MECHANISM OF GLYCINE ACCUMULATION IN THE FAT BODY OF THE SILKWORM *BOMBYX MORI* L.

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ABSTRACT

The manner in which glycine is transported by the fat body of the silkworm *in vitro* is shown. It is observed that glycine is transported against a concentration gradient and that a Michaelis Menten-type relationship exists between amino acid concentration and transport. The transport is inhibited by metabolic poisons (1). Complete inhibition of glycine uptake (1mM) is observed when equimolar concentration of L-methionine and L-proline are used and the transport mechanism seems to be stereospecific since D-methionine inhibited glycine absorption only partially. Further the effect of pH, antibiotics and heavy metal ions on glycine uptake support the view that glycine is transported by an active process.

INTRODUCTION

The fat body of the silkworm is physiologically an important organ and possesses in addition to other functions an efficient mechanism for accumulating glycine and chromatographic evidence indicates that glycine tends to remain free in the cell[1]. Christensen postulated that accumulation may occur either through chemical binding, involving no specific transport as such, or by an active process[2]. Heinz pointed out that if amino acids were to remain free in the cell, their concentration must be the result of an active transport[3]. The present investigations were designed to elucidate nature and characteristics of the glycine transporting mechanism in the fat body.

EXPERIMENTAL PROCEDURE

Twenty to thirty silkworm larvae (*Bombyx mori* L.) in the later stages of V instar were anaesthetized with ether and dissected in water and their fat body tissue was pooled together and the final homogeneous sample was used for the experiment, care being taken to remove all other tissues as completely as possible. The fat body was pre-incubated in Ringer's solution for 30 mts[4] and then allowed to take up glycine-2-C^{14} dissolved in Ringer's solution (specific activity – 5.2 mc/mM, 1.44 mgs – glycine-2-C^{14}—dissolved in 2.5 ml of 80% alcohol). The absorption of glycine was followed by the procedure detailed earlier[1].

To ascertain whether absorption is an active uptake process or mere exchange diffusion the following experiment was conducted. The fat body was pre-loaded with unlabelled glycine (200 mM; extracellular) for half an hour and subsequently transferred to Ringer's solution containing radio-active glycine. Controls were run simultaneously using Ringer's solution only for pre-incubation. For studying the uptake of glycine at 0°C, both the tissue material as well as the incubation mixture were kept in the cold for 3-4 hours prior to carrying out the experiment in the cold room itself

In all cases, the radioactivity in the supernatant was counted using a thin-window Geiger-Muller tube. The decrease in radioactivity of the suspension medium was found to be an adequate measure of glycine absorption for the following reasons: (1) No significant water transport occurred during incubation as evidenced from the unabsorbed dye, Evans Blue and from the dry weight of fat body before and after incubation; (2) No significant loss in radioactivity as $^{14}CO_2$ occurred during manometric experiments under identical conditions; (3) Most of the radioactivity in the tissue as well as supernatant was recovered in the glycine after 30 mts incubation.

RESULTS

1. *Concentrative uptake of glycine.*—The rate of glycine entry into the fat body was measured at varying external concentrations (0 01 mM – 3.0 M) and it was found to be very rapid. When substrate concentration was plotted against reaction velocity a hyperbolic saturation curve was obtained which can be explained by Michaelis-Menten formulation. The apparent Km value was found to be 0.03 mM, by the Lineweaver-Burk plot (Fig. 1). The distribution

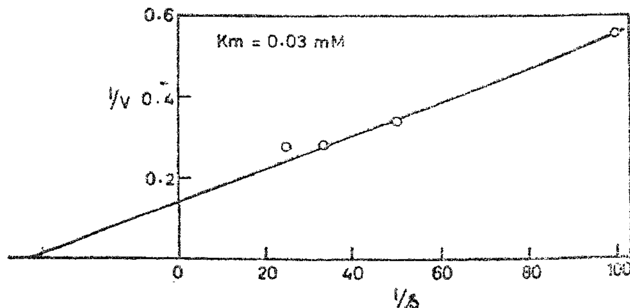

FIG. 1
Line Weaver—Burk plot for substrate concentration

ratio of intracellular to extracellular concentration exceeded unity (Table 1), a result that confirms the conclusion that factors other than passive diffusion contribute to the concentrative uptake of glycine by the fat body[5].

TABLE 1
Effect of Substrate concentration on glycine uptake

Glycine concentration		% uptake	Moisture content of the fat body	Distribution ratio = Gly/ml intra-cellular / Gly/ml extra-cellular
(a)	0.01 mM	8.5	74.0%	1.1
	0.02 mM	7.5		1.0
(b)	0.10 M	14.0		1.75
	1.00 M	18.0		2.25
	2.00 M	25.0	80.0%	3.12
	3.00 M	30.0		3.75

(a) and (b)—On two different days these experiments were performed using V instar larvae
Time: 4 minutes for (a)
10 minutes for (b).

2. *Exchange phenomenon.*—The intracellular accumulation of free amino acids against a concentration gradient may occur by an active transport or exchange diffusion with previously accumulated amino acid [6,7]. Eventhough the reaction rate observed in this instance was fast, from Fig. 2 it is clear that

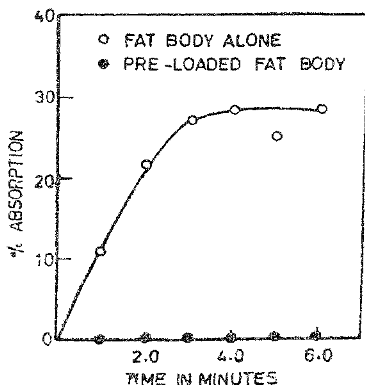

FIG. 2
Exchange of Glycine

the percentage uptake was negligible compared to the control when the fat body was pre-loaded by incubation in Ringer's solution, containing 200 mM of glycine and before its suspension in the incubation mixture containing labelled glycine. It is evident that the exchange diffusion between the unlabelled glycine in the fat body and the radioactive glycine in the outside medium was in fact negligible.

3. *Effect of pH and low temperature on glycine uptake.*—The relation of concentrative uptake of glycine to pH is illustrated in Fig 3 and the maximum activity was recorded between pH 7 to 8. Above pH 8, the percentage uptake of glycine in bicarbonate buffer was lower than in the phosphate buffer and glycine transport was optimum in the pH range 7 – 9.

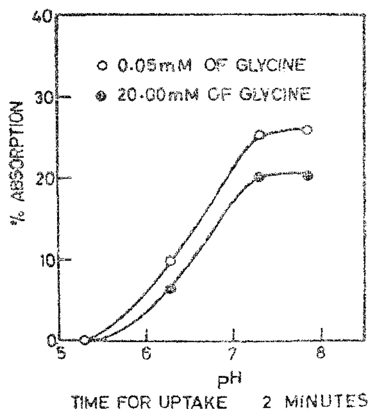

FIG. 3
Variation of Glycine uptake with pH

Christensen and Riggs[8] and others have shown in many tissues that the concentrative uptake of amino acids was negligible at low temperature (0°C). Present results (Table 2) show that glycine uptake was inhibited in cold by about 50% when compared to control uptake at 27°C.

4. *Effect of antibiotics, heavy metals on glycine transport.*— The effects of antibiotics on glycine uptake are shown in Table 3. Both chloromycetin and aureomycin inhibited strongly the absorption of glycine. This is in agreement with the results obtained by Gale, Gale and Paine in bacterial systems[9,10] and by Christensen in Ehrlich mouse ascites cells (8).

TABLE 2

Effect of Temperature (0°C) on glycine uptake (1mM)

	Control experiment % uptake of glycine at room temp. (27°C)	% uptake in the cold (0°C)	% inhibition in glycine uptake at (0°C)
(*a*)	8 54	4 83	45.0%
	±1.05	±0 97	
(*b*)	7.70	3.50	56 0%
	±1 44	±1.72	

(*a*) and (*b*)—larvae from different batches of V instar silkworm.
Time: 1 minute.

The result of the action of different concentrations of copper ions (Cu^{++}) and mercuric ions (Hg^{++}) on glycine transport is shown in Table 3 and it may be seen therefrom that the glycine uptake decreases in comparison with the control experiments Riggs *et al*[11] showed that a number of reagents like alanine, glutamate, Co^{++}, Cu^{++} or Zn decrease the glycine uptake without causing corresponding loss of potassium. Here, the concentration of potassium was not measured and get the inhibitory effect of heavy metal ions was evidenced in the external medium. Preliminary experiments indicate the dependence of the transport on sodium and potassium in the medium.

5. *Competition with other amino acids.*—When glycine was used 1 mM concentration, equimolar concentration of L-methionine and L-proline completely inhibited the glycine uptake (Tables 4 and 5) whereas higher concentrations of these amino acids upto a level of 10 mM did not reveal any effect on the transport of glycine. At lower concentrations (0.1 mM to 1.0 mM) only L-proline stimulated the glycine absorption and the effects of other amino acids were found to be negligible. Addition of L-leucine and L-histidine over a wide range of concentration did not show any effect on glycine uptake at 1 nM level. D-methionine, however, partially inhibited the concentrative uptake of glycine (Table 6).

TABLE 3

Effect of antibiotics and heavy metal ions on the uptake of glycine (1 mM) (Time—1 minute)

Substrate	% uptake	% inhibition	Substrate	% uptake	% inhibition	Substrate	% uptake	% inhibition
Glycine 1 mM	32.0		Glycine 1 mM	21.7		Glycine 1 mM	7.3	
Glycine + Chloromycetin 10 μg/ml. of incubation mixture	24.0	25.0	Glycine + 5 μg Cu^{++} ion/I M.	15 9	26.2	Glycine + 5 μg Hg^{++} per I. M.	4.3	41.1
			,, + 7.5 μg	11.0	49.3	,, + 15 μg Hg^{++}	5.5	24.7
Glycine + Aureomycin 10 μg/ml of incubation mixture	24 0	25.0	Glycine + 15.0 μg per I.M.	9.7	55.3			

I.M.– Incubation Mixture

TABLE 4

Effect of L-leucine and L-methionine on glycine uptake (1 mM)—(Time 1 minute)

	Gly 1 mM	Gly 1 mM + L-met 1 mM	Gly 1mM + L-leu 5 mM	Gly 1mM + L-leu 10 mM	Gly 1mM + L-met 1mM + L-leu 5mM	Gly 1 mM + L-met 1 mM + L-leu 10 mM
% uptake	18.80	0.0	21.86	25.1	22.95	23.44
with S E.	±1.55	±0.0	±2.75	±2.38	±5.56	±1.27

TABLE 5

Effect of L-histidine and L-proline on glycine uptake (1 mM)—(Time 2 minute)

	Gly 1mM	Gly 1mM + L-his 0.5 mM	Gly 1mM + L-his 1.0 mM	Gly 1mM + L-his 10 mM	Gly 1mM + L-pro 0.5 mM	Gly 1mM + L-pro 1.0 mM	Gly 1mM + L-pro 10.0 mM
% uptake	5.87	6.2	6.2	6.4	17.7	0.0	5.98
with S.E.	±1.65	±1.52	±0.75	±1.60	±4.186		±2.08

TABLE 6

Effect of D and L-methionine of glycine uotake (1 mM)—(Time 2 minutes)

	Substrate	Gly 1 mM + Met 1 mM	Gly 1 mM + Met 2 mM	Gly 1 mM + Met 4 mM	Gly 1 mM + Met 8 mM	Gly 1 mM + Met 10 mM
% uptake	Glycine alone	14.00				
	Glycine + L-met	0.00	14.07	15 0	16.7	12.1
	Glycine + D-met	6.7	10.00	7.7	----	6.5

DISCUSSION

In vitro studies of glycine transport by the fat body of the silkworm show that there is an active, in fact a concentrative uptake of the amino acid by the cells since it gets transported against a concentration gradient[12].

Furthermore, the system fulfils some of the other criteria for the transport of a solute, *viz.*, inhibition of the transport by metabolic inhibitors as well as by two of the wellknown competitive amino acids L-methionine and L-proline[13-18] It shows saturation kinetics. The transport appears to be stereospecific since D-methionine did not inhibit glycine uptake completely. Christensen *et al* have shown that D-methionine is a much less effective inhibitor of glycine uptake than is L-methionine[18]. Though the reaction rate is very rapid, it is shown that it cannot be accounted for by exchange diffusion. Hence, an active transport mechanism can be postulated for the transport of glycine by the fat body of the silkworm.

The effects of other amino acids on glycine transport not only indicate the different affinities the system has for different amino acids but also suggest the presence of at least two mechanisms operating at different Km values and differing in their amino acid affinities. This aspect will be considered in greater details in a subsequent paper.

The amino acid accumulating mechanism[5] presumably has a role in the maintenance of extracellular and intracellular compartments for amino acids. Bricteux-Gregoire and Florkin[19], for example, showed definite differences in the muscle-haemolymph distribution of amino acids in the silkworm, *Bombyx mori* L. Levenbook[20], on the other hand, presented evidence in favour of equal distribution of free amino *N* in fat body with that in haemolymph in *Prodenia eridenia*. But he does not rule out changes in individual amino acids Recently, Chen[21] revealed considerable differences observed in distribution of amino acids between salivary gland and haemolymph in *Drosophila* larvae. The metabolic dependence of absorption as well as release[1] argue strongly for their role in amino acid distribution. The exact mechanisms and their quantitative contribution *in vivo*, however, remain to be worked out. Such mecchnisms, *e.g.*, might be responsible for osmotic regulation encountered as in the release of histidine and methionine to counteract the hypoamino acidaemia in the silkworm consequent on silk synthesis[22].

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