UREA CYCLE IN THE SILKWORM, BOMBYX MORI

Tamio Inokuchi, Yasuhiro Horie, and Toshio Ito

Sericultural Experiment Station, Suginami-ku, Tokyo, Japan

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Information on the synthesis of urea in insects is limited (Gilmour, 1961; Chefurka, 1965), and the evidence for the existence of the ornithine cycle in insects is also scanty. In larvae of the silkworm, Bombyx mori L., Garcia et al. (1956) have suggested the presence of this cycle based on the identification of arginine, ornithine, citrulline, and urea, and Hayashi (1961) has reported the formation of urea from arginine, however, the direct biochemical demonstration is unavailable on this cycle. In the earlier nutritional studies of the silkworm arginine and proline have been demonstrated to be essential, in addition to other nine essential amino acids (Arai and Ito, 1964; Ito and Arai, 1965), but the subsequent experiment has revealed that proline is semi-essential (Arai and Ito, 1967). In the present paper it will be reported that proline requirement can be spared by some amino acids in urea cycle, and that a pathway from citrulline to proline via arginine and ornithine, with the formation of urea, occurs in the silkworm.

The larvae of the silkworm were reared on an amino-acid diet throughout. The methods of formulation of the diet and of rearing of larvae have been reported previously (Ito and Arai, 1966). In the nutrition tests the newly hatched larvae were reared for a period of 15 days. The nutritional tests showed that the growth retardation due to the deficiency of dietary proline was more or less reduced by adding either glutamic acid, ornithine, or arginine into the diet. The sparing effect of arginine for proline requirement

was lowered, when ornithine was added to the diet simultaneously. Furthermore, citrulline showed a sparing effect for arginine, whereas ornithine did not possess any sparing effect for arginine, as far as the diet contained a sufficient amount of proline. These nutritional results suggest that the pathways from citrulline to arginine and from arginine to ornithine operate actively in larvae of the silkworm.

Subsequently, the fourth-instar larvae, grown on amino-acid diets, were injected with radioactive compounds, and after 5 hours the whole larvae were homogenized in an ice-cold, 10 % trichloroacetic acid (TCA). The homogenates were centrifuged at 5°C, and the precipitate was washed three times with an ice-cold, 5 % TCA. The precipitate was then washed once with a mixture of ethyl ether and ethanol (1 : 1) and three times with ethyl ether, and was hydrolyzed with 6 N HCl at 110°C for 20 hours.

TCA-soluble fraction and three washings were combined, and washed with ethyl ether several times for removal of TCA. This fraction, containing free amino acids, was concentrated, and added to the top of a column of Dowex-50-H^+-X8 (200 - 400 mesh; 0.9 x 10 cm). The amino acids were eluted with 2 N ammonia, and the eluate was concentrated. This fraction is referred to as the free amino acid fraction.

Aliquots of the free amino acid fraction and of the hydrolyzate of protein were subjected to two-dimentional separation by thin-layer chromatography on silica-gel G. The ninhydrin-positive areas corresponding to arginine, proline, glutamic acid, and aspartic acid were scraped off the plate into tubes, and each amino acid was extracted with 80 % ethanol. Radioactivity was determined in a gas-flow counter.

When the free amino acid fraction of the larvae, which had been injected with L-citrulline-ureido-^{14}C, was chromatographed, arginine and urea contained a strong radioactivity (Fig. 1). When protein hydrolyzate of the same larvae was analyzed, a strong radioactivity was found in arginine (Table 1). Thus, it is concluded that the silkworm is capable of urea formation from

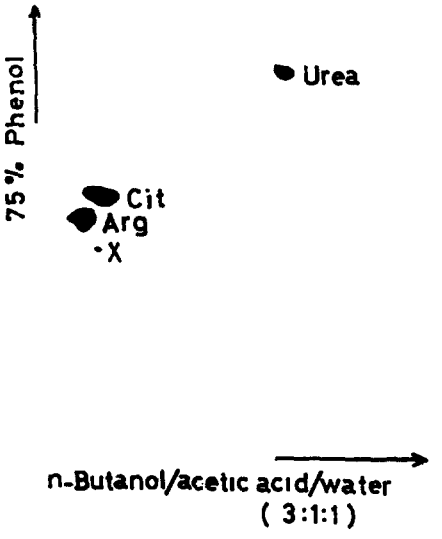

Fig. 1. Autoradiograph of free amino acid fraction of larvae after injection of L-citrulline-ureido-^{14}C.
X, un-identified compound.

Table 1. Specific activities of carbon from amino acids of hydrolyzed protein of larvae after injection of L-citrulline-ureido-^{14}C (1 μCi/larva)

Amino acid	Radioactivity (cpm/mg protein)	
	Arginine added in the diet	Without arginine in the diet *
Arginine	1539 ± 27.8	2221 ± 33.3
Proline	28 ± 3.9	31 ± 4.4
Glutamic acid	13 ± 2.6	5 ± 1.6
Aspartic acid	24 ± 3.5	23 ± 3.5

* Larvae were kept for 24 hours, then analyzed.

citrulline via arginine. It is interesting to note that higher radioactivity (1.44 times) is found in arginine from the larvae, which have been transferred to the diet lacking arginine only for 24 hours, as compared to the value obtained with the larvae still feeding the same complete diet containing arginine (Table 1). It is considered that the dietary condition regulates

the metabolic rate of citrulline to arginine, which is one of the essential amino acids.

Subsequently, the larvae were injected with L-arginine-^{14}C(U). When the free amino acid fraction was analyzed, it was found that ornithine was strongly labelled. Furthermore, the silkworm was found to be able to convert arginine to proline, from the analysis of larval protein (Table 2). Much higher incorporation into proline (3.29 times) was obtained on the diet lacking proline than the rate obtained with the complete diet containing proline. On the other hand, 76 % radioactivity was found in arginine of the protein, when the larvae were allowed to feed the diet lacking proline, as compared to the normal larvae. These results indicate that the silkworm metabolizes argi-

Table 2. Specific activities of carbon from amino acids of hydrolyzed protein of larvae after injection of L-arginine-^{14}C(U) (0.5 μCi/larva)

Amino acid	Radioactivity (cpm/mg protein)	
	Proline added in the diet	Withour proline in the diet *
Arginine	6195 ± 55.1	4739 ± 48.6
Proline	362 ± 13.5	1192 ± 24.4
Glutamic acid	27 ± 3.6	46 ± 4.8
Aspartic acid	20 ± 3.2	45 ± 4.8

* Larvae were kept for about three days, then analyzed.

Table 3. Specific activities of carbon from amino acids of hydrolyzed protein of larvae after injection of DL-ornithine-5-^{14}C (1 μCi/larva)

Amino acid	Radioactivity (cpm/mg protein)	
	Proline added in the diet	Without proline in the diet *
Arginine	50 ± 3.2	70 ± 3.7
Proline	2591 ± 22.8	4441 ± 28.2
Glutamic acid	31 ± 2.7	37 ± 2.7
Aspartic acid	37 ± 2.7	22 ± 2.3

* See the footnote of Table 2.

nine to proline more rapidly in the absence of dietary proline than in the presence of proline, which is a semi-essential amino acid.

Furthermore, a marked incorporation of ^{14}C into proline of the protein was demonstrated, after the injection of DL-ornithine-5-^{14}C, whereas only very slight recovery in arginine was obtained (Table 3). The incorporation rate into proline was also higher in the absence of dietary proline (1.71 times) than that on the complete diet. The data shown in Tables 2 and 3 indicate that the silkworm converts arginine to proline via ornithine rather rapidly. However, the incorporation of ^{14}C into citrulline after the injection of radioactive ornithine was found to be very little.

The present study revealed the occurrence of the metabolic pathway from citrulline to proline via arginine and ornithine, with the formation of urea, in larvae of the silkworm, not only from the nutritional tests but also from the direct radiochemical experiments. The present data indicate that the conversion of ornithine to citrulline hardly occur, or may occur very slightly, if any, in the silkworm. Thus, it is concluded that the urea cycle operates incompletely in this insect. The fact that the essentiality for proline is much lower than the usual ten essential amino acids is interpreted to reflect that the proline biosynthesis in the larval body fulfils only a part of proline requirement. It is most interesting that the metabolic activity from citrulline to proline is largely dependent on the dietary conditions, such as the presence or absence of the metabolite involved. Nutritional requirements are thus understood to be reflections of metabolic events or of metabolic regulations.

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