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Glycine Conversion to Sarcosine in the Aposymbiotic Weevil *Sitophilus oryzae* L.

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Abstract

^{14}C-glycine was incorporated into the diet of an aposymbiotic strain of *Sitophilus oryzae* larvae and resulted in the labelling of sarcosine, a methylated amino acid uncommon in insects, in 90% of the larvae analyzed. The total sarcosine amount significantly increased as a function of the larval mean weight. If measured in larvae which received not only ^{14}C-glycine but also unlabelled methionine, the total amount of sarcosine was significantly higher in larvae whose diet was supplemented by methionine compared to those with a normal diet. The early pupae showed a net significant decrease of the sarcosine level. All the results obtained confirmed those of Gasnier-Fauchet et al. (1986) and showed that sarcosine is synthesized by the methylation of glycine in an aposymbiotic strain of *Sitophilus oryzae*.

Keywords: sarcosine, symbiosis, *Sitophilus oryzae*, methylation, glycine

1. Introduction

In the destructive grain weevils *Sitophilus*, symbiosis was first described by Pierantoni (1927). Symbiotic bacteria were found at all stages of development (Mansour, 1930, 1934; Musgrave, 1964; Nardon, 1971). In larvae, the bacteria are harboured in a specialized organ called bacteriome. Their number is genetically controlled by the host (Nardon and Grenier, 1989). Viable aposymbiotic strains of *Sitophilus* have been obtained in our laboratory from some symbiotic strains by keeping imagos at 35°C and 90% relative humidity for about

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one month (Nardon, 1973). Thus, comparative physiological, nutritional and biochemical studies have been made possible between symbiotic strains and aposymbiotic strains from which they were derived. These studies have shown that in *Sitophilus oryzae* there are nutritional and biochemical interactions between the host and its symbiotes. On one hand the endocytobiotes can supply the host with several vitamins: pantothenic acid, biotin and riboflavin (Wicker, 1983). On the other hand, it has been demonstrated that the symbiotic bacteria are also involved in the protein metabolism of their host. Amino acid concentrations differ between symbiotic and aposymbiotic populations, in particular for methionine, which is in excess in wheat for *S. oryzae* (Gasnier-Fauchet and Nardon, 1986, 1987). In symbiotic weevils, methionine in excess is sulfoxidized and the index of methylation is three times lower than in the aposymbiotic ones. In the presence of symbiotes, this metabolic pathway is greatly exhausted by the direct action of bacteria, while in the absence of symbiotes, methionine is principally involved in the methylation of glycine to sarcosine, an amino acid which was found at a high level in the aposymbiotic larvae only. As sarcosine can be neither incorporated into proteins nor excreted by the insect, it is continuously accumulated during larval development (Gasnier-Fauchet and Nardon, 1986). The accumulation of sarcosine may be due to the absence or to a weak catabolism of sarcosine, the rate of which only diminishes during metamorphosis.

Sarcosine is uncommon in insects, since traces of this methylated amino acid have been reported in only two species (Firling, 1977; Mayer et al., 1975). In mammals the synthesis of sarcosine may result from the activities of two enzymes: one of these enzymes, dimethylglycine dehydrogenase, which is involved in the choline cycle, oxidizes dimethylglycine to sarcosine. The other enzyme, glycine N-methyltransferase, a cytoplasmic S-adenosylmethionine dependent methyltransferase, catalyzes the glycine transmethylation to sarcosine (Blumenstein and Williams, 1960; Heady and Kerr, 1973; Ogawa and Fujioka, 1982). Sarcosine biosynthesis has been investigated in the larvae of *S. oryzae*. Nutritional and enzymatic experiments have failed to demonstrate any dimethylglycine dehydrogenase activity in *S. oryzae* larvae. Nutritional experiments have led to hypothesize that sarcosine biosynthesis results from the methylation of glycine by a glycine N-methyltransferase-like activity (Gasnier-Fauchet et al., 1986), though until now, such an enzyme was unknown in insects. In order to confirm the pathway of sarcosine biosynthesis, the incorporation of ^{14}C-glycine into aposymbiotic *S. oryzae* was examined in the present study.

2. Materials and Methods

Insects

The insects were reared in wheat grains at 27.5°C and 75% relative humidity according to Laviolette and Nardon (1963). Adults were allowed to lay eggs for 2 days. Larvae developed inside the wheat-grain in four stages L1, L2, L3 and L4 separated from each other by larval molts. At the end of the fourth instar, larvae underwent a nymphal molt. When the insects were allowed to grow inside the wheat-grain which was not even opened, the young adults of the new generation emerged from the 32nd day after oviposition. One aposymbiotic strain of *S. oryzae* called SFR (SS) was used for the experiments. It was obtained from a symbiotic strain according to Nardon (1973). The aposymbiotic state of weevils was controlled: microscopic examination of the ovaries of the females showed the absence of apical bacteriome in ovary and of bacteria in ovocytes (Nardon, 1971).

Labelling of larvae

Wheat grains, each containing one larva, were opened to carefully deposit six microliters of ^{14}C-glycine or ^{14}C-serine solution (DOSITEK U-^{14}C glycine and U-^{14}C serine in HCl 0.01 N at 0.25 mCi/ml) in the grain around the larva. In one experiment, 3 μl unlabelled methionine (L-methionine-Merck) in HCl 0.01 N solution was also added to the grain. Then the grain containing the weevil was closed again and placed in a metabolism cage according to the procedure of Febvay and Bonnot (1990). The cage was sealed with extensible plastic film (Parafilm®). CO_2 produced by the larva was trapped in a filter, as shown in Fig. 1, in order to avoid $^{14}CO_2$ from spreading in the atmosphere. When the grains were taken out of the cage and opened again, larvae were carefully isolated from the grain and allowed to starve for one hour in order to drain off the digestive tract.

Amino acid analysis

Each larva was individually weighed and crushed with a pellet piston in a microtube of 1.5 ml (Bioblock) with 100 μl distilled water and 10 nmol glucosaminic acid as an internal standard. Proteins were precipitated with 5% (v/w) trichloroacetic acid. The mixture was centrifuged at 10,000 g at 4°C for 3 min. Lipids were eliminated by addition of two volumes of chloroform followed by centrifugation at 10,000 g at 4°C for 3 min. This last operation was repeated once. The final supernatant fraction was evaporated and taken

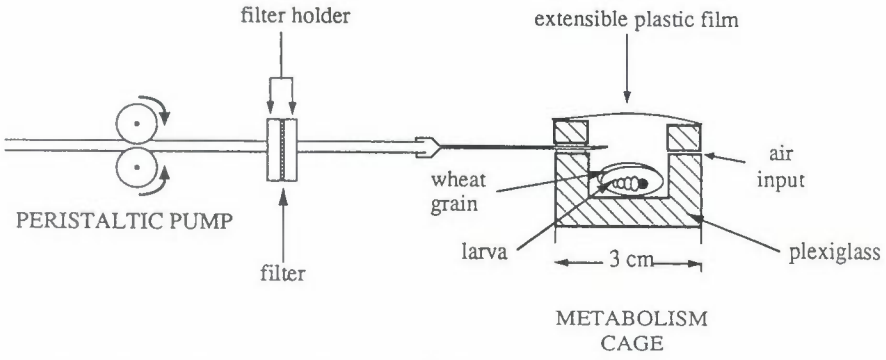

Figure 1. Experimental set-up used to trap $^{14}CO_2$

up in 0.05 M lithium citrate buffer pH 2.2. An aliquot of 50 μl was subjected to Ion Exchange Chromatography on an automatic amino acid analyzer (Beckman 6300), which measures total, both cold and radiolabelled, amino acid amounts. On the chromatogram the values of the elution time of the different peaks corresponding to each free amino acid and the ratio of peak heights measured at two different wavelengths were compared with those of the standard amino acid solutions according to Bonnot and Delobel (1970). By integrating the area under the peak corresponding to a given free amino acid, the total quantity of that amino acid was then determined.

Radiolabelled amino acid assay

Labelling was followed with a Flo-one radioactive β-detector (Radiomatic Instruments, scintillant:Lumaflow III). A quantitative determination was made every 3 sec. The background baseline level was subtracted in each chromatogram.

Statistical analysis

Anova procedure using the Macintosh software Statview (Abacus Concepts, Alpha System Diffusion) was used to analyze the significance of the differences.

3. Results

In order to compare the values obtained in our experiments with those of Gasnier-Fauchet et al. (1986), we used the same aposymbiotic strain, SFR(SS).

Insect development

The weight of 212 larvae of the strain SFR(SS) was measured for three weeks of their development time between the 15th (D15) and the 35th (D35) day after oviposition, and the mean weight was calculated every 5 days (Fig. 2). The

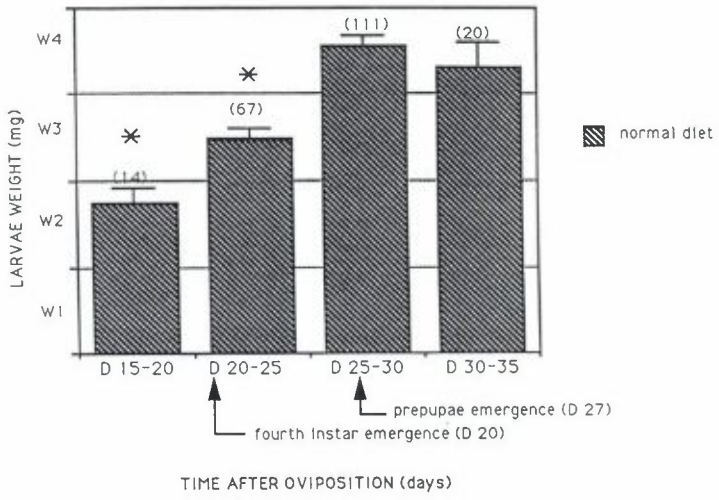

Figure 2. Evolution of the weights during third and fourth instar of *Sitophilus oryzae* larvae. Each box represents the mean ± standard error of the larvae weights during 5 days. Numbers in brackets represent the insect numbers in each class. *Values are significantly different from class D25–30.

mean weight showed an increase until the 25–30th days (D25–30). During that time the first prepupae appeared. Then the weight decreased slightly. The larvae inside the wheat grains are very susceptible to manipulation which lengthens their development time. Therefore we prefered to use weight rather than age to characterize the larvae.

Aposymbiotic larvae fed with wheat supplemented by ^{14}C-glycine

In the five different experiments 59 grains, each containing one-third- or fourth-instar larva, received 6 μl solution of ^{14}C-glycine. At the end of three experiments (2, 4 or 6 days later), a total of 55 insects were still alive. Eighteen larvae had become prepupae or pupae and 37 larvae could be analyzed.

Amino acid elution profile

A typical elution profile of free amino acids observed in an aposymbiotic larva supplemented with ^{14}C-glycine is shown in Fig. 3. The presence of sarcosine

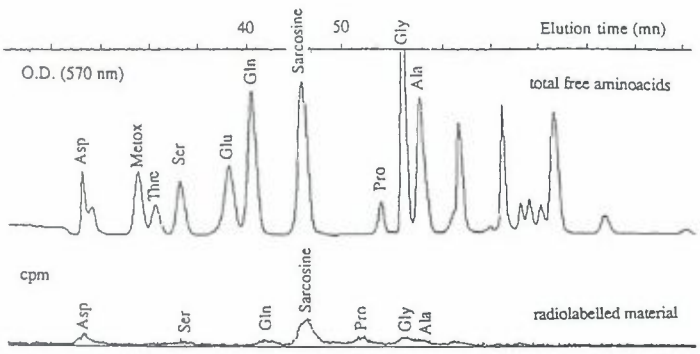

Figure 3. Elution profile of a *Sitophilus oryzae* radiolabelled larva which has been previously supplemented by ^{14}C-glycine. Cysthio = cystathionine.

was noticed in this aposymbiotic larva. The following experiments will specify the biosynthesis of sarcosine, a rare amino acid in insects.

Total sarcosine dosages

Total sarcosine was measured in the 37 above-mentioned larvae of *S. oryzae*. All larvae showed presence of sarcosine, the amount varying from 2.1 to 71.6 nmol/larva. If the larvae are put into 6 classes from 0–1 to 5–6 mg (W0 to W5) (Table 1), the rise of the mean amount of sarcosine was observed as a function of the weight from 10 to 31 nmol/larva.

The maximum of the mean amount of sarcosine was reached for class W3 (larvae of 3–4 mg weight). Then the amount of sarcosine decreased to 20 nmol/larva (Fig. 4A). If this weight is plotted on the insect weight curve of Fig. 3, it is noticeable that a 3–4 mg weight (class W3) is reached by fourth-instar

Table 1. Mean of total sarcosine amount in an aposymbiotic strain of *S. oryzae*, reared on wheat grains impregnated with ^{14}C-glycine (^{14}C-glycine diet)

Class	Weight (mg)	Mean Weight (mg)	Frequency	Mean sarcosine (nmol/insect)	Standard error
W0	0–1	0.77	5	10.4	4.81
W1	1–2	1.64	6	14.1	2.94
W2	2–3	2.49	6	21.3	5.51
W3	3–4	3.53	10	31.4	4.91
W4	4–5	4.26	10	20.4	5.73
P3	3–4	3.34	7	26.6	11.11
P2	2–3	2.51	2	2.4	0.38

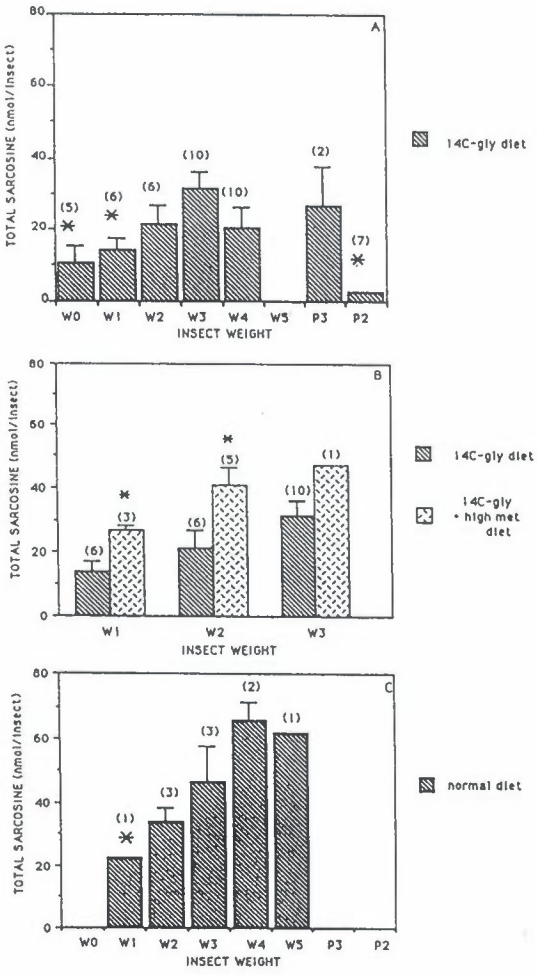

Figure 4. Sarcosine amounts measured as a function of the weights in *Sitophilus oryzae* larvae and pupae reared on wheat grains. W0... W5 represent larvae classes of weight 0–1 mg... 5–6 mg respectively. P_3 and P_2 represent pupae classes of weight 3–4 mg and 2–3 mg. Each box represents the mean ± standard error. Numbers in brackets represent the insect numbers in each class. (A) The grain containing the larva was opened twice: once during the fourth instar in order to deposit ^{14}C-glycine into the wheat grain and once for the sarcosine dosage. *values are significantly different from class W3 ($p < 0.05$ with PLSD Fischer test).(B) The grain containing the larva was opened twice: once time during the fourth instar in order to deposit ^{14}C-glycine and unlabelled methionine into the wheat grain and one time for the sarcosine dosage. *Inside a weight class, values are significantly different between the two diets ($p < 0.05$ with PLSD Fischer test).(C) The grain containing the larva was opened only one time for the sarcosine dosage. *Values are significantly different from class W4 ($p < 0.05$ with PLSD Fischer test).

25–30 day old larvae. As noted before, the emergence of the first prepupae occurred at the same time, 27 days after oviposition.

The total amount of sarcosine was also analyzed in nine pupae. All pupae showed the presence of sarcosine from 2.5 to 86.2 nmol/larvae. The pupae were divided into one class of 3–4 mg weight (P3) and one class of 2–3 mg weight (P2) (Table 1). The amount of sarcosine is shown in Fig. 4A. A net decrease can be observed between the two classes of pupae but it is not statistically significant because of the small number of pupae in class P2.

^{14}C-sarcosine and other radiolabelled amino acids assays

Radiolabelled amino acids have been measured in the larva feces but no measurable quantity of ^{14}C-sarcosine was detected.

Radiolabelled amino acids were analyzed in 35 larvae fed during 2, 4 or 6 days with wheat grains supplemented with ^{14}C-glycine. In the three experiments radiolabelled ^{14}C-glycine was detectable in 29 larvae, and 90% of these larvae contained radiolabelled sarcosine (Table 2).

Radiolabelled ^{14}C-sarcosine was also detectable in four among five prepupae or pupae containing ^{14}C-glycine at the end of the experiment.

The other radiolabelled amino acids detected were ^{14}C-serine (in 39% of the larvae), ^{14}C-proline (30%) and ^{14}C-alanine (26%).

Larvae fed with wheat supplemented by unlabelled methionine

In order to verify that biochemical synthesis of sarcosine occurs by glycine methylation as hypothesized by Gasnier-Fauchet et al. (1986), total free amino acid amounts were compared in the experiments when larvae were fed with wheat only supplemented with ^{14}C-glycine, and in one experiment in which 9 larvae received not only ^{14}C-glycine but also unlabelled methionine. The results showed (Fig. 5) that methionine did not cause any statistically significant

Table 2. Conversion of ^{14}C-glycine into ^{14}C-sarcosine by *S. oryzae* aposymbiotic larvae

	Experiment I	Experiment II	Experiment III	Total
Time of exposure to ^{14}C-GLY	2 days	4 days	6 days	
Number of larvae exposed to ^{14}C-GLY	5 larvae	16 larvae	14 larvae	35 larvae
Larvae with ^{14}C-GLY at the end of the experiment	3 larvae	12 larvae	14 larvae	29 larvae
Larvae with ^{14}C-SARC at the end of the experiment	3 larvae	9 larvae	14 larvae	26 larvae

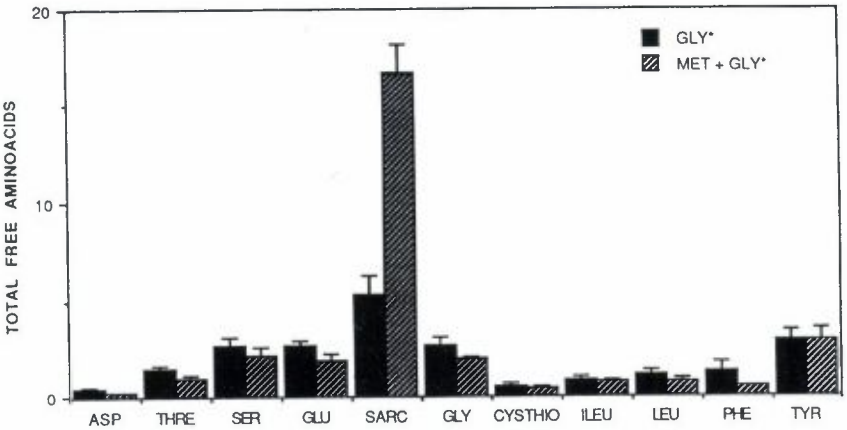

Figure 5. Total free amino acids in a *S. oryzae* larva fed with wheat supplemented by radiolabelled glycine with or without cold methionine.

Table 3. Mean of the total sarcosine amount in an aposymbiotic strain of *S. oryzae*, reared on wheat grains impregnated with ^{14}C-glycine and supplemented by methionine (^{14}C-gly + high met diet)

Class	Weight (mg)	Mean weight (mg)	Frequency	Total mean sarcosine (nmol/insect)	Standard error
W1	1–2	1.38	3	28.9	1.37
W2	2–3	2.63	5	40.7	5.45
W3	3–4	3.31	1	46.8	0

variation in the concentration of 9 free amino acids characteristic of *S. oryzae* haemolymph but, on the other hand, it induced a significant increase in the amount of sarcosine whose value was then multiplied by 2.5. This quantity of sarcosine was plotted as a function of the weight class in Table 3, and it was compared with the value obtained previously for the larvae supplemented only by ^{14}C-glycine. The results are shown in Fig. 4B. The values were significantly higher in larvae supplemented by methionine, compared to those with normal diet. When larvae mean weights increased from 1.38 to 3.31 mg, the sarcosine values also increased from 29 to 47 nmol/larva.

Aposymbiotic larvae fed with wheat not supplemented by ^{14}C-glycine

We have also measured the sarcosine in the larvae which received neither ^{14}C-glycine nor serine. Sarcosine was measured in 10 aposymbiotic fourth instar larvae weighing 1.80 to 5.10 mg. All larvae showed the presence of sarcosine from 22 to 72 nmol per larva. The larvae were distributed in 6 classes of weight,

Table 4. Mean sarcosine amount in an aposymbiotic strain of *S. oryzae*, reared on wheat grains (normal diet)

Class	Weight (mg)	Mean weight (mg)	Frequency	Mean sarcosine (nmol/insect)	Standard error
W0	0–1	–	0	–	–
W1	1–2	1.80	1	22.0	–
W2	2–3	2.30	3	34.0	4.62
W3	3–4	3.47	3	47.0	11.20
W4	4–5	4.60	2	66.0	5.52
W5	5–6	5.10	1	61.0	–

from 0–1 mg to 5-6 mg (W0 to W5), and the mean sarcosine concentration was calculated for each class (Table 4).

The mean sarcosine concentration was plotted as a function of the weight class and results are shown in Fig. 4C. The mean sarcosine concentration increased significantly from 22 to 66 nmol per larva for a mean weight increasing from 1.80 to 4.60 mg. As noted before for the larvae supplemented by ^{14}C-glycine, the amount of sarcosine decreased at the moment of the first prepupae emergence. Beyond this weight, the sarcosine amount decreased slightly.

4. Discussion

In this study, amounts of sarcosine were measured in *S. oryzae* larvae and pupae in order to compare with the results observed by Gasnier-Fauchet and Nardon (1986) in the same strain to complete the study. The total amount of sarcosine varied from 22 to 61 nmoles in our experiments and from 47 to 66 nmoles in those of Gasnier-Fauchet for the fourth instar larvae of comparable weights which have not received radiolabelled glycine. These amounts were similar in the two cases. When radiolabelled glycine was added, these total sarcosine amounts clearly decreased in all the weight classes. This may be explained by the fact that when the radiolabelled glycine was added, the larva were disturbed. This decrease in the amount of sarcosine following the addition of glycine in the diet, may also be due to the action of glycine on its own as noted previously (Gasnier-Fauchet, 1985). However, the addition of methionine and glycine at the same time was followed by a very important increase of the total amount of sarcosine.

The most important result contributing to elucidate the origin of sarcosine synthesis in aposymbiotic *S. oryzae*, is the labelling of sarcosine following the incorporation of ^{14}C-glycine. Although it has been previously hypothesized by Gasnier-Fauchet and Nardon (1986), evidence is presented in this work

supporting the existence of a biochemical pathway between the amino acids glycine and sarcosine in the insects. The analysis of the results allows two conclusions. Firstly the labelling of sarcosine after incorporation of ^{14}C-glycine was a rapid event: wheat grains opened only 48 hr after ^{14}C-glycine injection revealed that larvae already contained ^{14}C-sarcosine. Secondly the biochemical pathway between glycine and sarcosine was a major one since more than 90% of the labelled larvae contained ^{14}C-sarcosine. This was not the case for other amino acids like ^{14}C-serine, ^{14}C-proline and ^{14}C-alanine, which were found in less than 40% of the larvae containing ^{14}C-glycine. These two features of the ^{14}C sarcosine labelling support the concept of a direct and major pathway of sarcosine synthesis from glycine and methionine.

The emergence of ^{14}C-sarcosine, ^{14}C-serine and ^{14}C-phospholipids (unpublished results) after ^{14}C-glycine incorporation into *S. oryzae* diet proves the existence of the biochemical pathways 1, 2 and 3 in the choline cycle (Fig. 6), until now known only in mammals. Furthermore the reversibility of reaction 2 is

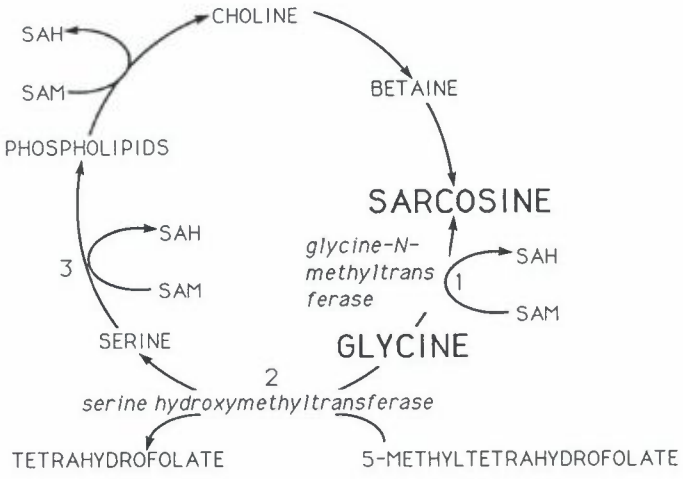

Figure 6. Choline cycle as known in mammals after Finkelstein (1975) and Lehninger (1975).

demonstrated in *S. oryzae* by the appearance of ^{14}C-glycine and ^{14}C-sarcosine after the incorporation of ^{14}C-serine into the diet (data not shown).

The results concerning the high level of sarcosine, following the synthesis and the accumulation of this amino acid in aposymbiotic larvae of *S. oryzae*, have already been fully developed and compared to the low levels observed in symbiotic larvae by Gasnier-Fauchet and Nardon (1984, 1986). Moreover,

these authors observed a high level of methionine sulfoxide in symbiotic compared to aposymbiotic strains, and they concluded that the elimination of bacteria in *S. oryzae* particularly affects the methionine metabolism. They showed that the symbiotes are involved in the oxidation of methionine into methionine sulfoxide. In the absence of symbiotes, the excess of methionine present in the wheat grains is transformed into sarcosine, which accumulates in aposymbiotic larvae. Our results confirm this sarcosine accumulation during the development of aposymbiotic larvae. Nevertheless the reason for such an accumulation during the larval life remains an unanswered question. A partial answer is given by analysis of the feces, where no ^{14}C-sarcosine was detected. Thus sarcosine is not eliminated in the feces. Furthermore it is known that this amino acid is not incorporated into proteins. As sarcosine accumulates, it could be possible that this amino acid is not reversely degraded into glycine, like it is in mammals (inverse of reaction 1 in the Fig. 6), because dehydrogenase, which converts sarcosine into glycine, is inhibited during the larval life.

Between larvae W3 and pupae P2, sarcosine amounts decreased more than 10 times. This observation is in full agreement with those of Gasnier-Fauchet and Nardon (1986), according to which sarcosine amounts decreased from about 45 nmol/larva to less than 5 nmol/larva between larvae at the end of the fourth instar and insects in end of pupae instar. Gasnier-Fauchet et al. (1986) suggested that this decrease of the sarcosine amounts after the pupal molt was the consequence of its elimination with the exuvial fluid during pupal ecdysis. It could also be possible that the dehydrogenase inhibited during the larval life, is derepressed at the pupal molt, transforming sarcosine into glycine. Finally sarcosine, and also glycine, could also be converted into CO_2, as reported for rats (Javilier et al., 1967). This could explain the great quantities of $^{14}CO_2$ eliminated by the larvae after ^{14}C-glycine incorporation. Thus sarcosine could be an energy storage molecule, and its catabolism could be used by the larvae for the molt which requires a large energy supply. The exact catabolic pathways of this amino acid still remain to be elucidated. A way to pursue this investigation could study the fate of ^{14}C-sarcosine after its incorporation in the *S. oryzae* diet.

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