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KINETIC CHARACTERISTICS OF GLYCINE TRANSPORT BY THE ISOLATED MIDGUT OF THE MARINE SHRIMP, *PENAEUS MARGINATUS**

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SUMMARY

1. Mucosal glycine influx occurred via a single carrier-mediated, active transport entry process (K_t = 0·36 mM; V_{max} = 0·42 μmoles/g.min) with an absolute Na^+ requirement.

2. Glycine transport inhibition by N_2 gas, NaCN, NaN_3, iodoacetate, 2,4-DNP, and ouabain was more extensive at 0·1 mM than at 1·0 mM glycine, suggesting a greater proportion of energy-dependent transport at lower amino acid concentrations.

3. Aliphatic neutral amino acids and histidine were more potent inhibitors of mucosal glycine influx than were aromatic neutral, and anionic amino acids.

4. Alanine was a fully non-competitive inhibitor of mucosal glycine entry, whereas proline appeared to be a fully competitive inhibitor of luminal glycine transfer.

5. D-Fructose added to the incubation medium restored normal glycine transport in the presence of alanine, indicating that the two amino acids most likely utilized separate, energy-consuming, mucosal transport processes.

6. A tentative model of glycine transport in the penaeid shrimp intestine is presented.

INTRODUCTION

Although crustaceans are very successful, ubiquitous, and of considerable economic importance to man, knowledge of their nutritional and digestive physiology has remained in a rather primitive state. Investigations have, to a great extent, been restricted to localization and identification of digestive enzymes (Vonk, 1960; van Weel, 1970), while scant attention has been given to an analysis of mechanisms in the gut for absorption of soluble food components.

There are three major regions of the crustacean gut: the foregut (mouth, esophagus and stomach), the midgut (digestive gland and intestine) and hindgut (rectum and anus). Unlike the foregut and hindgut, the midgut is not lined with a chitinized cuticle. Although the foregut and hindgut are somewhat permeable to organic compounds (Yonge, 1924, 1936; Dall, 1967; Speck & Urich, 1970), the major site of nutrient absorption in these animals is considered to be the midgut (Vonk, 1960; van Weel, 1970). Both the digestive gland tubules and intestine are lined with columnar

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epithelia bearing apical microvilli (Miyawake & Tanoue, 1962; Talbot, Clark & Lawrence, 1972), and the accumulation of sugars, amino acids and lipids by these cells in both organs have been demonstrated (Yonge, 1924; van Weel, 1955; Speck & Urich, 1970).

There has been, however, no detailed characterization of the uptake mechanisms for organic molecules in the crustacean gut. In this study the characteristics of glycine transport by the isolated shrimp midgut is investigated. A preliminary account has recently been published (Ahearn, 1973).

MATERIALS AND METHODS

Collection and maintenance of animals

Shrimp were collected at night off Haleiwa, Oahu, Hawaii by trawling at a depth of approximately 120 m. In the laboratory they were maintained at 24 °C in circulating, filtered sea water. The animals were fed daily with a prepared diet consisting of tuna, shrimp, soybean, ground corn and high gluten wheat flour. In most cases only animals that had been kept in the laboratory under the above conditions for a week or less were used for experimental purposes.

Incubation medium for midgut tissues

The salt composition of the incubation medium was based upon the composition of blood samples taken from shrimp within 2 h of capture. Samples were obtained by syringe puncture of the thin, intersegmental membrane between the cephalothorax and abdomen, and immediately frozen. After thawing, the blood was centrifuged at 10000 rev/min for 15 min to remove the clotted proteins and the clear serum was diluted 1:500 with distilled water for measurement of Na^+ and K^+ using a Coleman Flame Photometer (model 21). Serum chloride was measured with an Aminco-Cotlove chloride titrator (American Instruments Co.) and total osmotic pressure was determined with an Advanced Instruments osmometer calibrated with NaCl standards. Table 1 indicates that these three ions accounted for about 95 % of the total serum osmotic pressure: their concentrations were very close to those in sea water so an artificial sea water solution was considered an appropriate medium for midgut incubation. The saline solution proposed by Nicol (1960) with a chlorinity of 19‰, a freezing-point depression of $-1{\cdot}872$ °C and a pH of 8·1 was prepared. This solution had a total Na^+ concentration of 471 mM and an osmotic pressure of about 1000 mOsm/l. A Na^+-free medium was prepared by substituting choline chloride and choline bicarbonate for NaCl and $NaHCO_3$. In addition, $MgSO_4$ replaced $NaSO_4$ and NaF was deleted. The remaining salt composition of the Na^+-free medium was adjusted to maintain sea-water ionic ratios and total osmotic pressure.

Experimental procedures

Midguts were removed by severing from the digestive gland and rectum. They were then slit longitudinally to expose the absorptive epithelium. Faecal matter was rinsed off in saline solution and the intestines were gently blotted dry with paper tissue. Midguts were weighed to the nearest 0·1 mg on an analytical balance and were transferred individually to 5 ml of saline. After 5–10 minutes each midgut was trans

Table 1. *Ionic composition and total osmotic pressure of hemolymph serum*

Serum component	Ion concentration and osmotic pressure	n*
Na^+	468·0 ± 6·2 mEq/l†	10
K^+	11·2 ± 1·2 mEq/l	10
Cl^-	461·1 ± 8·7 mEq/l	10
Osmotic pressure	992·5 ± 9·7 mOsm/kg	10

* Number of pooled samples (2–4 individuals/sample).
† Mean ± 1 standard deviation.

ferred to 5 ml of saline to which was added either [^{14}C]glycine (U) or [^{3}H]glycine (2-^{3}H) (New England Nuclear, Corp.) together with inactive glycine to final concentrations between 0·05 to 10 mM and activities of 0·3–2·0 μCi/ml. The medium was oxygenated throughout the period of incubation. Medium samples taken before and after midgut incubation gave similar radioactivity measurements indicating that the tissue had not significantly altered the concentration of glycine in the solution during the exposure interval. Following incubation, the intestines were removed, rinsed rapidly (5 sec) in unlabelled saline and extracted for 48 h in 5 ml of 70 % ethanol. The temperature throughout the procedure was 24 °C.

To determine the viability of the preparations during the incubation, the oxygen consumption of six slit midguts was measured over a 4 h period, taking readings every 10 min with a Gilson Differential Respirometer. An approximately constant rate of oxygen uptake over this time interval (0·59 μl O_2/mg wet wt.h) indicated that the tissues had not significantly deteriorated under these conditions.

When measurements of serosal uptake of labelled glycine into midguts were made, the entire intestine was removed, flushed clean with saline medium using a syringe, and ligated at either end with surgical thread. Incubation in labelled saline was as described above, but in this instance, at the end of exposure the ligated intestines were slit longitudinally for final rinsing and then weighed before ethanol extraction. Samples of the extracts were counted in liquid scintillation spectrometers (Beckman LS100 and LS230) using a toluene-based cocktail and the external standard method for quench correction. When non-extractable radioactivity was to be measured, the entire midgut was dissolved in Protosol tissue solubilizer (New England Nuclear, Corp.) after two 48 h ethanol extractions and subsequently counted in the scintillation counters as described above.

Labelled compounds in ethanol extracts were separated and identified by thin-layer chromatography on plastic silica gel sheets (Eastman Kodak Co.). Extract samples were de-salted by passage through a Dowex-2 anion exchange column (chloride form). The eluant was evaporated to dryness and redissolved in 20 μl of 70 % ethanol for application to the silica gel sheets. The solvent system used was butanol/acetic acid/water (80:20:20). Sheets were scanned for distribution of radioactivity by cutting out 0·5 × 3·0 cm units of the separation path and transferring them directly into liquid scintillation vials for counting. On each chromatograph sheet an inactive glycine standard was run, its position being determined by spraying with ninhydrin.

Inulin space (adherent + intercellular volume) was measured for both the longitudinally slit midgut preparation (total tissue exposure) and ligated preparation

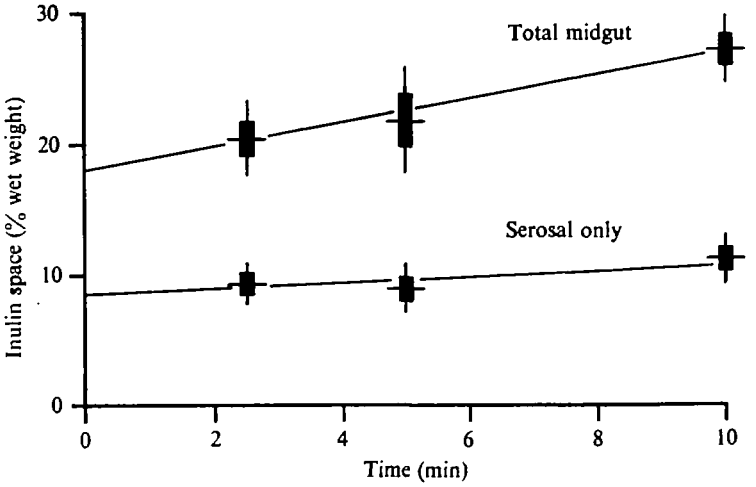

Fig. 1. Time course of [^{3}H]inulin uptake into longitudinally slit (mucosal + serosal exposures) and ligated (serosal exposure only) midgut preparations. Horizontal lines are means (5 midguts/mean), vertical bars are ± 1 standard error of the mean, and vertical lines are ± 1 standard deviation.

(serosal exposure only) by incubating the respective intestines in saline medium containing trace amounts of [^{3}H]inulin (New England Nuclear, Corp.). After exposure for predetermined intervals the midguts were removed from this solution, rinsed in unlabelled saline solution, and extracted in alcohol as described previously. After a 48 h ethanol extraction of [^{3}H]inulin activity, the tissues were rinsed rapidly in fresh alcohol and dissolved in Protosol. Extract and digest samples were added to Aquasol scintillation cocktail (New England Nuclear, Corp.) in proportions which formed thick gels and prevented the precipitation of labelled inulin. Radioactivity counting for both ethanol and digest samples was conducted as previously discussed. Inulin space (expressed as % tissue wet weight) was calculated according to Neame & Richards (1972) using the sum of activity present in both the tissue extracts and digests at each exposure interval. For all incubation periods, [^{3}H]inulin activity present in the Protosol digests did not exceed 6 % of the total.

RESULTS

Midgut morphology

The penaeid midgut (intestine) is a straight tube extending from its anterior connection with the digestive gland to the point where it joins the rectum in the posterior segments of the abdomen. It lies in the dorsal aspect of the abdominal cavity on top of the flexor muscles, but just beneath and invested by lateral branches of the dorsal blood vessel carrying oxygenated blood from the cephalothorax (Fig. 11A, Plate 1). Histologically, the penaeid midgut is a simple absorptive structure bearing only a single, tightly packed, luminal layer of columnar epithelium attached to a thin basal lamina (Fig. 11B–D, Plate 1). Beneath the basal lamina are circular and longitudinal muscle layers, connective tissue fibres and the serosal mesothelium.

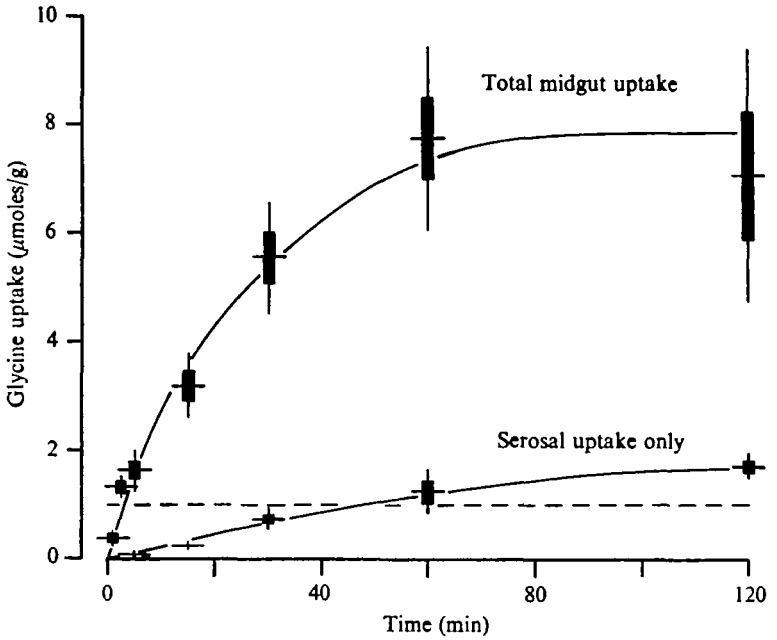

Fig. 2. Time course of [^{14}C]glycine uptake into entire midguts (mucosal + serosal sides) and into the serosal aspect alone of ligated midguts. Symbols and sample size/mean are as described in Fig. 1. The dotted line represents the glycine concentration of the exposure medium throughout the uptake period (μmoles/ml).

Determination of inulin space

Total tissue and serosal inulin spaces were measured for incubation periods of 2·5, 5 and 10 min (Fig. 1). Serosally exposed midgut preparations had similar inulin spaces, between 8 and 10 % of the tissue wet weight. In contrast, the longitudinally slit, total tissue preparations showed a gradual increase in inulin space from 20 to 27 % of the tissue wet weight over the time interval selected. Since this increase may have resulted from inulin entering cells, the true value of adherent + intercellular volume (total extracellular space) was estimated by extrapolating both inulin space curves back to zero time. The initial inulin spaces, derived in this manner, for both total midgut and serosally exposed preparations were approximately 18 and 9 % of the tissue wet weight, respectively. All subsequent amino acid transfer data were corrected for these extracellular inulin spaces, the final results thereby providing a measure of cellular transporting activities alone.

Time course of glycine uptake

The isolated midgut of *P. marginatus* rapidly accumulated glycine from a 1 mM solution, reaching steady-state with the exposure medium after 1 h (Fig. 2). At steady-state the tissue [^{14}C]glycine concentration was about eight times the level in the saline medium, suggesting the existence of a process which was capable of accumulating this amino acid against a considerable concentration gradient. In contrast, uptake of glycine via the serosal surface alone in ligated midguts, most likely representing transport activities of the longitudinal and/or circular muscles, was markedly slower and attained

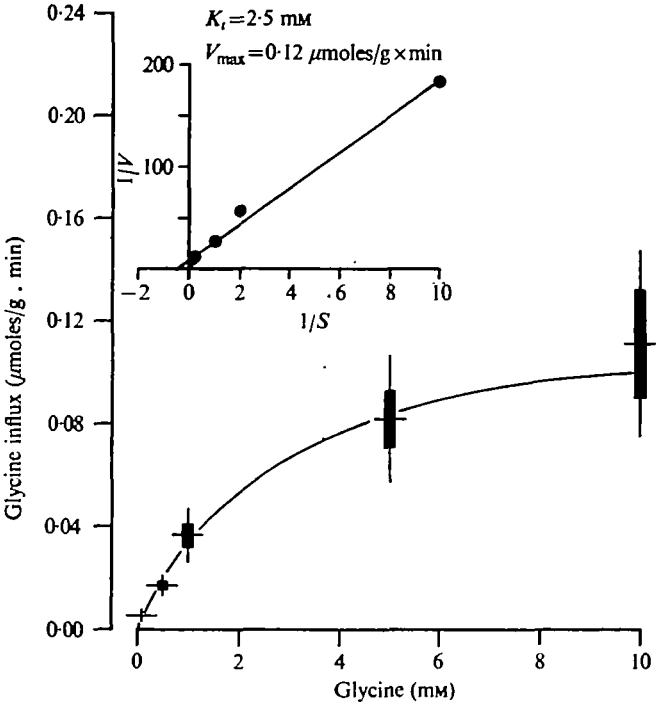

Fig. 3. Serosal influx of [^{14}C]glycine into ligated midguts as a function of glycine concentration. Symbols and sample size/mean for observed data in the main body of figure are as described in Fig. 1. The inset is a Lineweaver–Burk plot of mean serosal influx values. The curve drawn through the observed data was calculated from equation (1) in the text using the kinetic constants obtained from the double reciprocal plot.

a much lower tissue [^{14}C]glycine concentration at steady state. In the latter instance the tissue glycine concentration only slightly exceeded that in the medium.

To measure unidirectional influx into both the mucosal and serosal sides together and the serosal surface alone, short exposure periods were used; uptake was then approximately a linear function of time. Whole tissue [^{14}C]glycine uptake from a 1 mM solution was approximately linear for the first 2½ min of exposure with an influx of 0·290 μmoles/g.min. Influx from the serosal side alone, as measured over the first 15 min of uptake, amounted to 0·023 μmoles/g.min (Fig. 2). Since the serosal contribution to total tissue influx was only about 8 %, [^{14}C]glycine influx into isolated midguts was assumed to be mostly representative of mucosal cell activities alone. All subsequent total tissue influx measurements (unless otherwise stated) were conducted using 2½ min exposure periods, while serosal influx was determined using ten minute incubations.

The extent to which ethanol extractable activity was representative of total midgut activity was determined by exposing longitudinally slit intestines to [^{14}C]glycine for 5, 15, 30 and 60 min. For all time periods, greater than 98 % of the total tissue activity was released in the first alcohol extraction, indicating that reliable measurements of soluble activity could be obtained with a single extraction. Chromatograms of ethanol extractable activity for exposure periods up to 120 min yielded a single radioactivity peak with an R_f corresponding to that of standard glycine. The volatile fraction o

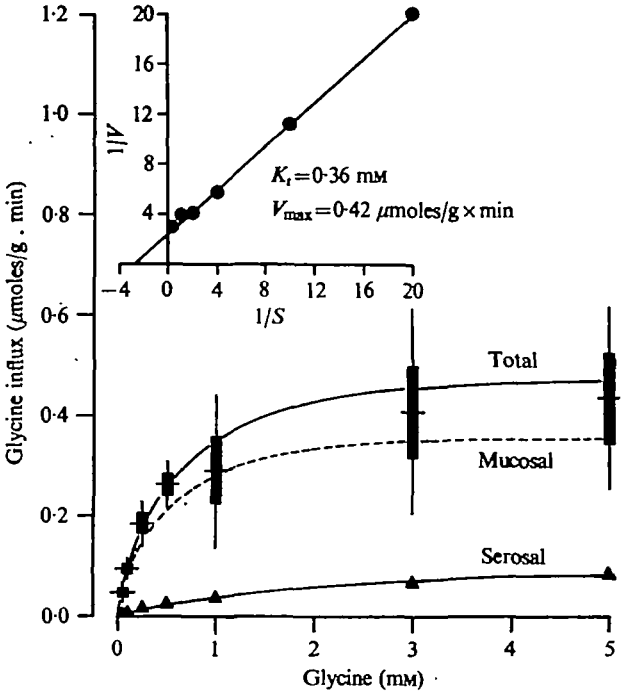

Fig. 4. [^{14}C]Glycine influx into entire midguts (mucosal + serosal sides) as a function of glycine concentration. Symbols and sample size/mean for observed data in main body of figure are as described in Fig. 1. Mucosal glycine influx (dashed line) was estimated by subtracting serosal entry at each glycine concentration from mean values of total midgut transfer. These estimated mucosal influx values are presented in a Lineweaver–Burk plot shown in the inset. The solid curve drawn through the observed total midgut influx data was calculated from the sum of two Michaelis–Menten functions using the kinetic constants obtained from this figure and from Fig. 3.

ethanol extracts (difference in activity before and after evaporation of a known volume of ^{14}C-labelled ethanol extract) proved to be less than 7 % of total ethanol activity for exposure times up to 60 min, suggesting that only minimal metabolism and conversion of labelled glycine to $^{14}CO_2$ had occurred during the incubation process. These results indicated that, due to the low rate of glycine metabolism, use of either [^{14}C]glycine (U) or [^{3}H]glycine (2-^{3}H) would yield similar transport data and would not introduce significant isotope artifacts.

Effect of external glycine concentration on glycine influx

Serosal influx of [^{14}C]glycine into ligated midguts of *P. marginatus* was a hyperbolic function of external glycine concentration (0·1–10 mM) (Fig. 3). This type of mediated transport across a biological membrane, by a process which saturates at higher substrate concentrations, can be described by the Michaelis–Menten equation:

$$V_s = \frac{V_{max}(S)}{(S)+K_t}, \qquad (1)$$

where V_S is serosal [^{14}C]glycine influx rate (μmoles/g.min), V_{max} is the maximal transport rate, K_t the glycine concentration resulting in half maximal influx, and (S) ıe glycine concentration in the incubation medium. Mean values of glycine influx at

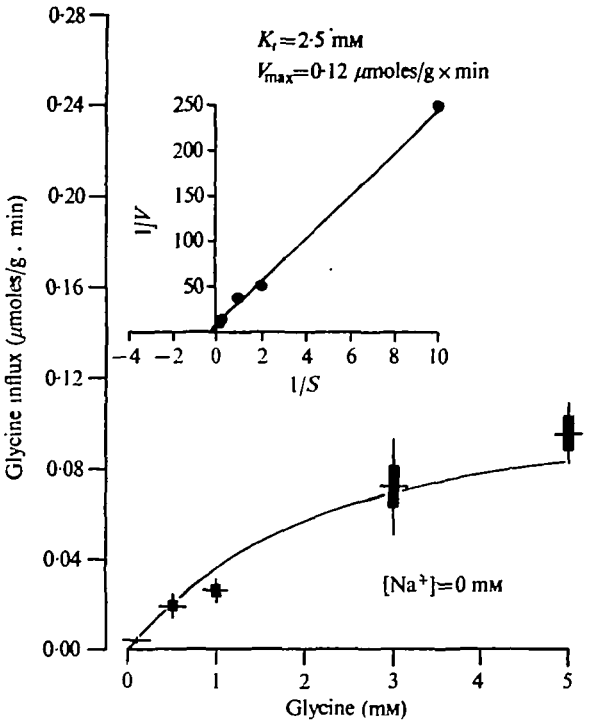

Fig. 5. Effect of Na^+ replacement by choline$^+$ on [^{3}H]glycine influx into entire midguts (mucosal + serosal sides) at a series of glycine concentrations. Symbols and sample size/mean are as described in Fig. 1. The inset is a double-reciprocal plot of mean total tissue influx values and the curve drawn through the observed data was calculated from equation (1) using these kinetic constants.

each glycine concentration are plotted in the inset in the manner of Lineweaver & Burk (1934) and indicate that the K_t and V_{max} for glycine entry on this aspect of the shrimp intestine were 2·5 mM and 0·12 μmoles/g.min, respectively.

Influx of labelled glycine simultaneously into mucosal and serosal intestinal surfaces of *P. marginatus* was also a hyperbolic function of external glycine concentration (0·05–5 mM) (Fig. 4). A Lineweaver–Burk plot of the estimated values of mucosal glycine transfer (inset, Fig. 4) provided kinetic constants for the luminal transport of this amino acid (K_t = 0·36 mM; V_{max} = 0·42 μmoles/g.min). These data indicate that total glycine influx into shrimp intestine through the combined serosal and mucosal pathways was the sum of two independent facilitated transport mechanisms illustrating saturation kinetics over the concentration range examined. Furthermore, significant diffusional entry of glycine by way of either midgut surface could not be established with the present techniques.

At an external glycine concentration of 0·05 mM, mucosal transfer amounted to 96 % of the total amino acid accumulated, while at 5 mM glycine it still accounted for 83 % of the total.

Effect of Na^+ replacement on glycine influx

In order to determine if glycine influx was partially or wholly dependent upon Na^+ in the incubation medium, this ion was completly replaced with choline$^+$. A prelimi

Table 2. *Effect of metabolic inhibitors and ouabain on total midgut* [^{14}C]*glycine influx**

Inhibition	1 mM glycine		0·1 mM glycine	
	Glycine influx (μmoles/g.min)	Inhibition (%)	Glycine influx (μmoles/g.min)	Inhibition (%)
N_2 gas	0·184 ± 0·033†	36·5	0·051 ± 0·017	45·7
1 mM-NaCN	0·192 ± 0·028	33·8	0·027 ± 0·011	71·2
10 mM-NaCN	0·190 ± 0·005	34·6	—	—
1 mM-NaN_3	0·174 ± 0·023	40·0	0·011 ± 0·005	88·0
1 mM iodoacetate	0·192 ± 0·028	33·9	0·020 ± 0·003	79·0
1 mM 2,4-DNP	N.S.‡	~ 100·0	N.S.	~ 100·0
0·1 mM ouabain	0·164 ± 0·014	43·4	0·039 ± 0·011	58·3
Control (no inhibitor)	0·290 ± 0·068	—	0·093 ± 0·010	—

* Midguts preincubated for 15 min in sea-water medium + inhibitor before exposure for 2·5 min to [^{14}C]glycine + inhibitor. Four to five midguts used for each experiment.

† ± 1 standard error of mean.

‡ Non-significant glycine influx.

nary experiment was conducted to determine the time course of total tissue [^{3}H]glycine influx in the Na^+-free medium. Influx under these conditions appeared to be a linear function of time for at least 15 min, so this time period was selected for further experiments in the Na^+-free medium.

Although total replacement of Na^+ with choline$^+$ resulted in a marked decrease in the simultaneous [^{3}H]glycine influx through the combined serosal and mucosal surfaces of the shrimp intestine (Fig. 5), the transfer process still exhibited a hyperbolic relationship with the external amino acid concentration. The effects on glycine entry were greatest at the lower amino acid concentrations (around 0·1 mM) where a 95 % reduction in observed influx occurred. In contrast, at 5 mM glycine in Na^+-free medium, greater than 22 % of the normal transport rate was measured. The percentage decrease in [^{3}H]glycine influx at each glycine concentration in Na^+-free medium, relative to the transport rate in normal Na^+ medium, therefore approximated the percent of total midgut influx occurring solely through the saturable mucosal mechanism. This indicates that most, if not all, of the effects of Na^+ removal were on the facilitated transport system on the luminal surface. Mean values of [^{3}H]glycine influx in Na^+-free medium are plotted in the manner of Lineweaver & Burk (1934) in the inset in Fig. 5 to obtain kinetic constants for total tissue amino acid entry under these conditions. Since the constants are identical to those exhibited by serosal influx in normal Na^+ medium (Fig. 3), it is concluded that the entry of glycine from the serosal side was a Na^+-independent process. Furthermore, these findings suggest a totally Na^+-dependent transport mechanism on the mucosal side of the intestine.

Inhibition of glycine influx

To determine the extent to which [^{14}C]glycine influx at two glycine concentrations (0·1 and 1·0 mM) was dependent upon cellular energy sources, midguts were exposed to a variety of metabolic inhibitors before and during measurement of amino acid transport. The results shown in Table 2 indicate that for all agents used (except 2,4-DNP) a much greater degree of transport inhibition occurred at 0·1 mM than took place at 1·0 mM glycine, suggesting that a higher percentage of glycine influx at the

Table 3. *Amino acid inhibition of total midgut* [^{14}C]*glycine influx**

Inhibitor	Glycine influx (μmoles/g.min)	Inhibition (%)
Neutral amino acids		
Alanine	0·032 ± 0·014†	89·1
Valine	0·022 ± 0·010	92·3
Methionine	0·018 ± 0·014	93·8
Leucine	0·013 ± 0·009	95·6
Cysteine	0·027 ± 0·009	90·8
Serine	0·053 ± 0·010	81·7
Threonine	0·070 ± 0·016	76·0
Tryptophan	0·155 ± 0·051	46·6
Phenylalanine	0·192 ± 0·036	33·9
Tryosine	0·298 ± 0·072	0·0
Imino acids		
Proline	0·097 ± 0·034	66·4
Anionic amino acids‡		
Aspartic Acid	0·194 ± 0·053	32·9
Glutamic Acid	0·227 ± 0·039	21·8
Cationic amino acids‡		
Lysine	0·071 ± 0·031	75·5
Arginine	0·085 ± 0·028	70·6
Histidine	0·037 ± 0·016	87·4
Control (no inhibitor)	0·290 ± 0·068	—

* Both glycine and inhibitor present at 1 mM. Five midguts used for each experiment.
† ± 1 standard error of mean.
‡ pH adjusted to 8·0 with either 2 N-NaOH or 1 N-HCl.

lower concentration depended on a transport process which was either directly or indirectly linked to cellular energy sources. There was considerably more variation in the effects of the transport inhibitors at the lower amino acid concentration than that shown at the higher level, perhaps indicating the relative sensitivity to the agents used. The most potent inhibitor used at both glycine concentrations was 1 mM 2,4-DNP. Ouabain, a known inhibitor of (Na^+-K^+)-activated ATPase, reduced [^{14}C]glycine influx significantly at both glycine concentrations, suggesting a possible link between the regulation of cellular Na^+ concentration by the Na^+-pump and the entry of amino acid.

Several amino acids were tested as potential inhibitors of total intestinal [^{14}C]glycine influx in saline medium when both glycine and inhibitor concentrations were 1 mM (Table 3). All amino acids tested, except tyrosine, reduced glycine transport to some extent, but considerable variation occurred in the magnitude of inhibition. Among the neutral amino acids, the aliphatic species were very strong inhibitors, while the aromatic compounds were considerably less effective. Proline proved intermediate in its effects on glycine influx. The least potent amino acid inhibitors were the acidic species, aspartic and glutamic acid, illustrating only 32·9 and 21·8% reduction in glycine transport at the concentration used. The basic amino acids were also divided in their effects on glycine transfer, with histidine a powerful inhibitor, but lysine and arginine somewhat less so.

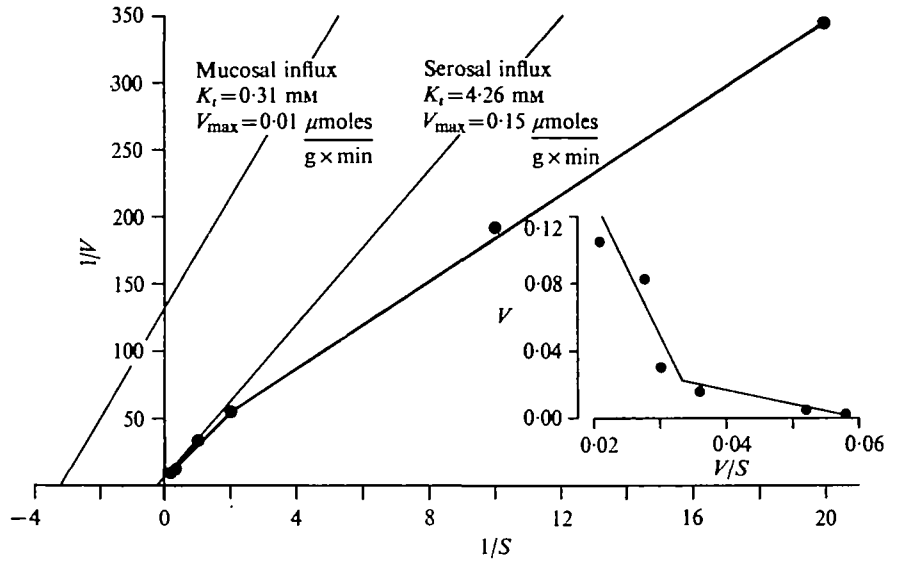

Fig. 6. Effect of 1 mM alanine on total tissue (mucosal + serosal sides) [^{3}H]glycine influx at a series of glycine concentrations, drawn according to Lineweaver & Burk (1934) and Hofstee (1959) (inset). Filled circles represent mean influx values (n = 4–5 midguts/mean). Narrow lines, representing mucosal and serosal glycine influx in the presence of alanine, were calculated according to Neal (1972) as described in the text.

Mechanism(s) of amino acid inhibition of glycine influx

To determine the mechanism(s) of inhibition shown by a neutral amino acid and an imino acid on glycine transport, whole tissue [^{3}H]glycine influx at a variety of glycine concentrations (0·05–5·0 mM) was measured in the presence of 1 mM alanine in one experiment and in the presence of 1 mM proline in another. Preliminary studies examining the time course of whole tissue [^{3}H]glycine influx (1 mM) in the presence of 1 mM alanine or 1 mM proline indicated that transfer was a linear function of time for at least 10 min when alanine was added and for at least 5 min when proline was included. Therefore, these respective incubation periods were selected for further measurement in the presence of these inhibiting amino acids.

Total tissue glycine influx in the presence of 1 mM alanine, representing the sum of mucosal and serosal facilitated transport, was plotted according to the method of Lineweaver & Burk (1934), showing a biphasic relationship between the variables (Fig. 6). To verify the existence of the non-linear double-reciprocal plot, the data are shown in the inset in the manner of Hofstee (1959), which expands the portion of the curve occurring at the higher glycine concentrations. Both graphical methods produced biphasic curves composed of two linear segments, one occurring between 0·05 and 0·5 mM glycine, while the other segment was present from 0·5 to 5·0 mM glycine. Neal (1972) described a method of resolving biphasic Lineweaver–Burk plots into their component systems, assuming that the two linear segments result from the superposition of two uptake mechanisms, both exhibiting Michaelis–Menten kinetics, but with different constants. By knowing the slopes and vertical axis intercepts of the two linear segments of the Lineweaver–Burk plot, the kinetic constants of the individual transport systems can be calculated.

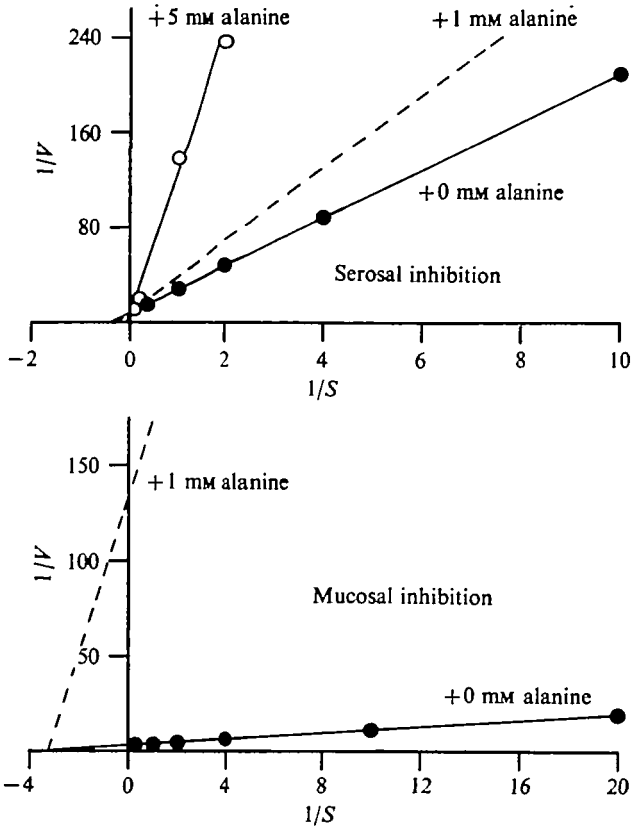

Fig. 7. Effect of alanine on carrier-mediated [^{3}H]glycine influx through the independent mucosal and serosal midgut surfaces. Values of glycine entry recorded in the absence of alanine are from Figs. 3 and 4, in the presence of 1 mM alanine are from Fig. 6, and in the presence of 5 mM alanine are from a separate experiment using ligated midguts (n = 4–5 midguts/mean).

Applying this method of calculation to the present data for [^{3}H]glycine influx in the presence of 1 mM alanine resulted in the resolution of the biphasic double-reciprocal plot into two independent slopes (narrow lines in Fig. 6), representing mucosal and serosal [^{3}H]glycine influx, respectively, each derived from a separate set of kinetic constants. Comparison of the kinetic constants for facilitated mucosal and serosal glycine transport in the presence (Fig. 6) and absence (Figs. 3, 4) of 1 mM alanine demonstrates that at this concentration alanine proved to be a potent inhibitor of both mucosal and serosal glycine entry, but appeared to exert different inhibitory effects on the two intestinal surfaces. A double-reciprocal plot of facilitated mucosal [^{3}H]glycine influx at various glycine concentrations in the presence and absence of 1 mM alanine is illustrated in the lower portion of Fig. 7. These lines show similar horizontal axis intercepts (common K_t) and dissimilar vertical axis intercepts (different V_{max}) indicative of non-competitive inhibition. Therefore, in *P. marginatus* midgut, alanine was a strictly non-competitive inhibitor of mucosal [^{3}H]glycine influx, the equation describing its interaction with glycine transfer being:

$$V_M = V_{\max_1}\left(\frac{K_{t_1}}{(I)+K_{t_1}}\right)\left(\frac{(S)}{(S)+K_{t_1}}\right), \qquad (2)$$

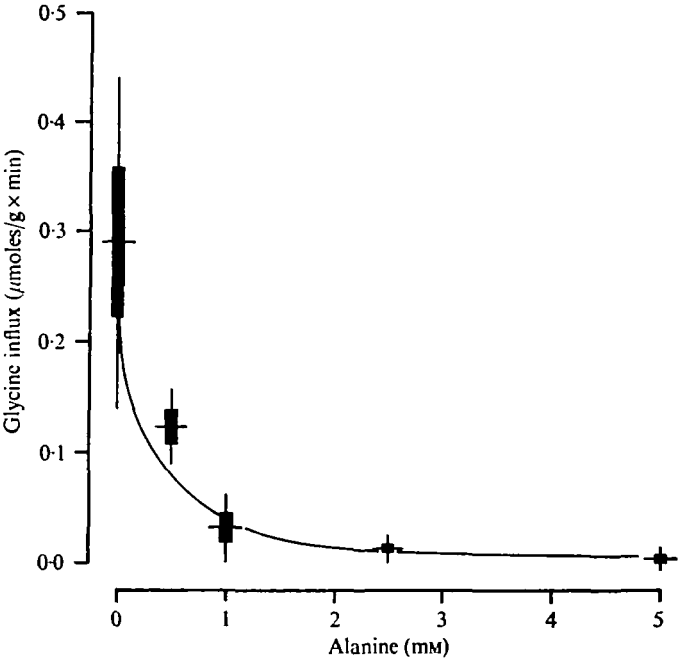

Fig. 8. Effect of increasing alanine concentration on 1 mM [^{14}C]glycine influx into entire midguts (mucosal + serosal sides). Symbols and sample size/mean are as described in Fig. 1. The solid line drawn through the observed data was calculated by combining equations (2) and (3) and using the mucosal and serosal alanine inhibitory constants described in the text.

where V_M is mucosal influx in the presence of alanine; V_{max_1} and K_{t_1} the kinetic constants for uninhibited mucosal glycine entry; (S) the glycine concentration; (I) the alanine concentration; and K_{i_1} the mucosal alanine inhibitory constant. The K_{i_1} for for alanine inhibition of mucosal glycine transport was calculated according to Webb (1963) and was determined to be 0·02 mM alanine.

In the upper portion of Fig. 7 is a double-reciprocal plot of carrier-mediated serosal glycine influx at various glycine concentrations in the presence and absence of 1 and 5 mM alanine. Serosal glycine influx was competitively inhibited by alanine, showing a change in K_t from 2·5 mM in the uninhibited state to 4·26 and 20 mM in the presence of 1 and 5 mM alanine, respectively. In contrast, glycine influx V_{max} was unaffected by the presence of the inhibitor. The equation describing the strictly competitive inhibition exhibited by alanine on serosal glycine influx in *P. marginatus* midgut is

$$V_S = \frac{V_{max_2}(S)}{(S)+K_{t_2}\left(1+\frac{(I)}{K_{i_2}}\right)}, \tag{3}$$

where V_S is serosal glycine influx in the presence of alanine; V_{max_2} and K_{t_2} the kinetic constants for uninhibited serosal glycine entry; (S) the glycine concentration; (I) the alanine concentration; and K_{i_2} the serosal alanine inhibitory constant. In this case the serosal K_{i_2} for 1 and 5 mM alanine inhibition was 1·68 and 1·06 mM, respectively (average K_{i_2} = 1·37 mM), as calculated from Webb (1963).

Total intestinal [^{14}C]glycine influx from a 1 mM solution was measured in the presence of increasing alanine concentrations (Fig. 8). A marked decrease in glycine entry

Table 4. *Effect of exogenous unlabelled sugars on total tissue [^{3}H]glycine influx in the presence and absence of alanine*

Compounds in incubation medium	Incubation period (min)	Glycine influx (μmoles/g.min)*
1 mM glycine	2·5	0·289 ± 0·037
1 mM glycine + 1 D-glucose	2·5	0·290 ± 0·040
1 mM glycine + 1 mM alanine	10	0·081 ± 0·013
1 mM glycine + 1 mM alanine + 1 mM D-glucose	10	0·083 ± 0·012
1 mM glycine + 1 mM alanine + 5 mM D-glucose	10	0·075 ± 0·007
1 mM glycine + 1 mM alanine + 5 mM D-fructose	10	0·381 ± 0·033

* Mean values ± 1 S.E.M. Four to five midguts used for each experiment.

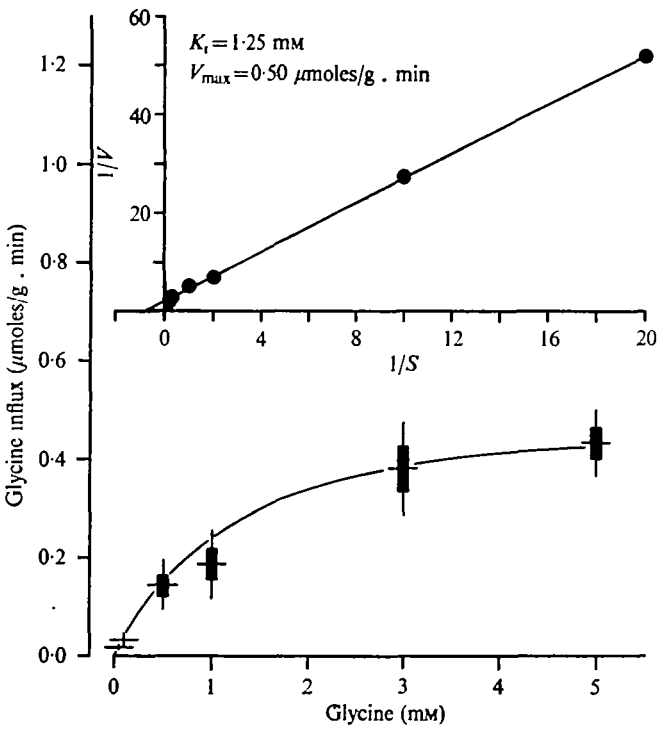

Fig. 9. Effect of 1 mM proline on total tissue (mucosal + serosal sides) [^{3}H]glycine influx at a series of glycine concentrations. Symbols and sample size/mean are as described in Fig. 1. Mean values of total tissue glycine influx are shown in the inset in a Lineweaver–Burk plot, the resulting kinetic constants representing the sum of mucosal + serosal glycine transport in the presence of the inhibitor. The solid line drawn through the observed data was calculated by combining equations (1) for serosal influx and (3) for mucosal influx and using the mucosal proline inhibitory constant described in the text.

occurred between 0 and 1 mM alanine which was followed by a more gradual reduction in influx until the maximum inhibitor concentration (5 mM alanine) was reached. At 5 mM alanine the entry rate of glycine was not significantly different than zero, indicating complete inhibition by alanine at this concentration.

The effect of potentially metabolizable exogenous sugars on the alanine-induced non-competitive inhibition of mucosal glycine influx was determined. Table 4 shows that unlabelled 1 mM D-glucose in the incubation medium not only failed to stimulate control levels of glycine influx in the absence of alanine, but had no effect at either 1 or 5 mM on the influx reduction of glycine due to the presence of the inhibiting amino acid. However, 5 mM D-fructose did relieve the alanine-induced inhibition of glycine entry and, in fact, produced a 32 % increase in transfer rate of glycine over the control value.

Total tissue [^{3}H]glycine influx at a variety of glycine concentrations was measured in the presence of 1 mM proline (Fig. 9). The results indicate that this imino acid induced a change in glycine transfer K_t, but not an appreciable alteration in V_{max} (compare Figs. 4 and 9). The single-segmented Lineweaver–Burk plot of these data presented in the inset of Fig. 9 suggests that the analysis used to differentiate the inhibitory effects of alanine on serosal and mucosal glycine transport cannot be utilized to determine the effects of proline. To determine if proline exerted an inhibitory effect on glycine transport through the serosal surface alone, [^{3}H]glycine influx (10 min incubations) in ligated midguts was measured at two glycine concentrations (0·1 and 1·0 mM) in the presence of 1 mM proline. Comparison of glycine entry rates at both concentrations in the presence and absence of proline indicated that this imino acid exerted little, if any, effect on serosal glycine movements (0·1 mM glycine = control 0·009 ± 0·001 μmoles/g.min, test 0·010 ± 0·003 μmoles/g.min; 1·0 mM glycine = control 0·028 ± 0·008 μmoles/g.min, test 0·034 ± 0·006 μmoles/g.min) (mean ± 1 S.E.M.; n = 5). Therefore the alteration in total tissue [^{3}H]glycine influx K_t in the presence of 1 mM proline observed in Fig. 9 was most likely a result of a strictly competitive mucosal inhibition of glycine transfer by proline. The proline K_i for mucosal inhibition of glycine transport was calculated from the slope of the Lineweaver–Burk plot in Fig. 9 as described by Webb (1963) and was determined to be 0·52 mM.

DISCUSSION

Functional comparisons with mammalian gut

In the mammalian intestine the mucosal brush border membrane possesses concentrative transport mechanisms which accumulate cellular amino acids to concentrations considerably in excess of those in the intestinal lumen or blood. Once accumulated to such high cellular concentrations these amino acids are then held to flow (by simple diffusion and/or facilitated diffusion) into blood capillaries through the lateral and serosal columnar epithelial cell membranes. From the capillaries these nutrients eventually reach the general blood circulation. In the crustaceans, and particularly in decapod shrimp such as *P. marginatus*, the oxygenated blood supply to the absorptive intestinal epithelium is derived from the dorsal blood vessel and its lateral branches (Fig. 11A, B, Plate 1) by a posteriorly directed blood flow. In contrast to the arterial system, the venous system is non-vascular and blood returning to the heart bathes all organs in the haemocoel. As shown in the present investigation, glycine and possibly other amino acids and compounds of nutritive importance are apparently accumulated to relatively high concentrations in the crustacean mucosal epithelial cells, but instead of diffusing into blood capillaries investing the intestinal matrix as in

the mammals, these compounds may flow directly into the general venous circulation which immediately bathes the entire intestine. It may be for this reason that the ovaries in shrimp develop and mature in very close proximity to the intestine over its entire length (Fig. 11A, Plate 1), presumably benefiting from local high concentrations of nutritive compounds diffusing across the intestinal wall.

Na^+ requirement for mucosal glycine influx

The active mucosal glycine transport mechanism in the marine shrimp midgut exhibited an absolute Na^+ dependency (Fig. 5). Although the functional role of Na^+ in active intestinal transport of organic molecules is still somewhat in dispute (Schultz & Curran, 1970; Kimmich, 1973), the requirement for this ion appears to be widespread throughout very diverse animal groups. Glycine transport in both the rabbit ileum (Peterson, Goldner & Curran, 1970) and the chicken small intestine (Nelson & Lerner, 1970) has also been shown to occur by way of Na^+-dependent processes. In addition, alanine (Field, Schultz & Curran, 1967) and lysine (Munck & Schultz, 1969) are also transported by the rabbit intestinal mucosal membrane by processes, at least in part, requiring Na^+.

Amino acid inhibition of glycine influx

The aliphatic neutral amino acids and histidine were shown to be the strongest inhibitors of glycine influx in the midgut of *P. marginatus* (Table 3). One member of this potent inhibitory group, alanine, exhibited fully non-competitive inhibition of luminal glycine transfer (Fig. 7). Inhibition of this type is usually attributed to interference with the production or utilization of cellular energy and is most commonly observed in studies using metabolic poisons such as cyanide or 2,4-DNP (Neame & Richards, 1972). However, recently a considerable body of information has accumulated which indicates that non-metabolizable, actively transported sugars non-competitively inhibit the intestinal transport of amino acids in mammals (Newey & Smyth, 1964; Reiser & Christiansen, 1969; Bihler & Sawh, 1973). The authors explain this effect as resulting from a competition between the sugars and amino acids for a common cellular energy source. Often the addition of a metabolizable substrate to the incubation medium, such as glucose or fructose, relieves this type of inhibition by making available sufficient energy reserves to supply the needs of both transport processes. Similarly, the non-competitive inhibition of alanine on glycine influx also appeared to result from competition for a limited supply of energy between two active transport processes, one serving the transfer of glycine and the other the transfer of alanine. Although exogenous D-glucose did not relieve the alanine-induced inhibition of glycine influx, D-fructose added to the incubation medium did restore normal glycine transport, presumably by providing sufficient energy supplies through its catabolism for the simultaneous transfer of both alanine and glycine. Due to the restoration of normal glycine influx in the presence of alanine by the addition of D-fructose, allosteric interactions between the binding process of alanine and that of glycine for two independent carrier systems, without the involvement of energy limitations, seem minimal.

Proline proved to be a competitive inhibitor of mucosal glycine influx (Fig. 9), thereby implicating the existence of a shared imino acid–glycine carrier system in this intestinal surface. In contrast, proline appeared to be without effect on serosal glycine,

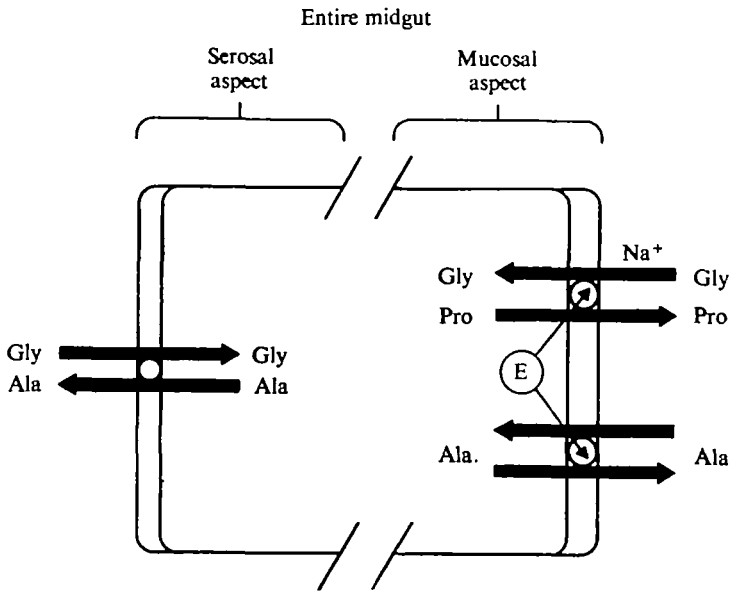

Fig. 10. Proposed model for midgut glycine transport in *Penaeus marginatus*. Amino acid abbreviations stand for glycine (GLY), alanine (ALA), and proline (PRO), respectively, while E represents a shared cellular energy supply which serves both transport processes through an unknown transduction mechanism.

transfer. The presence of a carrier-mediated transport mechanism which is shared by the imino acids and glycine has been previously described in mammalian kidney (Scriver & Wilson, 1967; Hillman & Rosenberg, 1969) and mammalian intestine (Munck, 1966; Peterson *et al.* 1970). Moreover, in these tissues, glycine is transported by a second mechanism which is shared by alanine and other neutral amino acids. In renal tubule preparations there is a third system for glycine transport that is inhibited by lysine (Hillman, Albrecht & Rosenberg, 1968) but not by proline or alanine. In contrast to the shared imino acid–glycine transfer process identified in mammalian tissues, proline and glycine are apparently largely transported by distinct systems in the chicken small intestine (Nelson & Lerner, 1970; Burrill & Lerner, 1972). In the present investigation mucosal glycine transport appeared to occur by only a single, proline-shared, carrier-mediated transfer process (Figs. 4, 9). Had there been at least two facilitated processes (with sufficiently different K_t values) involved in the luminal membrane translocation of labelled glycine, a biphasic Lineweaver–Burk plot would have resulted from the mucosal influx values after subtraction of serosal transfer (Fig. 4) (Hillman *et al.* 1968; Mohyuddin & Scriver, 1970). At the present time, however, the existence of two mucosal glycine transport systems with K_t values less than an order of magnitude apart and both apparently unshared by alanine cannot be totally excluded.

The mechanism(s) of glycine influx inhibition exhibited by the other amino acids tested (Table 3) remains unknown at present, but in each case the decrease in glycine entry may, to some extent, have been the result of competition for a common energy supply by the simultaneous activities of more than one midgut transport process.

However, competitive interactions between these compounds and glycine for one or more shared carrier systems with similar K_t values may have equally been responsible for the inhibition observed.

Model for midgut glycine transport

Fig. 10 represents a tentative model of midgut glycine transport characteristics in *P. marginatus* summarizing the results obtained in the current investigation. At present it is unclear which membrane(s) was (were) rate-limiting for glycine entry into the serosal aspect of the intestine. The outermost cellular layer of the serosal mesothelium may not actually have represented a permeability barrier to glycine penetration and the rate-limiting step may perhaps have been a function of membranes associated with the muscle layers and/or the serosal surface of the luminal columnar epithelium. Because the serosal transport properties cannot be conclusively attributed to a specific cellular membrane, the model in Fig. 10 presents these properties as functions of some unidentified membrane in the 'serosal aspect' of the midgut. These serosal transport characteristics were mainly established to allow the differentiation of mucosal influx properties. The influx characteristics of the 'mucosal aspect' of the intestine were most likely functions of the brush border membrane of the mucosal epithelium.

The present study has revealed the operation of two apparently separate transport processes functioning on the luminal aspect of the shrimp intestine. One of these carrier-mediated systems served the concentrative transfer of glycine (Fig. 2), was Na^+-dependent (Fig. 5), was shared by proline (Fig. 9), and at least at low luminal glycine concentrations, required a cellular energy input (Table 2). The second mucosal system also appeared to have an energy requirement and operated as an alanine transport vehicle. The simultaneous transport of both amino acids by their respective carrier systems involved a direct competition between these processes for a common energy supply. This competition led to a reduction in the transfer capability of the imino acid–glycine carrier system during the operation of the alanine carrier system. When sufficient energy supplies were available (e.g. through the catabolism of exogenous D-fructose), normal transport function was restored. No conclusive data are yet available to definitely establish the exclusiveness of each of these two processes. Although a small portion of glycine influx may have occurred through the alanine system, alanine appeared to be essentially excluded from the major, proline-shared, glycine entry process. If alanine competed with glycine for both a common energy supply as well as for attachment to a common carrier site, a mixed type of inhibition between the two would have occurred where both K_t and V_{max} for glycine entry were altered in the presence of alanine. Since Fig. 7 indicates only a change in glycine influx V_{max} under these conditions, alanine apparently did not exhibit a significant degree of mucosal competitive inhibition with glycine, indicating that the great majority of mucosal transport of these two amino acids by their respective carrier systems was unshared.

Although the serosal transport properties of the shrimp midgut were of indefinite origin, they were strikingly different to those shown by the mucosal surface. Facilitated serosal glycine transfer occurred by a process which was apparently unshared by proline (Fig. 9), but shared by alanine (Figs. 6, 7), was Na^+-independent (Fig. 5), and was incapable of accumulating glycine to concentrations markedly in excess of

those in the incubation medium (Fig. 2). Determination of the physiological consequences, if any, of such disparate transport processes on each surface of the shrimp midgut as they may relate to the transmural movements of nutritionally important compounds, must await further experimentation.

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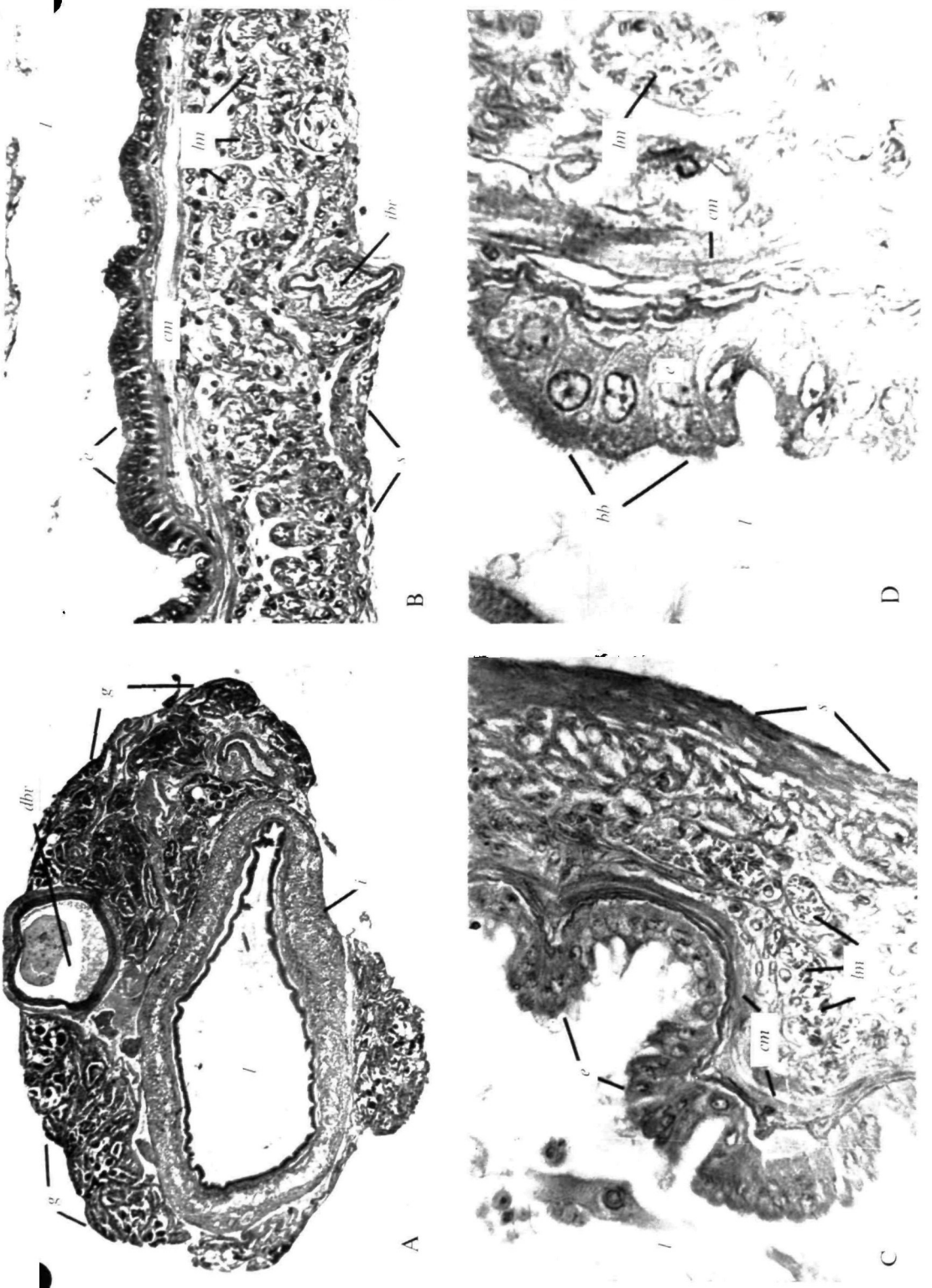
EXPLANATION OF PLATE

Fig. 11. Cross-sections through midgut and associated organs of *P. marginatus* at various levels of magnification. Tissues were fixed in sea water Bouin's solution and stained with hematoxylin and eosin. *l*, Intestinal lumen; *g*, gonad; *dbv*, dorsal blood vessel; *i*, intestine; *e*, columnar epithelium; *cm*, circular muscle layer; *lm*, longitudinal muscle layer; *ibv*, intestinal blood vessel; *s*, serosal mesothelium; *bb*, brush border of columnar epithelium.

(A) Low magnification of association between intestine, gonads, and dorsal blood vessel in mature female shrimp. ×25.

(B) and (C) Sections of intestine illustrating component tissues. ×200 and ×400.

(D) Columnar epithelial cell layer illustrating brush border membrane. ×1200.



G. A. AHEARN

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