For DEP treatment vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 7.5 and incubated with 10 mM DEP for 10 minutes. Influx in the absence of any driving forces ( $\text{pH}_i = \text{pH}_o = 7.5$ ), was considered to be apparent diffusion (open triangles).

5

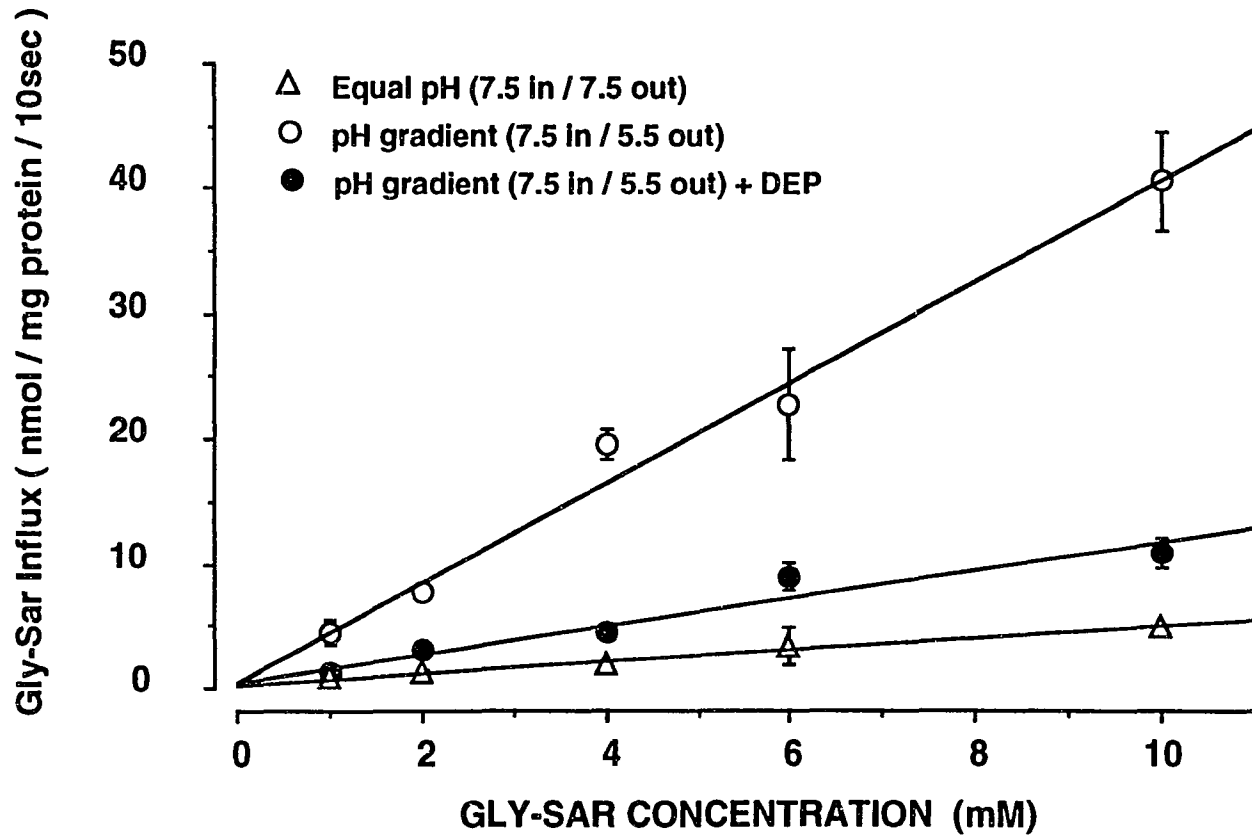


Figure 6. Effect of 0.1 mM external substrates on 0.01 mM  $^{14}\text{C}$ -Gly-Sar influx into tilapia BBMV. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM HEPES/ Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. External media had 100 mM KCl, 100 mM mannitol and 20 mM MES/Tris, at pH 5.5 and 0.1 mM respective test substrate.



**CIS-INHIBITION**

**6**

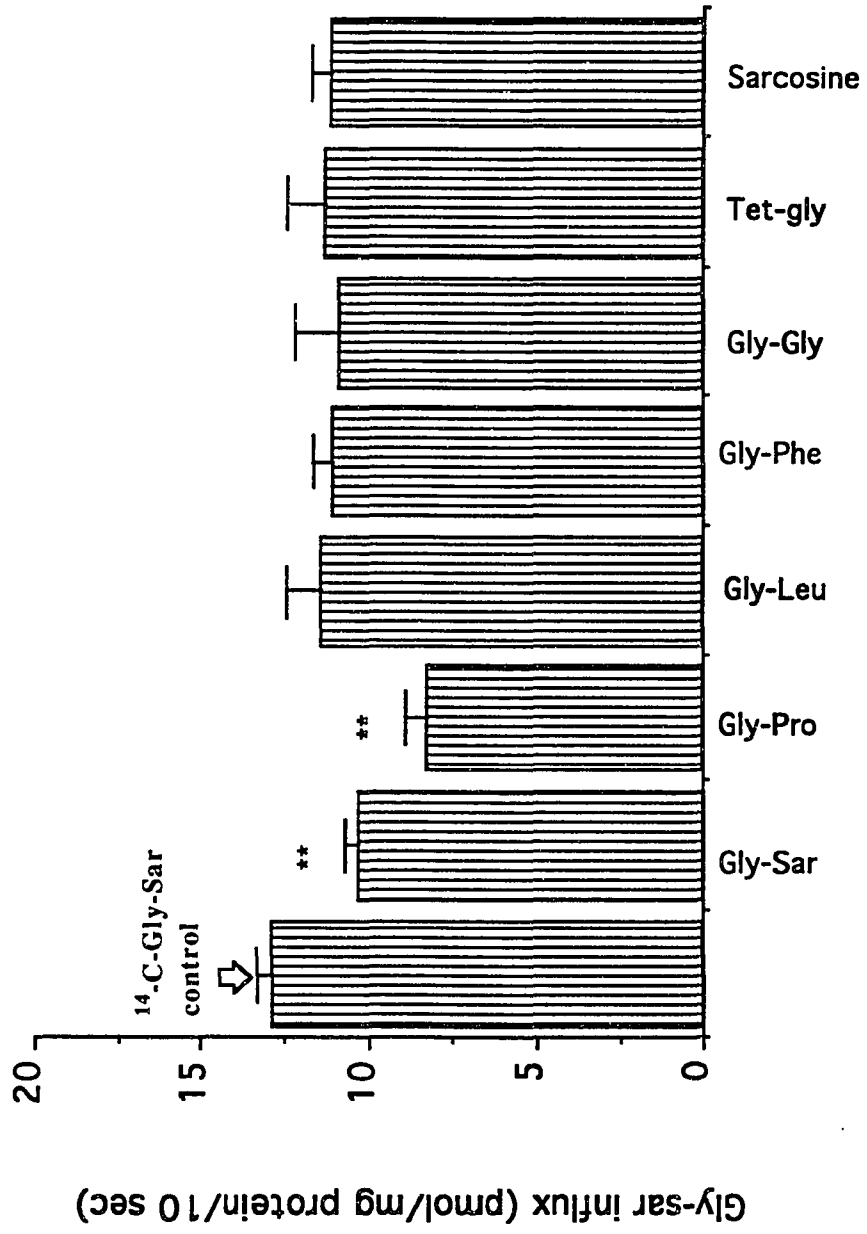


Figure 7. Trans-stimulation of  $^{14}\text{C}$ -Gly-Sar influx (10 sec) by the efflux of preloaded intravesicular 0.1 mM test substrates. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris at pH 7.5, 50  $\mu\text{M}$  valinomycin and any one of 0.1 mM test substrate. External media contained 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris, at pH 5.5 and 0.01 mM  $^{14}\text{C}$ -Gly-Sar.

7 TRANS-STIMULATION

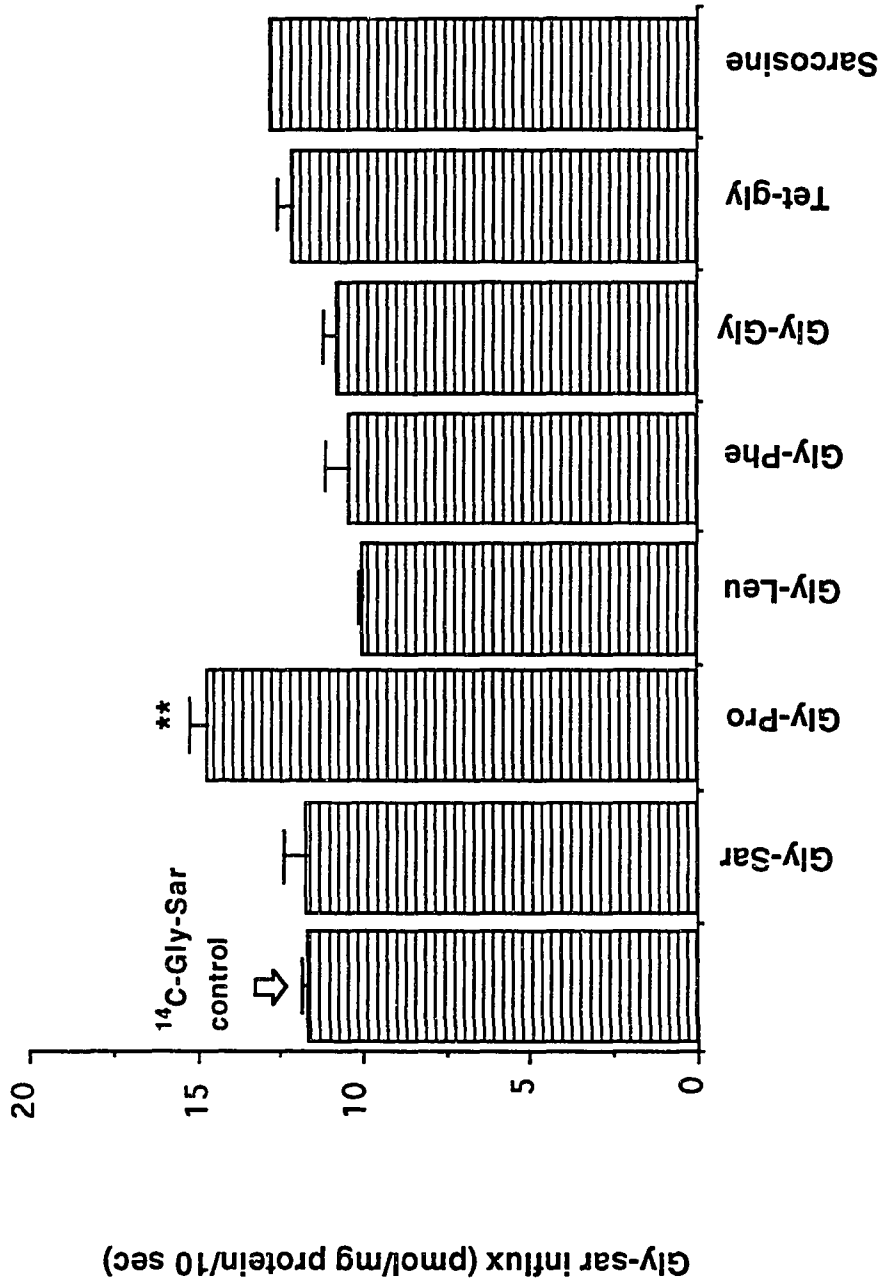


Figure 8. Dipeptide uptake model in fish gut. Model shown illustrates the three possible dipeptide uptake mechanisms present in BBMV of carnivorous and herbivorous fish gut epithelial cell: (1) A saturating, high affinity, low capacity, proton dependent peptide carrier, (2) A low affinity, high capacity, non-saturable (up to 10 mM), DEP sensitive, proton-dependent carrier, and (3) Simple diffusion. Absorbed dipeptides may be hydrolyzed to component amino acids within the cell or could leave the cell intact. Basolateral efflux mechanisms shown here are hypothetical, based on what is known in mammalian systems.

# M O D E L

## Di-peptide Transport Processes in teleost intestine

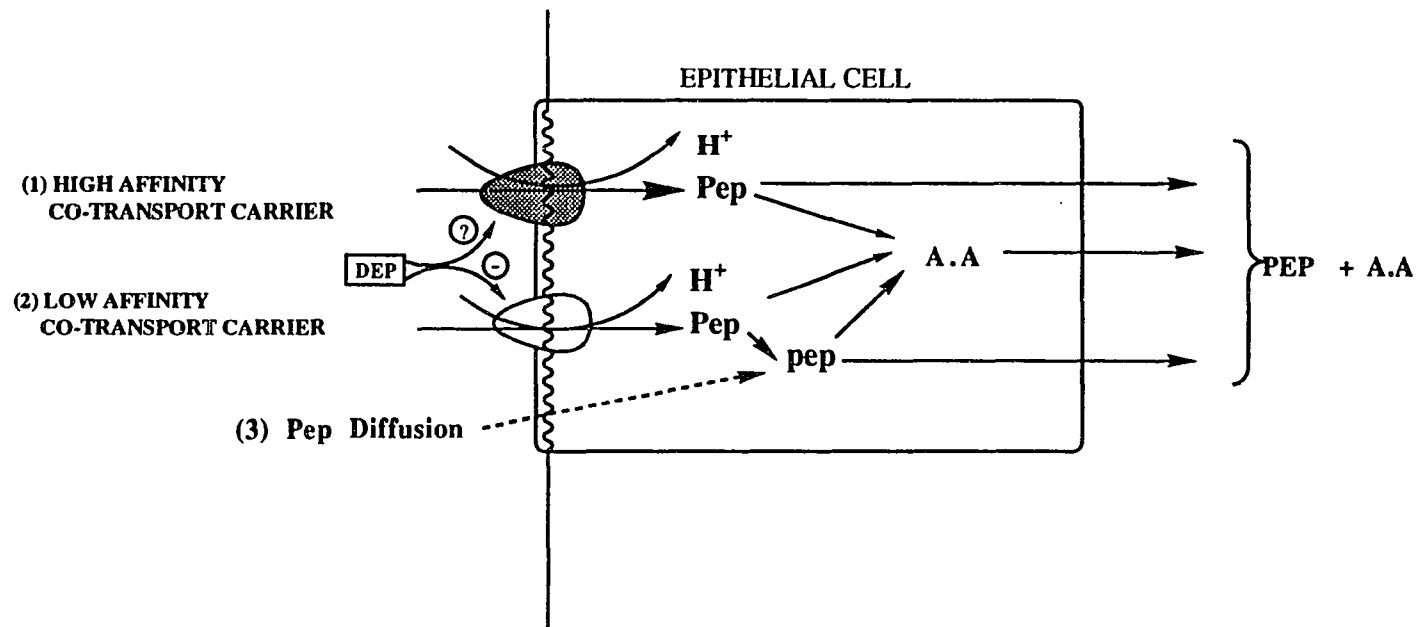


Table 1. Illustration of different peptide transport components and their relative contribution at various external peptide concentrations to total peptide uptake in tilapia gut epithelial cell BBMV. Total measured influx and calculated kinetic constant values (equation 1) were used to determine the percentage of total  $^{14}\text{C}$ -Gly-sar influx attributed to each transport component at a variety of external peptide concentrations.

Table: 1

<b>GLY-SAR CONCENTRATION (MM)</b>	<b>TOTAL %</b>	<b>DIFFUSION %</b>	<b>HIGH AFFINITY CARRIER %</b>	<b>LOW AFFINITY CARRIER %</b>
<b>0.001</b>	<b>100</b>	<b>6</b>	<b>44</b>	<b>50</b>
<b>0.01</b>	<b>100</b>	<b>6</b>	<b>44</b>	<b>50</b>
<b>0.02</b>	<b>100</b>	<b>6</b>	<b>44</b>	<b>50</b>
<b>0.04</b>	<b>100</b>	<b>6</b>	<b>42</b>	<b>52</b>
<b>0.05</b>	<b>100</b>	<b>6</b>	<b>42</b>	<b>52</b>
<b>0.1</b>	<b>100</b>	<b>6</b>	<b>40</b>	<b>52</b>
<b>1.0</b>	<b>100</b>	<b>8</b>	<b>22</b>	<b>70</b>
<b>5.0</b>	<b>100</b>	<b>9</b>	<b>10</b>	<b>81</b>

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## CHAPTER 3

BASOLATERAL DIPEPTIDE TRANSPORT BY THE INTESTINE  
OF A TELEOST FISH

## INTRODUCTION

Intestinal transepithelial transport of intact peptides *in vivo* has been demonstrated in rat (10), hamster (6), guinea-pig (14), ruminants (20) and man (9). These earlier studies showed that peptides were transported by systems different from that of free amino acids (4). Dipeptide transport is of greater quantitative significance than free amino acid transport during early growth (8). Intestinal perfusion studies showed, di- and tripeptide uptake was less inhibited than free amino acid uptake following starvation (18). This transcellular route of dipeptide transfer involved entry through the brush border membrane, passage through the cell and finally exit out across the basolateral membrane. Isolated membrane vesicles have been used widely as an *in vitro* model system for studying intestinal transport. Recent studies using membrane vesicles were concerned with the mode of dipeptide transport across the mucosal brush border membrane into cells. Brush border membrane vesicles (BBMV) isolated from mammalian small intestine have demonstrated that dipeptides can be transported intact by proton-gradient-energized transport systems (3). In teleost gut epithelia proton-gradient-energized, glycyl-glycine (19) and glycylsarcosine (15) transport, as well as cation independent, facilitated diffusion of glycyl-L-phenylalanine (11) have been demonstrated using intestinal BBMV.

Even though much evidence has accumulated describing brush border uptake mechanisms, very little is known about dipeptide transport through intestinal epithelial cells or across epithelial basolateral membranes. At present, no basolateral efflux studies have been reported for teleost gut and very few studies have been conducted with mammalian intestinal tissue, using either intestinal basolateral membrane vesicles (2) or Caco-2 cell monolayers (16, 17, 13).

For this reason, in this study we have used the biologically stable dipeptide,  $^{14}\text{C}$ -glycyl-sarcosine, to determine the characteristics of gastrointestinal basolateral peptide transport in the herbivorous teleost, tilapia (*Oreochromis mossambicus*).

## MATERIALS AND METHODS

### Collection and maintenance of animals

African tilapia (*Oreochromis mossambicus*) were obtained from the Marine and Research Training Center (M.R.T.C.) on Oahu, Hawaii and maintained in large holding tanks in the Department of Zoology at the University of Hawaii.

### Preparation of basolateral membrane vesicles

Fish were killed by a blow to the head and intestines removed for preparation of basolateral membrane vesicles (BLMV). In tilapia, the upper half of the intestine was used for preparation of BLMV. Vesicles were prepared using a modified percol gradient technique (1). Briefly, upper

intestinal tissue was washed twice in a buffer (PBS) containing: 136.8 mM NaCl, 2.68 mM KCl, 1.47 mM Na<sub>2</sub>HP0<sub>4</sub>, 0.5 mM phenylmethylsulphonyl fluoride (PMSF) adjusted to pH 7.4 with NaOH. Then 4-5 mg of scraped mucosa and pieces of intestine were put in a buffer (MSS) containing: 250 mM sucrose, 2 mM TRIS. HCl and 0.5 mM PMSF, and homogenized using a polytron for 2 minutes. The resulting suspension was centrifuged at 3,000 g for 15 minutes and the supernatant at 20,500 g for 20 minutes. The white fluffy pellet from the high speed spin was resuspended in 6 ml of MSS buffer solution. To this white fluffy suspension 4.2 ml of percol was added and the final volume brought to 35 ml with MSS buffer and centrifuged at 48,000 g for one hour. The resulting gradient was fractionated into 2 ml samples and three fractions (3,4 and 5) were collected. Combined fractions were resuspended in 35 ml of appropriate transport buffer and homogenized ten times using a Potter-Elvehjem homogenizer and centrifuged at 48,000 g for 1 hour. The purified membrane pellet was resuspended in sufficient transport buffer to provide 8-10 mg/ml. protein content. Protein content of the preparation was assessed with the Bio-Rad protein assay.

#### Transport measurements

Transport studies using intestinal BLMV were conducted at 23<sup>o</sup> C using the Millipore filtration technique (5). <sup>14</sup>C-Gly-Sar was provided by Dr. F. H. Leibach, Dept. of Cell and Molecular Biology, Medical College of Georgia. Long-term <sup>14</sup>C-Gly-Sar uptake experiments were initiated by mixing 20 ul membrane suspension with 180 ul radiolabeled incubation medium. Composition of the incubation medium varied with the nature of the experiments. Uptake of <sup>14</sup>C-Gly-Sar was

terminated by injecting 20 ul of reaction mixture into 2 ml of ice cold stop solution (same composition as the incubation medium without radiolabelled solute). This was then filtered onto a Millipore filter (0.65um) and washed with another 5 ml of ice-cold stop solution. Filters containing the washed vesicles were placed in scintillation cocktail and counted in a Beckman LS-8100 scintillation spectrometer.

Short-term influx experiments were initiated by mixing 5 ul of vesicle sample with 45 ul of radiolabeled incubation medium containing variable concentrations of unlabeled solute. Solute influx was terminated by injecting 20 ul of reaction mixture into 2 ml of ice-cold stop solution. This was then filtered, washed, and counted as described earlier. Carrier-mediated  $^{14}\text{C}$ -Gly-Sar influx kinetics were determined by a curve-fitting procedure in which influx data were computer fitted, using an iterative, nonlinear method to the Michaelis-Menten kinetic equation.

$$J_{oi} = \{ ( J_{\max} * [S] ) / ( K_t + [S] ) \} \quad (1)$$

In this equation  $J_{oi}$  was  $^{14}\text{C}$ -Gly-Sar influx in picomoles per milligram protein per 10 sec,  $[S]$  was external  $^{14}\text{C}$ -Gly-Sar concentration in mM;  $J_{\max}$  was maximal  $^{14}\text{C}$ -Gly-Sar influx;  $K_t$  was the concentration of S that yielded one half  $J_{\max}$ . All isotope transport values were corrected for a "vesicle blank" obtained by adding the incubation medium and vesicles directly to the respective stop solution, filtering onto Millipore filters, and counting. Each experiment was repeated using membrane vesicles prepared from different fish to confirm consistent experimental findings. Within a given experiment, each point represented the mean of 3 replicates and their standard error values. Significant

differences between values were determined by student's t test.

Specificity of the  $^{14}\text{C}$ -Gly-Sar carrier was assessed by measurement of the influx of 1 mM  $^{14}\text{C}$ -Gly-Sar in the presence of several external unlabelled dipeptides (cis inhibition) with an inwardly-directed hydrogen gradient.

## RESULTS

### Enzyme assays

Spectrophotometric assays of marker enzymes were used to establish the relative purity of the membrane preparation. Alkaline phosphatase activity was assayed at room temperature using Sigma kit 104. Na-K-stimulated adenosinetriphosphatase was measured by the coupled assay reaction. Table 2 shows enzyme activity and enrichment data for BLMV from tilapia upper intestine. In addition  $^3\text{H}$ -D-Glucose uptake by BLMV was blocked by Phloretin, but not by phloridzin (data not shown). These results suggest the presence of a highly purified basolateral preparation with minimal contamination by brush border membrane elements.

### Osmotic reactivity of BLMV

To confirm closure of BLBV prepared by this method and substantiate that dipeptide transport by these vesicles was into an osmotically reactive space, 60 minute equilibrium uptake of 1 mM  $^3\text{H}$ -D-Glucose was assessed at a series of transmembrane osmotic gradients. Vesicles were loaded with

300 mM mannitol, 20 mM Hepes/Tris at pH 7.5, 50  $\mu$ M valinomycin and incubated in external media containing 1mM  $^3\text{H-D-Glucose}$ , 100 NaCl, 100 mM mannitol, 0-800 mM sucrose and 20 mM Mes/Tris at pH 5.5. Fig. 9 indicates that uptake decreases as a linear function of the reciprocal of medium osmolarity, indicating that these vesicles exhibit osmotic reactivity. Extrapolation of this relationship to the vertical axis shows no equilibrium binding at infinite osmolarity.

Time course of  $^{14}\text{C-Gly-Sar}$  transport in herbivorous tilapia intestinal BBMV

Effect of a pH

In order to assess whether the transport of  $^{14}\text{C-Gly-Sar}$  was specifically activated by a proton gradient, uptake of 1 mM  $^{14}\text{C-Gly-Sar}$  was measured with vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either, 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 or 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Figure 10 illustrates the effects of a transmembrane proton gradient, on the time course of 1 mM  $^{14}\text{C-Gly-Sar}$  uptake by tilapia upper intestinal BLMV. Gly-Sar uptake was characterized by a slow, hyperbolic, time course and was higher when  $\text{pH}_i (7.5) > \text{pH}_o (5.5)$  than when  $\text{pH}_i = \text{pH}_o = 7.5$ . These data show that protons had a marked effect on the transport of this dipeptide.

To further assess whether a proton gradient or the absolute external proton concentration played a role in this



transport, uptake of 1 mM  $^{14}\text{C}$ -Gly-Sar was measured with vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 or 20 mM Mes/Tris at pH 5.5 and, 50  $\mu\text{M}$  valinomycin. Transport was initiated by exposing 20  $\mu\text{l}$  of vesicle suspension to 180  $\mu\text{l}$  of transport medium having either, 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5, 100 mM KCl or 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Fig. 11. shows that Gly-Sar uptake in tilapia was not proton gradient dependent, but rather dependent on the absolute external proton concentration and that internal BLMV proton concentration did not play a role in this uptake process. This observation was further assessed by lowering the external proton concentration in a step-wise fashion. Fig. 12. illustrates that lowering the external hydrogen ion concentration (pH 5.5 to 6.5 to 7.5) showed step-wise reduction in the uptake of this dipeptide. These data clearly show that (1) external pH had a marked effect on transport of this dipeptide and (2) absolute proton concentration and not the proton gradient was involved in the uptake of this dipeptide across the BLMV in tilapia gut.

#### Effect of a membrane potential

The effect of a  $\text{K}^+$ -generated diffusion potential (inside negative) on  $^{14}\text{C}$ -Gly-Sar transport was studied in the presence of an inwardly-directed hydrogen ion gradient. In this experiment vesicles were preloaded with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris at pH 7.5 and, 50  $\mu\text{M}$  valinomycin. Transport was initiated by exposing 20  $\mu\text{l}$  of vesicle suspension to 180  $\mu\text{l}$  of transport medium having either (1) 100 mM KCl, 100 mM mannitol or (2) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Imposing a membrane potential under these conditions

did not stimulate uptake of  $^{14}\text{C}$ -Gly-Sar in tilapia BLMV compared to the uptake in short-circuited conditions. This demonstrates electroneutral transport of this dipeptide by tilapia intestinal BLMV (Fig 13).

#### Effect of a sodium gradient

The effect of  $\text{Na}^+$  ions on the uptake of  $^{14}\text{C}$ -Gly-Sar in tilapia was studied in the presence of an inwardly-directed hydrogen ion gradient along with a  $\text{K}^+$  generated diffusion potential (inside negative) condition. In these experiments vesicles were preloaded with 100 mM KCl, 100 mM mannitol, 20 mM Hepes/Tris, at pH 7.5, and, 50  $\mu\text{M}$  valinomycin. Transport was initiated by exposing 20  $\mu\text{l}$  of vesicle suspension to 180  $\mu\text{l}$  of transport medium having either (1) 100 mM KCl, 100 mM mannitol; (2) 100 mM choline chloride, 100 mM mannitol; or (3) 100 mM sodium chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5. Addition of a 100 mM inwardly-directed  $\text{Na}^+$  gradient along with an outwardly-directed  $\text{K}^+$  generated diffusion potential (inside negative) did not stimulate  $^{14}\text{C}$ -Gly-Sar uptake over that induced by a  $\text{K}^+$  generated diffusion potential (inside negative) alone. These results suggest that  $^{14}\text{C}$ -Gly-Sar uptake in tilapia BLMV was  $\text{Na}^+$  independent. (Fig 14)

#### Kinetics of $^{14}\text{C}$ -Gly-Sar influx in tilapia intestinal BBMV

Apparent influx of  $^{14}\text{C}$ -Gly-Sar into tilapia intestinal BLMV was measured over a concentration range of 0.5 mM to 10 mM in the presence of an inwardly-directed hydrogen ion gradient ( $\text{pH}_{\text{in}}$  7.5/  $\text{pH}_{\text{out}}$  5.5) for 6 sec at  $23^\circ\text{C}$  to simulate normal physiological cell conditions of the intact animal. Membrane vesicles were pre-loaded with 100 mM mannitol,

100 mM KCl and 20 mM Hepes/Tris at pH 7.5 and, 50  $\mu$ M valinomycin. Influxes for this concentration range are shown in Fig. 6 and were used in equation (1) to generate transport kinetic constants. This analysis yielded the following apparent Michaelis-Menten values for a saturable, low affinity system:  $K_t = 14.55 \pm 1.37$  mM,  $J_{max} = 4472 \pm 2.8$  pmol/mg protein/sec. Lowering extravesicular pH to 7.5 had marked effect on both apparent affinity and capacity of the transporter,  $K_t = 26.90 \pm 9.35$  mM,  $J_{max} = 1755 \pm 40$  pmol/ mg protein/ sec (lower line, Fig 15).

#### Specificity of the $^{14}$ C-Gly-Sar carrier in tilapia gut

In order to investigate whether other dipeptides could inhibit the influx of  $^{14}$ C-Gly-Sar, entry of the labelled substrate was measured in the presence of several unlabelled dipeptides. In these studies membrane vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris at pH 7.5 and, 50  $\mu$ M valinomycin. Influx of 1 mM  $^{14}$ C-Gly-Sar into tilapia BLMV was measured with an inwardly-directed hydrogen ion gradient for 6 sec at 23 $^{\circ}$  C in the presence of 10 mM test peptide concentration. Results showed a significant ( $p < 0.01$ ) inhibition of Gly-Sar influx by other dipeptides employed in this experiment (Fig. 16).

DEP (diethylpyrocarbonate) has been shown to inhibit proton-coupled dipeptide transport in intestinal BBMV of rabbit (7), eel (19) and tilapia (15). In order to assess the effect of DEP on  $^{14}$ C-Gly-Sar influx in tilapia intestinal BLMV, a 10 mM concentration of DEP was used in uptake studies. In this experiment membrane vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris at pH 7.5, 50  $\mu$ M valinomycin and incubated with 10 mM

DEP for 10 minutes, washed and resuspended in inside buffer. In the presence of 10 mM DEP, total  $^{14}\text{C}$ -Gly-Sar influx was not significantly different from that of untreated vesicles (Fig. 16).

## DISCUSSION

The results of this study document for the first time, the presence, mechanism and driving forces for dipeptide (glycylsarcosine) transport in fish gastrointestinal BLMV. There has previously been only one study using basolateral membrane vesicles to study dipeptide uptake (2). In this study it was shown that Gly-Pro transport in BLMV was coupled to protons. But this study failed to compare uptake under proton gradient ( $\text{pH}_{\text{in}} 7.5 / \text{pH}_{\text{out}} 5.5$ ) and equal proton conditions ( $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 5.5$ ). Another dipeptide study using caco-2 cell monolayers reported that pH dependence of the apical and basolateral transporters were different (13). These authors cited Dyer's (1990) work (2) and claimed that further studies were needed to conclude whether the basolateral dipeptide transporter was independent of the proton gradient. In our study we further tested this phenomenon and found that in tilapia BLMV  $^{14}\text{C}$ -Gly-Sar transport was not activated by a proton gradient, but rather activated by absolute proton concentration and occurred in an electroneutral fashion.

In tilapia  $^{14}\text{C}$ -Gly-Sar uptake by intestinal BBMV appeared to be mediated by a carrier system. This carrier was characterized as a low affinity, high capacity type showing the following kinetic constants:  $K_t = 15\text{mM}$ ,  $J_{\text{max}} = 4472 \text{ pmol/ mg protein/ sec}$ . Lowering the extravascular

proton concentration (pH 7.5), had a marked effect on the transport parameters of the carrier ( $K_t = 27$  mM,  $J_{max} = 1755$  pmol/ mg protein/ sec). These results further confirm that proton concentration directly influences basolateral carrier affinity and maximal transport capacity of this dipeptide in tilapia BLMV.

In intestinal cells proton-coupled dipeptide absorption could result in intracellular acidification. This acidification might result in activation of the basolateral dipeptide transporter, resulting in efficient transport of dipeptides across the basolateral membrane into blood thus maintaining a luminal-cytoplasm dipeptide gradient for further uptake. Our data showed that  $^{14}\text{C}$ -Gly-Sar transport in tilapia BLMV was activated by absolute proton concentration. Previously, brush border influx of protons coupled to dipeptides (glycylglycine) has been shown to acidify mammalian enterocytes (12). It is possible that this cellular acidification can enhance efflux, by activating the basolateral carrier. Electroneutrality observed during transport and the fact that Gly-Sar exists predominantly as a dipolar zwitterion with zero net charge in the pH range tested, further supports proton activation rather than proton coupled transport of this dipeptide by BLMV. In addition this activation showed a step-wise decrease with lowering of extravesicular proton concentration under equal internal and external vesicle proton concentrations.

Cis-inhibition experiments show that the basolateral carrier exhibited a broad specificity, accepting a wide variety of peptides. A similar broad specificity basolateral dipeptide transporter has recently been demonstrated in Caco-2 cells (13).

Results of this study and our brush border study (15) lead to a model for transcellular dipeptide transport in teleost intestinal epithelium (Fig 17). In teleosts brush border uptake of dipeptides into the intestinal epithelial cells occurs by specialized transport systems with relatively high specificity (15). There are at least two dipeptide transport systems present in teleost intestinal epithelial brush border: (1) A proton-dependent, high affinity, low capacity, saturable carrier system; and (2) A proton-dependent, low affinity, high capacity, non-saturable, carrier system. These two systems apparently contribute to more than 90% of total dipeptide uptake in teleost gut. The basolateral transporter in tilapia gut epithelial cells was distinctly different from either apical transporter. This transport process showed: (1) activation, not energization by protons, (2) electroneutral transport of dipeptides across the basolateral membrane, (3) broad specificity, (4) low affinity, and (5) high capacity.

At present, no one has attempted to see whether the presence of plasma peptides differ between healthy and diseased animals or whether dietary derived-peptides are responsible for pathological symptoms. The major question is whether this mode of protein digestion and absorption plays a major role in the well-being of an animal. Our results show clear evidence for absorption and transport of intact dipeptides to the blood in teleosts. The importance of this transport system in fish nutrition remains to be investigated.

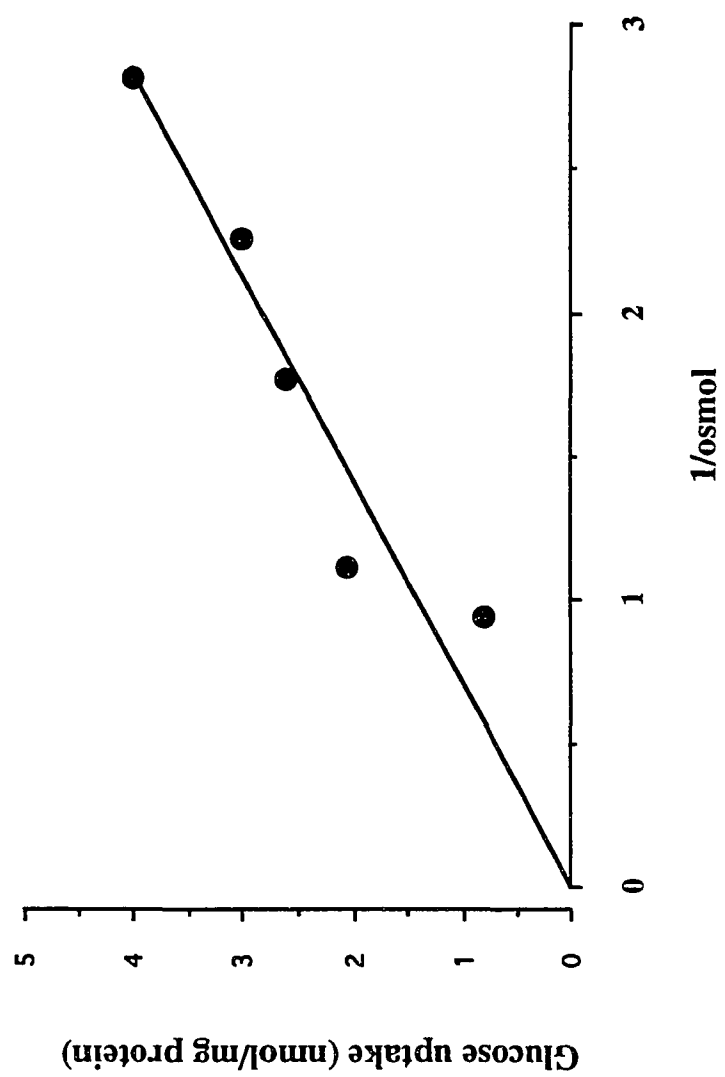
**Table 1. Enzymatic Characterization of tilapia (Oreochromis mossambicus)  
Intestinal basolateral membrane vesicles**

<b>Enzyme</b>	<b>Activity of the homogenate</b>	<b>Activity of the BLMV</b>	<b>Purification factor</b>	<b>Enzyme recovery %</b>
<b>Na/K-ATPase</b>	<b>1.63±0.24</b>	<b>19.8±1.86</b>	<b>12.1±0.8</b>	<b>21</b>
<b>Alkaline Phosphatase</b>	<b>19.9±0.48</b>	<b>14.46±0.73</b>	<b>0.8±0.3</b>	<b>1.5</b>

Values are mean ± SE. Enzyme activities are in  $\mu\text{mol/ mg protein/ hour}$ . Purification factors are calculated from the mean enzyme activities of the homogenate and vesicles. Enzyme measurements were performed on three different membrane preparations.

Figure 9. Osmotic reactivity of basolateral membrane vesicles. Vesicles were loaded with 300 mM mannitol, 20 mM Hepes/Tris at pH 7.5, 50  $\mu$ M valinomycin and incubated in external media containing 1mM  $^3$ H-D-Glucose, 100 NaCl, 100 mM mannitol, 0-800 mM sucrose and 20 mM Mes/Tris at pH 5.5.





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Figure 10. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into intestinal BLMV of tilapia. Effect of proton gradient. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5, or (2) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5

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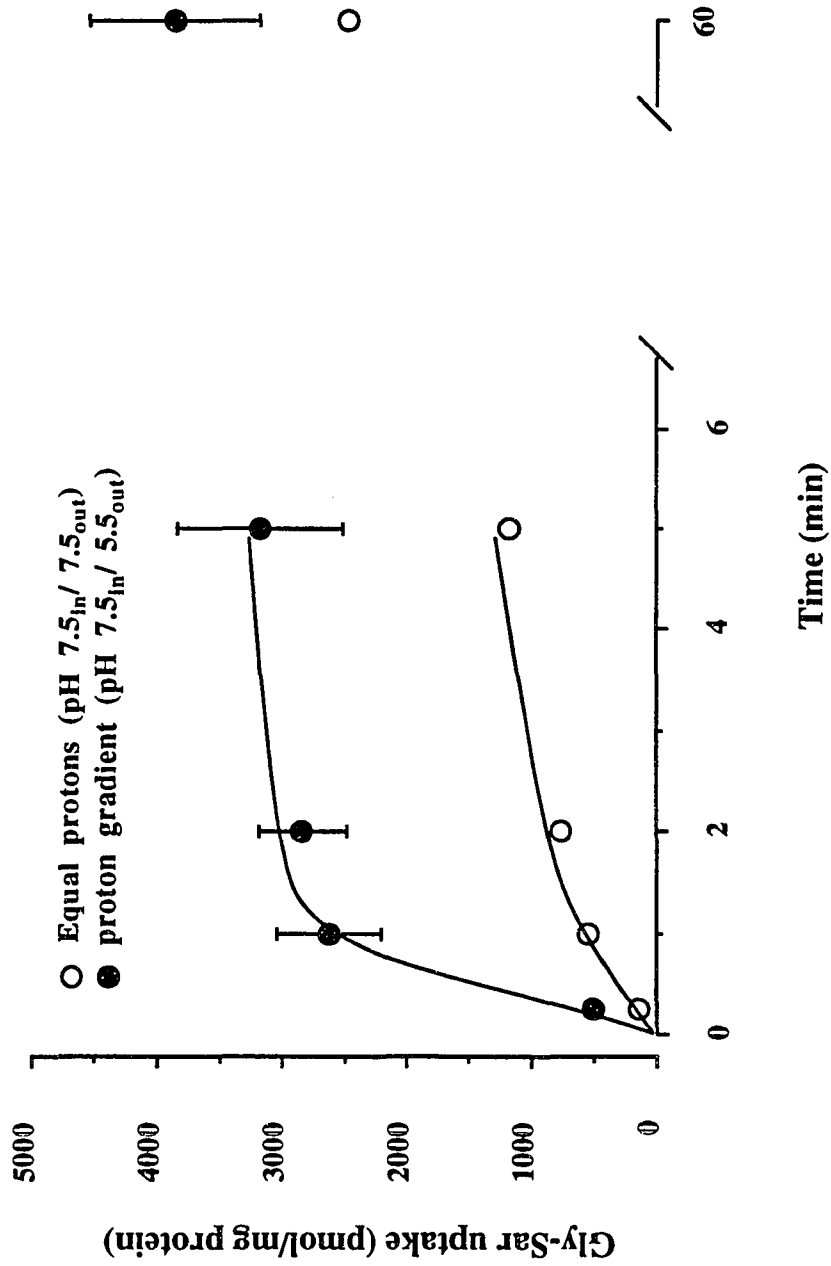
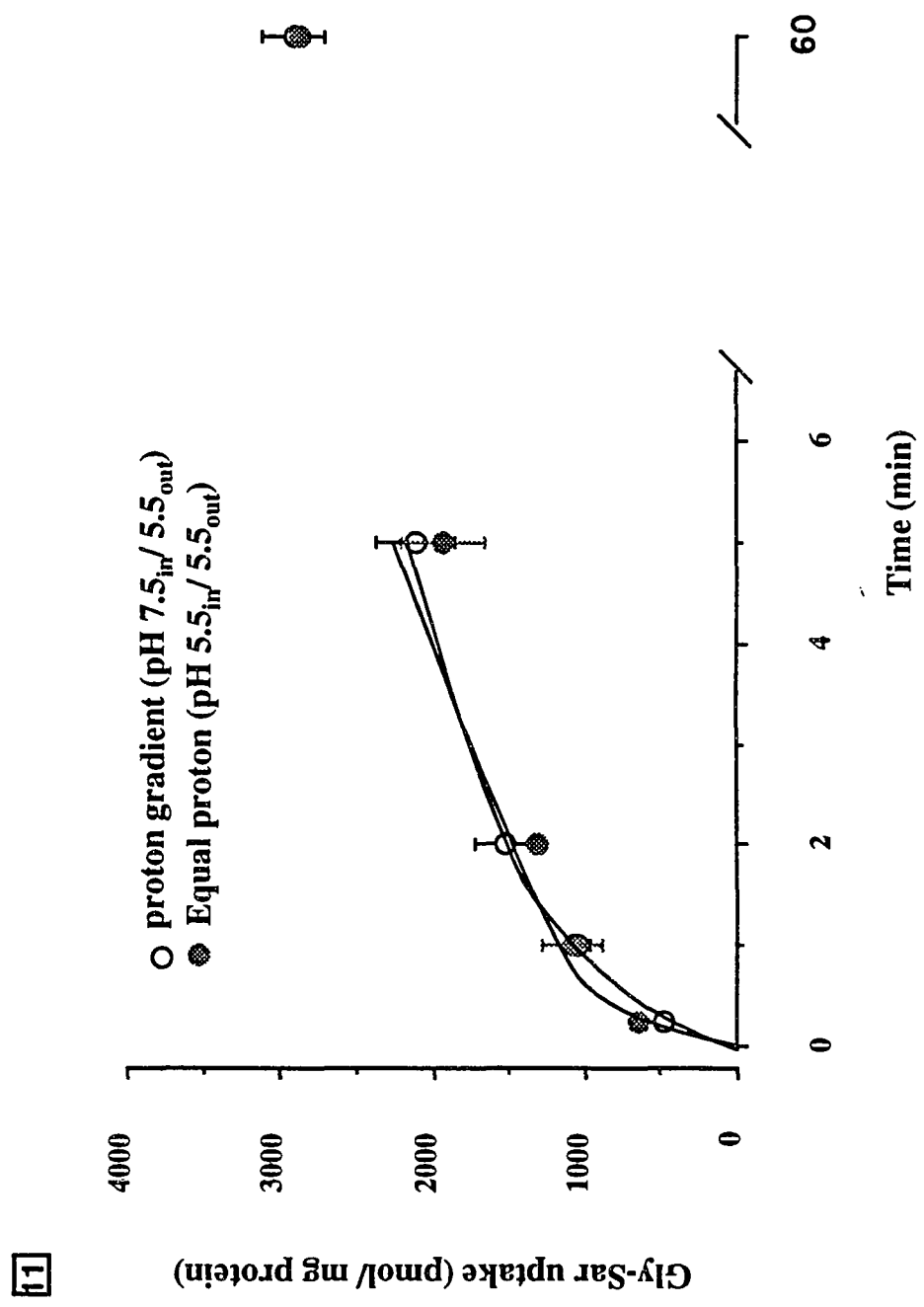


Figure 11. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into intestinal BLMV of tilapia. Effect of external proton concentration. Vesicles preloaded with 100 mM KCl, 100 mM mannitol having either: 1) 20 mM Hepes/Tris at pH 7.5, or 2) 20 mM Mes/Tris at pH 5.5 and 50  $\mu\text{M}$  valinomycin. Outside media was 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5



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Figure 12. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into intestinal BLMV of tilapia. Effect of different external proton concentration. Vesicles preloaded with 100 mM KCl, 100 mM mannitol having either 1) 20 mM Hepes/Tris at pH 7.5, 2) 20 mM Mes/Tris at pH 6.5 or 3) 20 mM Mes/Tris at pH 5.5 and 50  $\mu\text{M}$  valinomycin. Outside media was identical to that of internal media.

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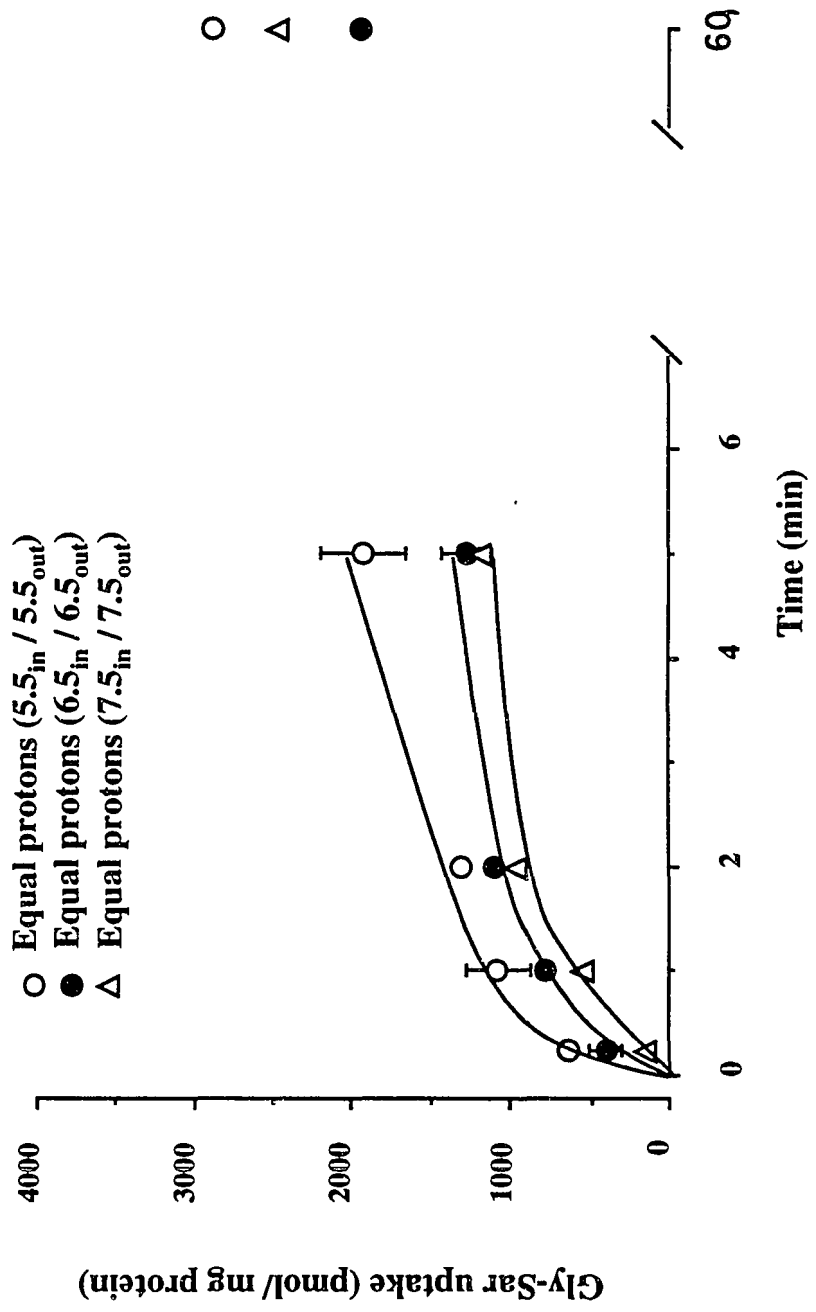


Figure 13. Effect of membrane potential. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50 uM valinomycin. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (2) 100 mM choline chloride, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5.



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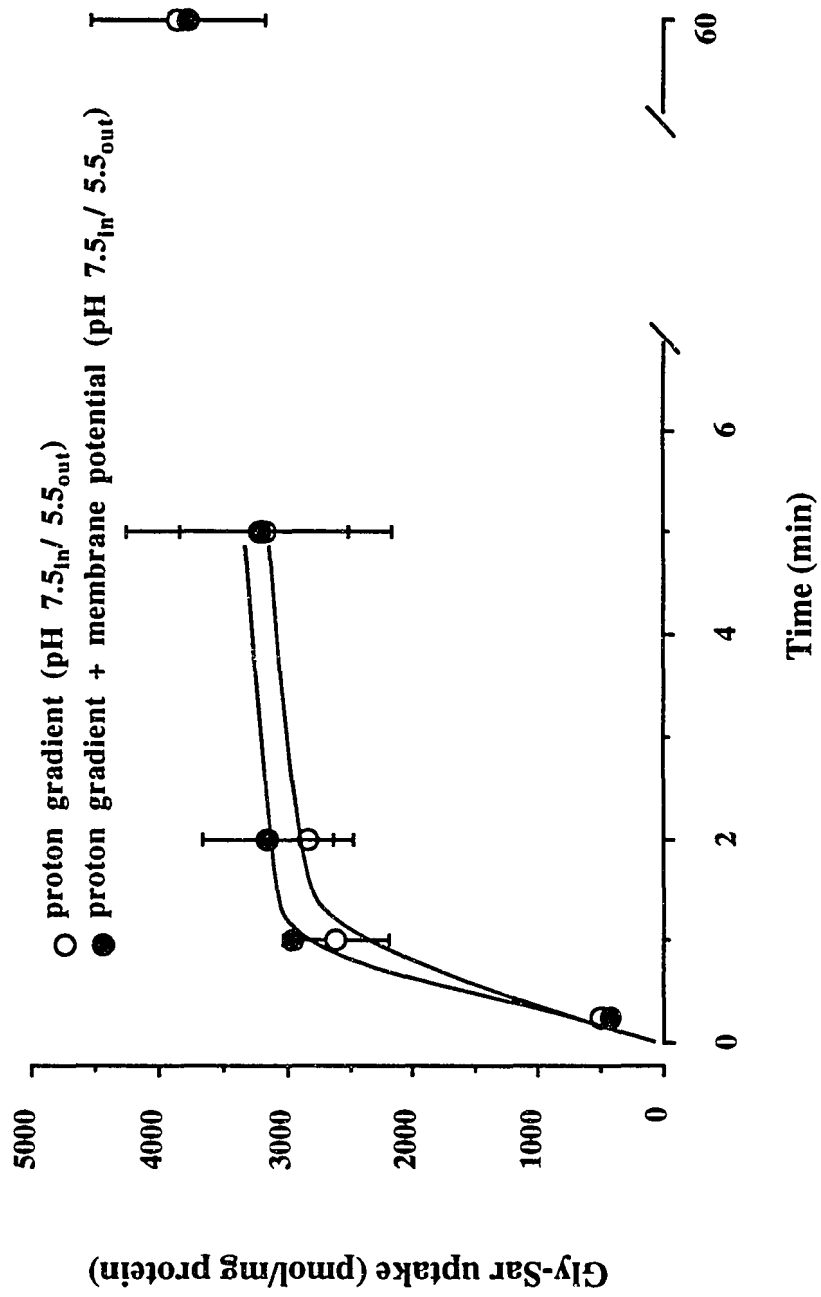


Figure 14. Effect Na<sup>+</sup> gradient. Vesicles preloaded with 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50 uM valinomycin. Outside media were: (1) 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (2) 100 mM mannitol, 100 mM NaCl and 20 mM Mes/Tris, at pH 5.5.

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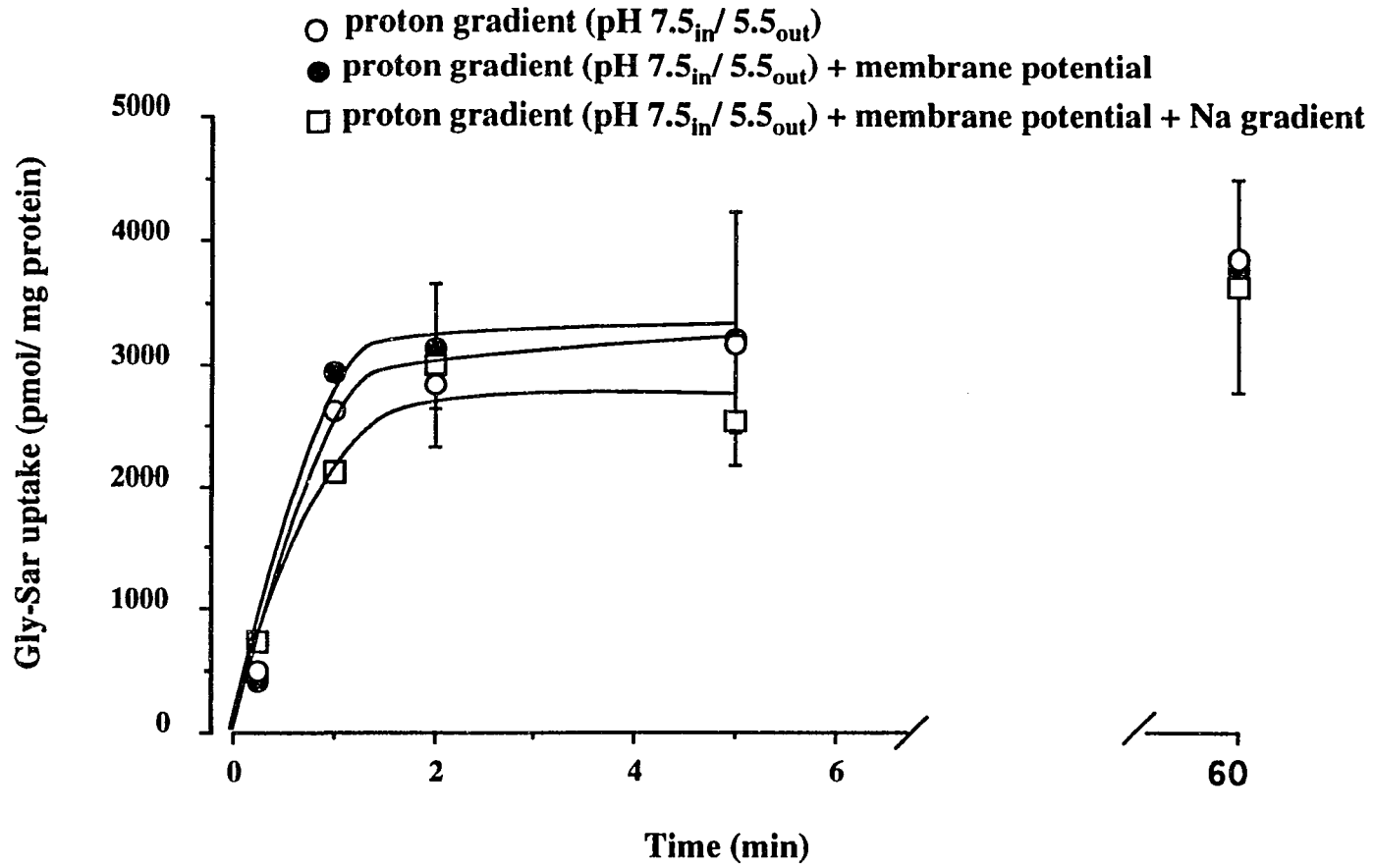
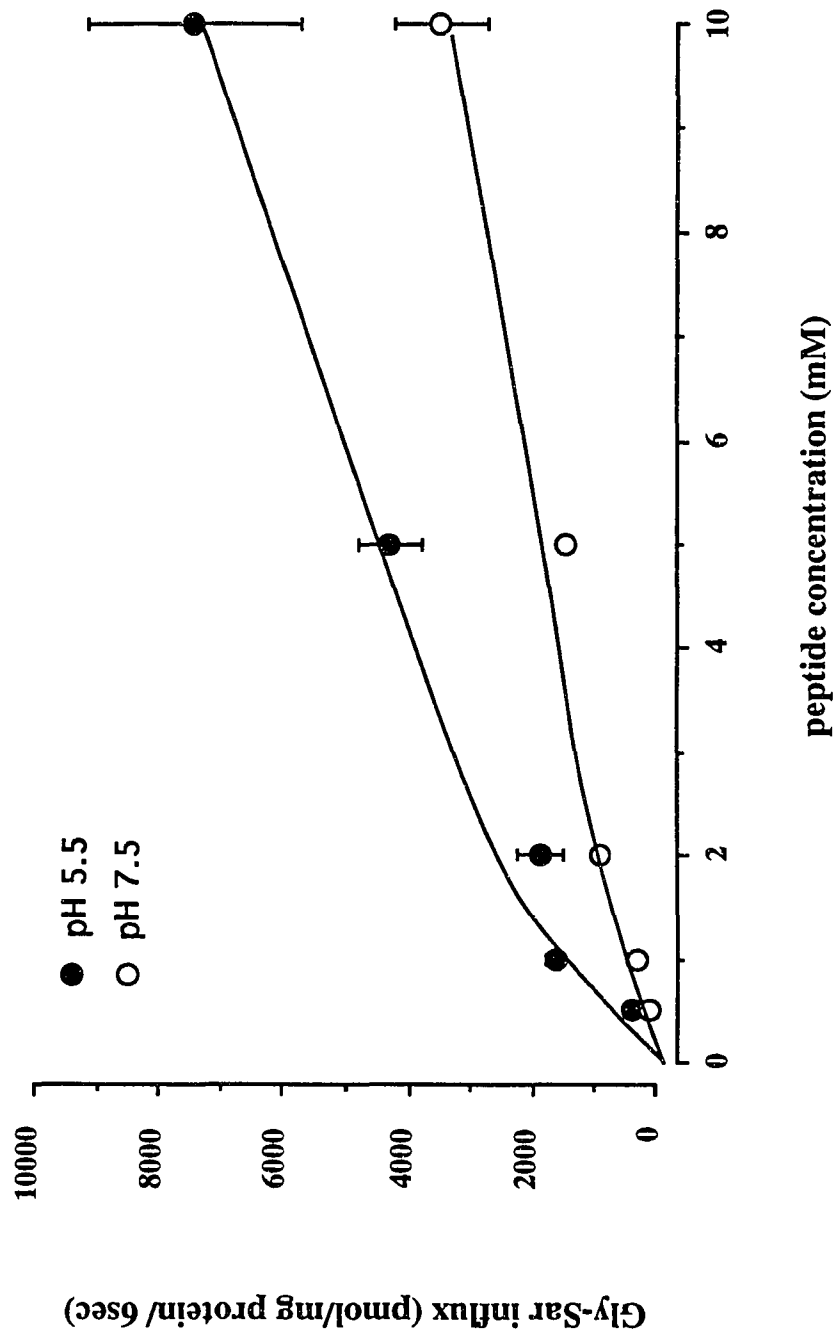


Figure 15. Uptake of  $^{14}\text{C}$ -Gly-Sar into tilapia BLMV as a function of substrate concentration. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM Hepes/ Tris, at pH 7.5 and 50  $\mu\text{M}$  valinomycin. External media either had 100 mM KCl and 20 mM Hepes/Tris, at pH 7.5, or 100 mM KCl, 100 mM mannitol and 20 mM Mes/Tris, at pH 5.5, with  $^{14}\text{C}$ -Gly-Sar concentration ranging from 0.5 to 10 mM. Data for total dipeptide influx in the different conditions were used in equation (1) to generate the respective transport kinetic constants under these conditions



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Figure 16. Effect of 10 mM external substrates on 1 mM  $^{14}\text{C}$ -Gly-Sar influx (6 sec) into tilapia BLMV. Vesicles were pre-loaded with 100 mM mannitol, 100 mM KCl and 20 mM HEPES/ Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. External media had 100 mM KCl, 100 mM mannitol and 20 mM MES/Tris, at pH 5.5 and 10 mM respective test substrate.

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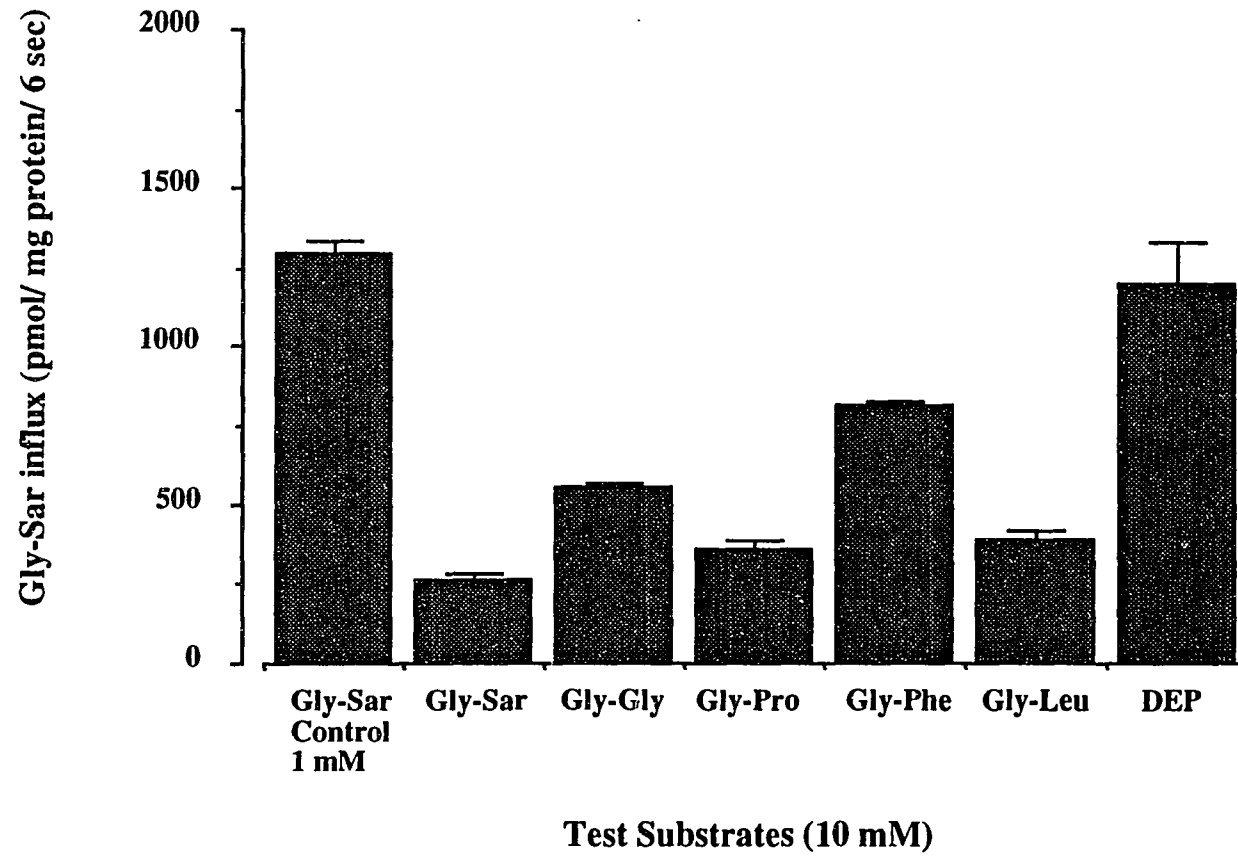


Figure 17. Transcellular Dipeptide transport model in fish gut. Model shown illustrates the three possible dipeptide uptake mechanisms present in BBMV of herbivorous fish gut epithelial cell: (1) A saturating, high affinity, low capacity, proton dependent peptide carrier, (2) A low affinity, high capacity, non-saturable (up to 10 mM), DEP sensitive, proton-dependent carrier, and (3) Simple diffusion (15).

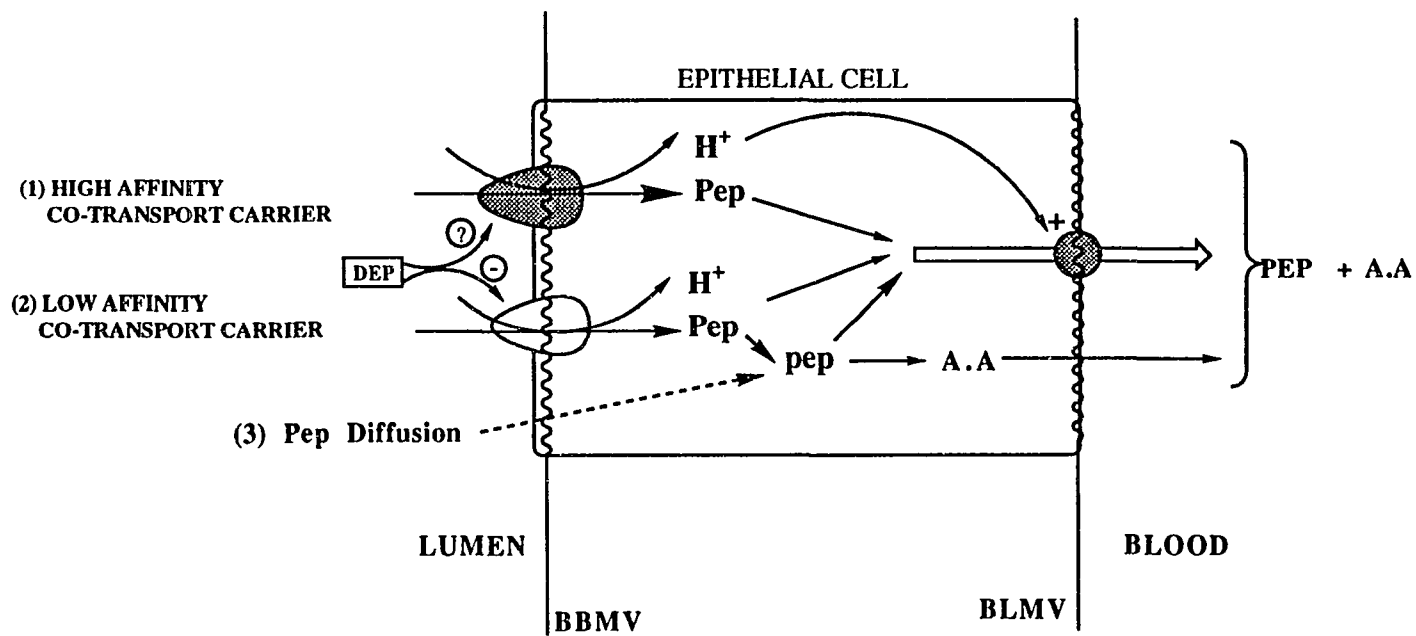
Absorbed dipeptides may be hydrolyzed to component amino acids within the cell or could leave the cell intact. A basolateral dipeptide transporter in tilapia gut epithelial cells is distinctly different from the apical transporter. This basolateral carrier process exhibits a broad specificity and illustrates: (1) activation by protons, (2) electroneutral transport, and (3) low affinity, high capacity transport kinetics.



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# MODEL

## Di-peptide Transport Processes in teleost intestine



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## CHAPTER 4

DIPEPTIDE TRANSPORT BY CRUSTACEAN  
HEPATOPANCREAS

## INTRODUCTION

In crustaceans, the hepatopancreas is a large branching gut diverticulum involved in the absorption of nutrients. Recently, a number of studies focused on the characterization of sugar (1) amino acid (3) and vitamin (13) transport by isolated hepatopancreatic brush border membrane vesicles (BBMV) of the lobster (*Homarus americanus*). These studies indicate that lobster hepatopancreatic BBMV possess at least seven distinct transport proteins for nutrient transport (4). They also point out that, hepatopancreatic brush border membrane transporter properties are modified, compared to those of the vertebrate system, to accommodate the very low pH occurring at the absorption site of the gut(2, 9).

A proton-gradient-energized co-transport of di- and tripeptides has been shown to be present in the intestine of mammals (7) and teleosts (15, 17). Advances continue rapidly in the field of dipeptide absorption and its probable contribution to the well-being of the animal (8). But, so far, the characteristics of proton coupled dipeptide transport systems in the crustacean hepatopancreas, where a 3 pH unit proton gradient (pH 4 to 7) normally exists from lumen to the cell, have remained unexplored.

For this reason in the present study we used  $^{14}\text{C}$ -glycyl-sarcosine ( $^{14}\text{C}$ -Gly-Sar), which is generally very resistant to hydrolysis and appears intact in mammalian (6) and teleost (15) intestinal tissues following absorption, to determine the characteristics of hepatopancreatic dipeptide transport in lobsters (*Homarus americanus*).

## MATERIALS AND METHODS

### Collection and maintenance of animals

Live Atlantic lobsters (*Homarus americanus*) were purchased from commercial dealers in Hawaii and maintained in sea water at 10° C.

### Preparation of brush-border membrane vesicles

Hepatopancreatic brush border membrane vesicles (BBMV) were prepared from fresh tissue removed from lobsters using a magnesium-precipitation technique described previously (1). Briefly, the tissue was homogenized in a solution containing 60 mM D-mannitol having 12 mM (Tris) HCl, 10 mM EGTA and 0.1 mM PMSF (buffer 1). The suspension was centrifuged at 27,000 g for 30 minutes. The resulting pellet was re-suspended in 250 ml of the above buffer solution using a Potter-Elvehjem homogenizer and magnesium chloride was added and mixed with the homogenate to a final concentration of 10 mM. The resulting solution was allowed to stand on ice for 15 minutes (step 1). The suspension was centrifuged at 3,000 g for 15 minutes and the supernatant at 27,000 g for 30 minutes (step 2). The pellet from the high speed spin was re-suspended in 35 ml of the above buffer

solution using a Potter-Elvehjem homogenizer. Step 1 and step 2 were repeated on this homogenate and the resulting pellet was re-suspended with the Potter-Elvehjem homogenizer in 10 ml of ice-cold transport buffer of appropriate composition. The final suspension was centrifuged at 27,000 g for 30 minutes. The purified membrane pellet was re-suspended in sufficient transport buffer to provide 6-8 mg/ml. protein content. Protein content of the preparation was assessed with the Bio-Rad protein assay.

#### Transport measurements

Transport studies using hepatopancreas BBMV were conducted at 15°C using the Millipore filtration technique (10).  $^{14}\text{C}$ -Gly-Sar was provided by Dr. F. H. Leibach, Dept. of Cell and Molecular Biology, Medical College of Georgia. Long-term  $^{14}\text{C}$ -Gly-Sar uptake experiments were initiated by mixing 20 ul membrane suspension with 180 ul radiolabeled incubation medium. Composition of the incubation medium varied with the nature of the experiments. Uptake of  $^{14}\text{C}$ -Gly-Sar was terminated by injecting 20 ul of reaction mixture into 2 ml of ice cold stop solution (same composition as the incubation medium without radiolabelled solute). This was then filtered onto a Millipore filter (0.65um) and washed with another 5 ml of ice-cold stop solution. Filters containing the washed vesicles were placed in scintillation cocktail and counted in a Beckman LS-8100 scintillation spectrometer.

Short-term influx experiments were initiated by mixing 5 ul of vesicle sample with 45 ul of radiolabeled incubation medium containing variable concentrations of unlabeled solute. Solute influx was terminated by injecting 20 ul of

reaction mixture into 2 ml of ice-cold stop solution. This was then filtered, washed, and counted as described earlier. Carrier-mediated  $^{14}\text{C}$ -Gly-Sar influx kinetics were determined by a curve-fitting procedure where, the individual influx values for all replicates were computer fitted, using an iterative, nonlinear method to the Michaelis-Menten kinetic equation:

$$J_{oi} = ( J_{\max} * [S] ) / ( K_t + [S] ) \quad (1)$$

In this equation  $J_{oi}$  was  $^{14}\text{C}$ -Gly-Sar influx in picomoles per milligram protein per 10 sec,  $[S]$  was external  $^{14}\text{C}$ -Gly-Sar concentration in mM;  $J_{\max}$  was maximal  $^{14}\text{C}$ -Gly-Sar influx; and  $K_t$  was the concentration of S that yielded one half  $J_{\max}$ . All isotope transport values were corrected for a "vesicle blank" obtained by adding the incubation medium and vesicles directly to the respective stop solution, filtering onto Millipore filters, and counting. Each experiment was repeated using membrane vesicles prepared from different lobsters to confirm consistent experimental findings. Within a given experiment, each point represented the mean of 3 replicates and their standard error values. Significant differences between values were determined by student's t test.

Specificity of the  $^{14}\text{C}$ -Gly-Sar carrier was assessed by measurement of the influx of 0.1 mM  $^{14}\text{C}$ -Gly-Sar in the presence of 1.0 mM concentration of several external unlabelled dipeptides (cis inhibition). All experiments employed an inwardly-directed hydrogen ion gradient.



## RESULTS

### Osmotic reactivity of BBMV

To substantiate that dipeptide transport by these vesicles was into an osmotically reactive space, 60 minute equilibrium uptake of 0.1 mM  $^{14}\text{C}$ -Gly-Sar was assessed at a series of transmembrane osmotic gradients. Vesicles were loaded with 200 mM mannitol, 100 mM KCl 20 mM Hepes/Tris at pH 8.5, 50 uM valinomycin and incubated in external media containing 0.1 mM  $^{14}\text{C}$ -Gly-Sar, 200 mM mannitol, 100 mM KCl 20 mM Mes/Tris at pH 5.5 and 0-600 mM sucrose. Fig. 18 indicates that uptake decreases as a linear function of the reciprocal of medium osmolarity, indicating that these vesicles exhibit osmotic reactivity. Extrapolation of this relationship to the vertical axis shows no equilibrium binding at infinite osmolarity.

### Time course of $^{14}\text{C}$ -Gly-Sar uptake in lobster hepatopancreas BBMV

#### Effect of a pH gradient

In order to assess whether the transport of  $^{14}\text{C}$ -Gly-Sar was specifically activated by a proton gradient, uptake of 0.1 mM  $^{14}\text{C}$ -Gly-Sar was measured with vesicles preloaded with 100 mM KCl, 200 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50 uM valinomycin. Figure 19 illustrates the effects of transmembrane hydrogen ion gradient on the time course of 0.1 mM  $^{14}\text{C}$ -Gly-Sar uptake by hepatopancreas BBMV. Gly-Sar uptake was characterized by a slow, hyperbolic, time course and was higher when  $\text{pH}_i$  (7.5) >  $\text{pH}_o$  (5.5) than when  $\text{pH}_i = \text{pH}_o = 7.5$ . These data show that external pH had a marked effect

on transport of this dipeptide and maximal peptide transport was attained with an inwardly-directed hydrogen ion gradient.

#### Effect of a membrane potential

The effect of a  $K^+$ -generated diffusion potential (inside negative) on 0.1 mM  $^{14}C$ -Gly-Sar transport was studied in the presence of an inwardly-directed hydrogen ion gradient. In this experiment vesicles were preloaded with 100 mM KCl, 200 mM mannitol, 20 mM Hepes/Tris at pH 7.5 and, 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 200 mM mannitol or (2) 100 mM choline chloride, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5. Imposing a membrane potential under these conditions resulted in increased uptake of  $^{14}C$ -Gly-Sar into hepatopancreas BBMV compared to the uptake in short-circuited conditions (Fig 20).

#### Effect of a sodium gradient

The effect of  $Na^+$  ions on the uptake of 0.1 mM  $^{14}C$ -Gly-Sar by hepatopancreatic BBMV was studied in the presence of an inwardly-directed hydrogen ion gradient along with a  $K^+$  generated diffusion potential (inside negative). In these experiments vesicles were preloaded with 100 mM KCl, 200 mM mannitol, 20 mM Hepes/Tris, at pH 8.5, and, 50  $\mu$ M valinomycin. Transport was initiated by exposing 20  $\mu$ l of vesicle suspension to 180  $\mu$ l of transport medium having either (1) 100 mM KCl, 200 mM mannitol; (2) 100 mM choline chloride, 200 mM mannitol; or (3) 100 mM sodium chloride, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5. Addition of a 100 mM inwardly-directed  $Na^+$  gradient along with an

outwardly-directed  $K^+$  generated diffusion potential (inside negative) inhibited membrane potential dependent  $^{14}C$ -Gly-Sar uptake. This reduced  $H^+$ /peptide uptake was possibly due to shorting out of diffusion potential by  $Na^+$  entry. These results suggest that  $^{14}C$ -Gly-Sar uptake in hepatopancreas BBMV is  $Na^+$  independent (Fig 21).

#### Kinetics of $^{14}C$ -Gly-Sar influx in hepatopancreas BBMV

Influx of  $^{14}C$ -Gly-Sar into hepatopancreas BBMV was measured over a concentration range of 0.05 mM to 20 mM in the presence of an inwardly-directed hydrogen ion gradient ( $pH_{in}$  8.5/  $pH_{out}$  5.5) for 10 sec at  $15^{\circ}C$ . Membrane vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM HEPES/Tris at pH 8.5 and 50  $\mu M$  valinomycin. Data for total dipeptide influx were used in equation (1) to generate transport kinetic constants for a Gly-Sar carrier system. This analysis yielded the following Michaelis-Menten values for a saturable, carrier mediated transport system:  $K_t = 5.99 \pm 0.42$  mM, (b)  $J_{max} = 5006 \pm 148.7$  pmol/ mg protein/ 10 sec (Fig 22).

Specificity of the  $^{14}C$ -Gly-Sar carrier in lobster hepatopancreas.

#### Cis inhibition

In order to investigate whether other dipeptides could inhibit the influx of  $^{14}C$ -Gly-Sar, entry of the labelled substrate was measured in the presence of several unlabelled dipeptides. In these studies membrane vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM

Hepes / Tris at pH 8.5 and 50  $\mu$ M valinomycin. Influx of 0.1 mM  $^{14}\text{C}$ -Gly-Sar into hepatopancreas BBMV was measured with an inwardly-directed hydrogen ion gradient for 10 sec in the presence of 1 mM test peptide concentration. Results showed a significant ( $p < 0.01$ ) inhibition of Gly-Sar influx by Gly-Pro, Gly-Gly, Gly-Leu and Gly-Phe employed in this experiment. Results also show that presence of 10 mM diethylpyrocarbonate (DEP), an inhibitor of carrier-dependent dipeptide transport in intestinal BBMV of rabbit (12) and teleost (15), almost totally abolished 0.1 mM  $^{14}\text{C}$ -Gly-Sar uptake by hepatopancreas BBMV (Fig 23).

Effect of external proton concentration on  $^{14}\text{C}$ -Gly-Sar influx into lobster hepatopancreatic BBMV

In these studies membrane vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 8.5 and 50  $\mu$ M valinomycin. Influx of 0.1 mM  $^{14}\text{C}$ -Gly-Sar into hepatopancreas BBMV was measured with an inwardly-directed hydrogen ion gradient for 10 sec in the presence of different external proton concentrations. Data for total dipeptide influx was fitted to a Michaelis-Menten equation to generate proton transport kinetic constants for the Gly-Sar carrier system. This analysis yielded the following Michaelis-Menten values for a saturable, high affinity system:  $K_t = 1.99$  nM and  $J_{\text{max}} = 71$  pmol/ mg protein/ 10 sec. Results of this study show the hyperbolic nature of proton-dependent Gly-Sar influx and suggest (16) a cotransport stoichiometry for Gly-Sar transfer by hepatopancreas BBMV of 1 H / 1 Gly-Sar (Fig 24).

## DISCUSSION

This report demonstrates for the first time that the epithelium of the crustacean hepatopancreas has the ability to transport dipeptides. The results demonstrate that a transmembrane proton gradient provides the driving force for dipeptide transport in lobster hepatopancreas BBMV. Downhill movement of protons along their electrochemical gradient, providing a driving force for electrogenic dipeptide uptake, has been demonstrated in mammalian (6) and teleost intestinal epithelia (15,17). Our data also showed that  $^{14}\text{C}$ -Gly-Sar transport in lobster hepatopancreas BBMV was electrogenic. The electrogenicity observed was likely due to cotransport of a proton with an electroneutral dipeptide.

Lobster hepatopancreas BBMV exhibited a high capacity, low affinity carrier with a  $K_t$  value of 6 mM. Previously, high and low affinity transport carriers for different peptides have been shown in mammals (5,14) and teleosts (15). Because the lobster is an omnivore, it is possible that its absorptive epithelium is exposed to high concentrations of dipeptides. For this reason it is of advantage for the animal to have a low affinity high capacity carrier that would enable it to absorb large quantities of dipeptides as they are broken down and released from proteins by proteases. This low affinity  $K_t$  value quantitatively resembles reported values of analogous low-affinity carriers described in intestinal tissue of other carnivorous mammals (11).

There are number of studies that have focused on the effect of pH on the absorption of nutrients by lobster hepatopancreas. A drop in external vesicular pH has been shown to stimulate both glucose (1) and amino acid (3)

transport in hepatopancreatic BBMV. But the effect of pH on these nutrients was different. Amino acids were protonated at low pH and became the preferred substrate for transport, while for glucose, protonation of the glucose carrier resulted in increase binding affinity of the carrier to glucose, resulting in increase glucose uptake (9). On the other hand inositol transport by the hepatopancreatic epithelium was shown to be maximal at pH values near neutrality (13). These studies indicate that there might be preferential nutrient absorption at different pH values by this animal as digestion of a meal proceeds.

Our study with dipeptides shows that lobster hepatopancreatic BBMV possess a high affinity proton binding site with an apparent 1:1 coupling stoichiometry between protons and Gly-Sar during transfer. In these animals, sea water is ingested during food intake. This can cause gut luminal pH to fall towards alkaline pH having a very low proton concentration. As peptides would be early break-down products during protein digestion, it would be beneficial to have peptide carriers with high affinity proton binding sites that would enable efficient absorption of peptides even at a very low proton concentration. At these low proton concentrations, a high dipeptide gradient across the brush border membrane may be responsible for driving the dipeptide into the cell.

Results reported here show that lobster hepatopancreas BBMV exhibited significant inhibition of 0.1 mM  $^{14}\text{C}$ -Gly-Sar influx by a 1 mM concentration of several other dipeptides (Fig 23). Investigations using labelled Gly-Sar or Gly-Pro indicated that both share the same transport system in rabbit intestine (7), rabbit kidney (6) and teleost intestine (15). The

findings here, illustrating inhibition of Gly-Sar by several other dipeptides, suggest that a broadly specific, shared peptide carrier was involved in the transport of these dipeptides in lobster hepatopancreas BBMV. Such a shared Gly-Sar transport system with other neutral dipeptides has been demonstrated in mammals (11).

The results obtained in the present investigation with lobster hepatopancreas BBMV indicate that peptide transport in these animals displayed the following characteristics. Brush border membrane vesicles from hepatopancreas were qualitatively similar in ionic requirements and electrogenicity to those of teleosts and mammals. Lobster hepatopancreas BBMV displayed  $^{14}\text{C}$ -Gly-Sar transport that was  $\text{H}^+$ -gradient-dependent and sodium-independent. In hepatopancreas  $^{14}\text{C}$ -Gly-Sar influx occurred by one transport processes: a low affinity carrier system with the following kinetic constants (a)  $K_t = 6 \text{ mM}$ , (b)  $J_{\text{max}} = 5000 \text{ pmol/ mg protein/ 10 sec}$ . The carrier exhibited a high affinity proton binding site with an apparent  $1\text{H} : 1\text{Gly-Sar}$  stoichiometry during transfer.

In summary this study is the first demonstration of the characteristics of a crustacean hepatopancreatic dipeptide transport mechanism. The significance of this class of nutrients in invertebrates deserves more research effort to assess the nature of the contribution dipeptides make to invertebrate nutrition and development.

Figure 18. Osmotic reactivity of basolateral membrane vesicles. Vesicles were loaded with 200 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris at pH 8.5 and 50  $\mu$ M valinomycin and incubated for 60 minutes in external media containing 0.1 mM  $^{14}$ C-Gly-Sar, 200 mM mannitol, 100 mM KCl and 20 mM Mes/Tris at pH 5.5 and 0-600 mM sucrose.



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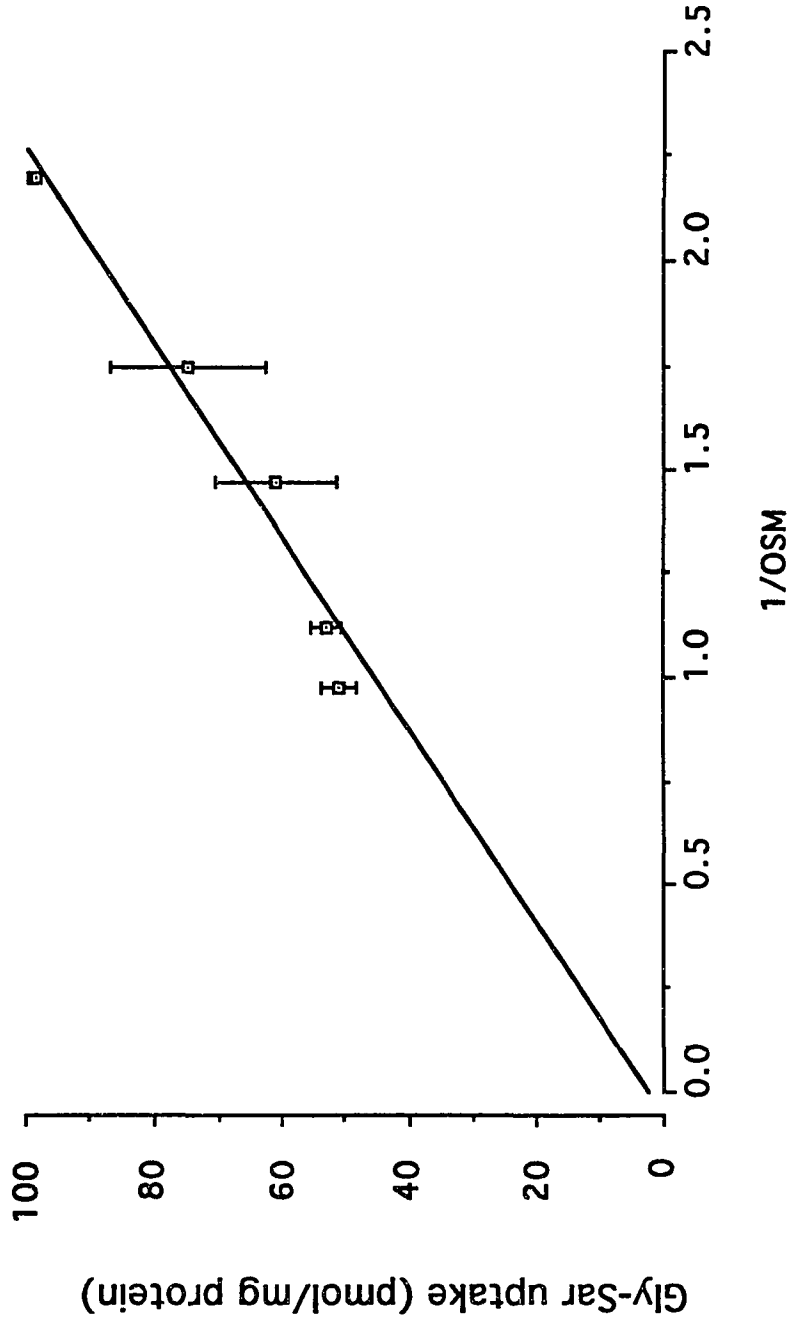


Figure 19. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into hepatopancreas BBMV. Effect of pH gradient. Vesicles preloaded with 100 mM KCl, 200 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (1) 100 mM KCl, 200 mM mannitol and 20 mM Hepes/Tris at pH 5.5, or (2) 100 mM KCl, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5.

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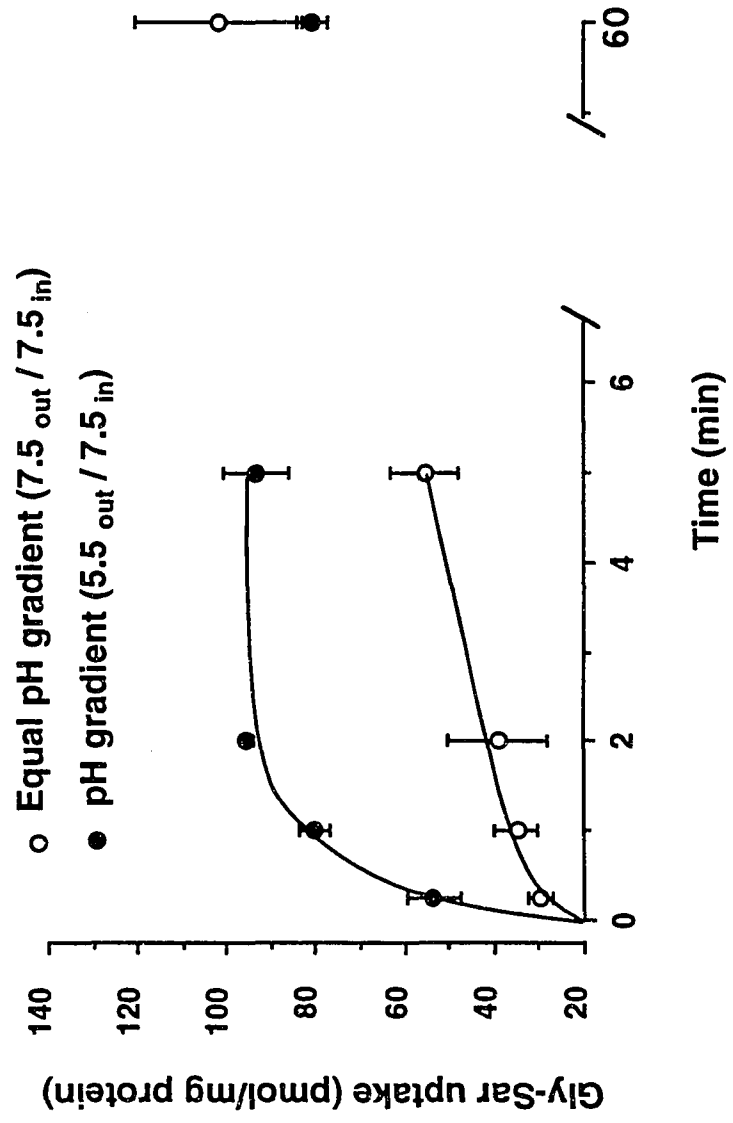


Figure 20. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into hepatopancreas BBMV. Effect of membrane potential. Vesicles preloaded with 100 mM KCl, 200 mM mannitol and 20 mM Hepes/Tris at pH 7.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (1) 100 mM KCl, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (2) 100 mM choline chloride, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5.

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- pH gradient (5.5<sub>out</sub> / 7.5<sub>in</sub>)
- ▲ pH gradient (5.5<sub>out</sub> / 7.5<sub>in</sub>) + mem. potential

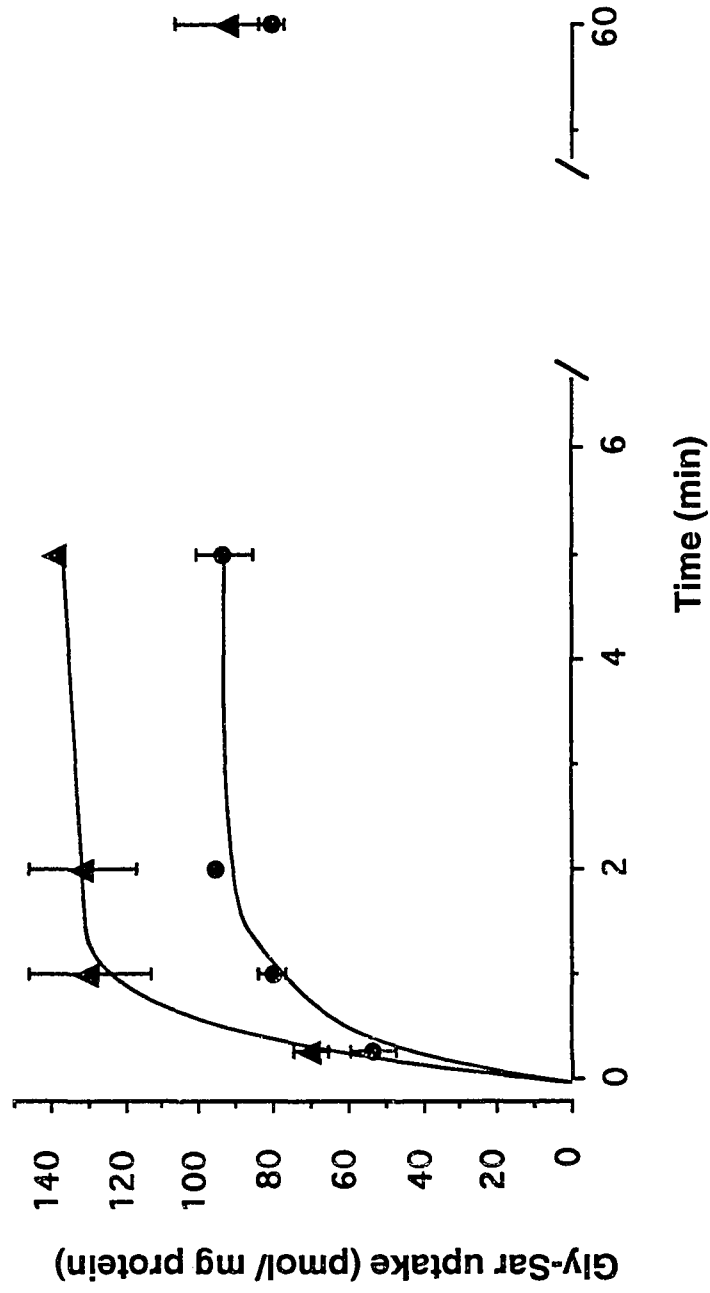


Figure 21. Time course of  $^{14}\text{C}$ -Gly-Sar uptake into hepatopancreas BBMV. Effect of membrane potential and  $\text{Na}^+$  gradient. Vesicles preloaded with 100 mM KCl, 200 mM mannitol and 20 mM Hepes/Tris at pH 8.5 and 50  $\mu\text{M}$  valinomycin. Outside media were: (a) 100 mM KCl, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (b) 100 mM choline chloride, 200 mM mannitol and 20 mM Mes/Tris at pH 5.5, or (c) 200 mM mannitol, 100 mM NaCl and 20 mM Mes/Tris at pH 5.5 .

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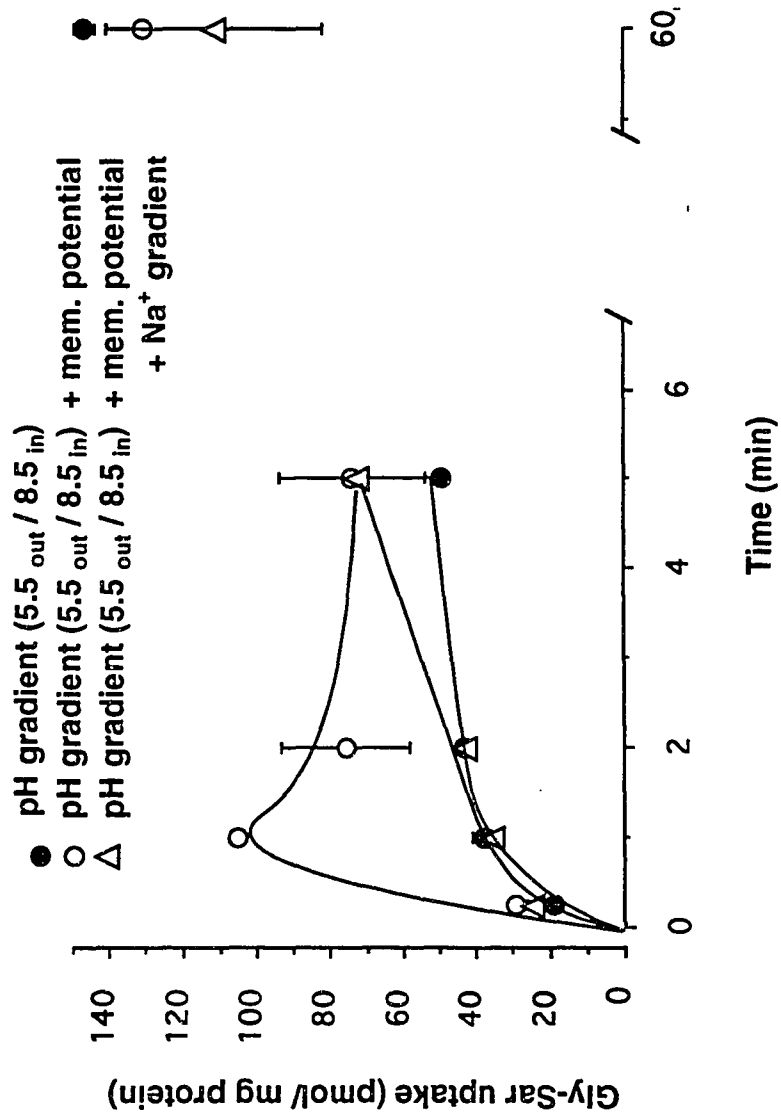
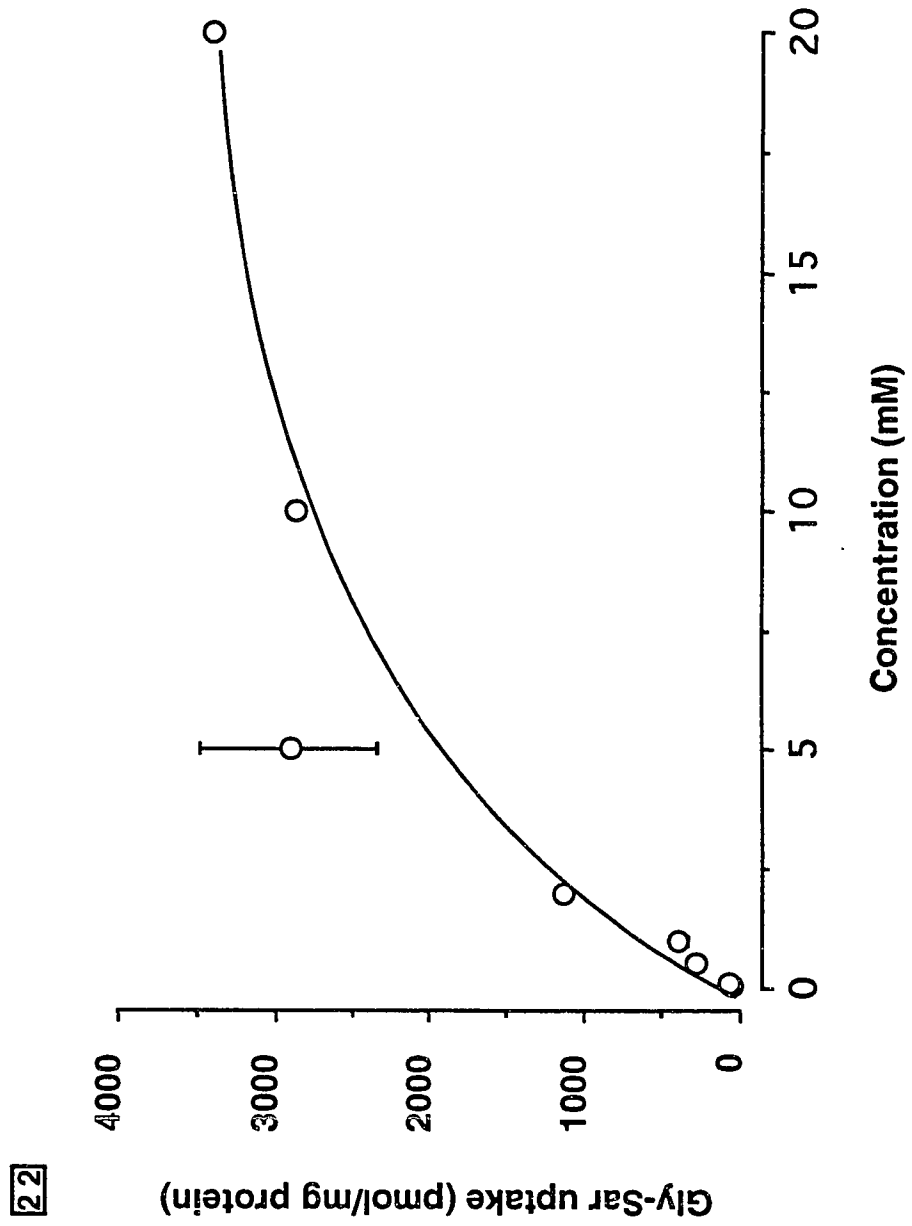


Figure 22. Influx of  $^{14}\text{C}$ -Gly-Sar into hepatopancreas BBMV as a function of substrate concentration. Vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM HEPES/Tris, at pH 8.5 and 50  $\mu\text{M}$  valinomycin. External media had 100 mM KCl, 200 mM mannitol and 20 mM MES/Tris, at pH 5.5, with  $^{14}\text{C}$ -Gly-Sar concentration ranging from .05 to 20.0 mM. Data for total dipeptide influx was used in equation (1) to generate transport kinetic constants for Gly-Sar carrier system.





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Figure 23. Effect of 1 mM external substrates on 0.1 mM  $^{14}\text{C}$ -Gly-Sar influx into hepatopancreas BBMV. Vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 8.5 and 50  $\mu\text{M}$  valinomycin. External media had 100 mM KCl, 200 mM mannitol and 20 mM Mes/Tris, at pH 5.5 and 1 mM respective test substrate. For DEP treatment, vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM Hepes/Tris at pH 8.5 and incubated with 10 mM DEP for 10 minutes.

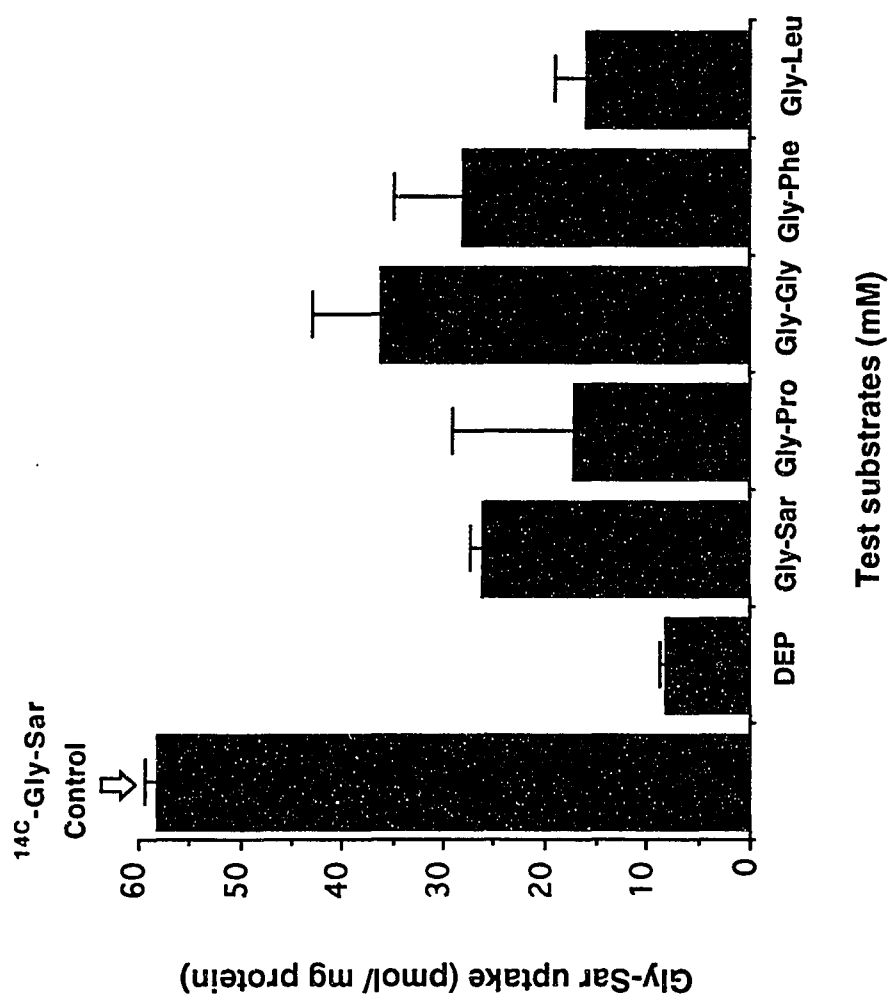
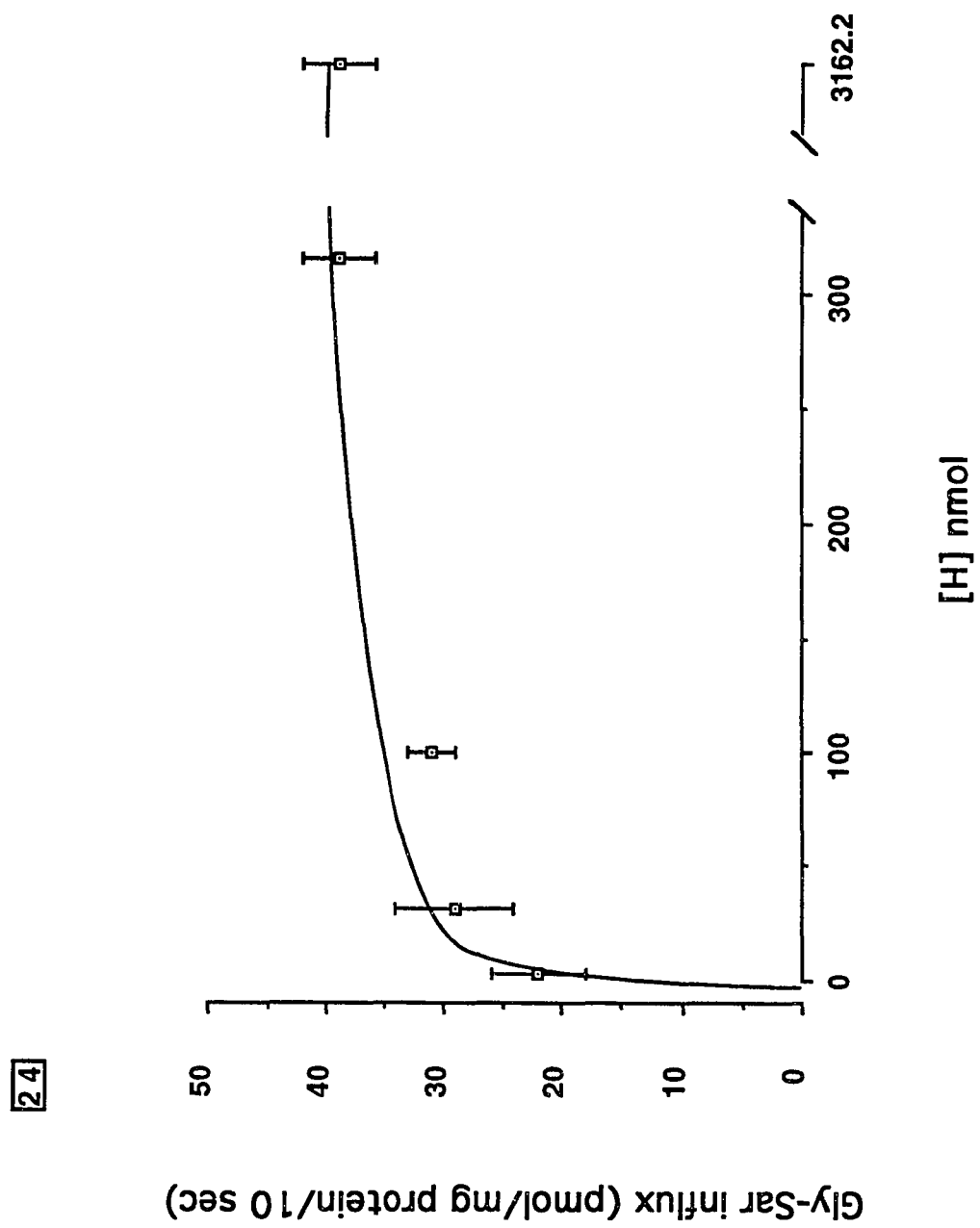


Figure 24. Effect of external proton concentration on 0.1 mM  $^{14}\text{C}$ -Gly-Sar influx into hepatopancreas BBMV. Vesicles were pre-loaded with 200 mM mannitol, 100 mM KCl and 20 mM Hepes / Tris at pH 8.5 and 50  $\mu\text{M}$  valinomycin. External media had 100 mM KCl, 200 mM mannitol and 20 mM Hepes/ Tris or Mes/Tris, at pH 7.5, 6.5, 6.0, 5.5 and 4.5.



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## CHAPTER 5

## CONCLUSIONS

These studies show that intestine of herbivorous tilapia (*Oreochromis mossambicus*), intestine and pyloric ceca of the carnivorous rock fish (*Sabastes caurinus*) and hepatopancreas of the lobster (*Homarus americanus*) are capable of absorbing dipeptides from the gut contents prior to their being hydrolyzed to individual amino acids. Brush border transmembrane dipeptide transport in all these tissues were found to be stimulated by transmembrane proton gradient, electrogenic and sodium independent. These observations suggest that both teleost intestinal, and crustacean hepatopancreatic, transport of dipeptides qualitatively resembles those of peptide transport paradigm shown for mammals.

Transport process in tilapia show both saturable and unsaturable components within the concentration of peptides employed in these experiments. Analysis of influx data in tilapia BBMV support the existence of two different carrier system. One exhibiting high affinity, low capacity and the other low affinity, high capacity. It is possible to have differential distribution of these carriers along the gut to ensure efficient absorption of amino nitrogen in this herbivorous animal. But, several interesting question also could be raised against this finding. Are the two different carriers described in this study really two different proteins or different functional status of the same protein. On the other hand, can it be a chance that this non saturable

transport process resulted due to "chance specificity" of a different carrier accepting Gly-Sar.

In tilapia brush border Gly-Sar transport was inhibited by DEP (diethylpyrocarbonate), a specific inhibitor of proton coupled transport system. In addition, Gly-Sar influx in tilapia showed cis-inhibition and trans-stimulation by Gly-Pro only. This suggest that these two peptides are transported by the same carrier. Similar findings to these were demonstrated in the intestine and kidney of mammals.

Basolateral dipeptide transport in tilapia occurred by facilitated diffusion and showed activation by proton concentration rather than proton gradient. The basolateral transporter was not inhibited by DEP and exhibited broad specificity. These findings show that the peptide transporters in brush border an basolateral are distinctly different in tilapia intestinal epithelial cells.

This study is the first demonstration of dipeptide transport in the gut of an invertebrate. Transport kinetics into BBMV from hepatopancreas showed low affinity high capacity carrier system with high affinity proton binding sites. This being a carnivorous animal, presence of a low affinity high capacity system with high affinity proton binding sites, would enable this animal efficient absorption of high concentration of dipeptides, even at high pH, resulting from large amounts of protein brake down in the tubular lumen soon after the meal.

Lobster BBMV dipeptide transporter was inhibited by DEP. A finding that is observed in all our brush border transport studies. The hepatopancreatic brush border transporter showed broad specificity. Mammalian transport studies using intact tissues have shown, Gly-Sar transport being inhibited by several different dipeptides. In tilapia the transport of Gly-Sar was inhibited only by Gly-Pro. This specificity differences may be due to the different affinity type transporters found in these species. Qualitative similarity of dipeptide transport in mammals, teleost and crustaceans show possible conservation of the functional structure of dipeptide transporter across phyla.