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Nitrogen and Free Amino Acids in the Haemolymph of the *Philosamia*-Pupae induced to Diapause by Brain Extirpation*

Sohji TAKAHASHI

Zoological Institute, College of Science, University of Kyoto

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It is well known that the pupae of lepidopterous insects are caused to diapause by the deficiency of the brain hormone (WILLIAMS, 1946, 1947, ICHIKAWA and NISHIITSUTSUJI, 1951, ICHIKAWA and ISHIZAKI, 1958, FUKUDA, 1958). In nature, *Philosamia cynthia ricini* does not enter diapause in any stage of its life cycle, but it is artificially induced to diapause by means of the brain extirpation (ICHIKAWA, 1956). The respiration of the brainless pupae thus produced falls down gradually and reaches the minimum level about 2 weeks later (KAWAI and ICHIKAWA, 1959). This situation remains so long as the pupae do not receive the brain implantation or administration of the brain hormone (ICHIKAWA and ISHIZAKI, 1961, 1963). But, if such pupae are provided with the active brains, the respiration rises again and the adult differentiation ensues. Consequently, the brainless pupae of the present species are to be equivalent in the physiological conditions as the diapausing pupae of other species.

Knowledge is essential for studies of insect physiology of comparing the metabolism between the brainless diapausing pupae and the normal or awakened pupae. In the present report, therefore, changes of the total nitrogen, protein nitrogen, non-protein nitrogen and free amino acids in the haemolymph are dealt with.

I wish to express my hearty thanks to Prof. Mamori ICHIKAWA under whose supervision the present study was carried out.

Materials and Methods

Pupae of *Philosamia cynthia ricini* were used as materials. Experiments were performed at 25°C. Arbitrary developmental stages were made with reference to the cuticle coloration in the case of young pupae and the degree of adult formation specified by dissection in the case of old pupae, as is shown in Table 1.

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Table 1. Developmental Stages of *Philosamia*-Pupae.

Stages	Days after pupation (25°C)	Remarks
0	0	Within 24 hours after pupation.
1	1—2	Cuticle brownish yellow. Remarkable degeneration of the silk glands.
2	3—5	Cuticle dark brown and becomes hard.
3	6—8	Slight dissociation of fat body cells.
4	9—11	Complete dissociation of fat body cells, and visible development of pigmented eyes. Brain enlarges and softens.
5	12—14	Development of wings, legs and antennae. Pattern of wing faintly discernible. Marked entwining of tracheae around the brain.
6	15—16	Color pattern of wing distinct.
7	17—18	Adult emergence.

Preparation of test animals: The brain was extirpated after ICHIKAWA's method (1959) between 6 and 12 hours after pupation. The brainless pupae thus operated were kept at 25°C. They received brain implantation later, and were also placed at this temperature.

Sampling and fractionation of haemolymph: Samples of haemolymph were obtained from the pupae through the incision made on the thorax. When the adult formation proceeded, the animals were beheaded and bled. When needed, samples of larval haemolymph were collected by means of cutting the coxal segments of thoracic legs.

Haemolymph of 10 specimens in respective stage was collected in each centrifuge tube with a special caution about sex, and centrifuged at $500 \times g$ for 10 minutes. The surface layer of fat and the bottom layer of cells precipitated were discarded. The middle layer alone was used for analysis, because total nitrogen was to be contained here.

Nitrogen analysis: Nitrogen content was determined by the micro-KJELDAHAL's method. Ten percent trichloroacetic acid (TCA) was added to 0.5 ml of the middle layer to give a 5% final concentration. This was centrifuged at $2000 \times g$ for ten minutes. The precipitate was separated from the supernatant, and one drop of 50% TCA in 50% ethanol was added to the supernatant. The precipitate thus obtained was mixed with the former precipitate. The precipitate was estimated as protein fraction and the supernatant, non-protein fraction.

Amino acid analysis: All of the amino acids were to be contained in non-

protein fraction. Therefore, with the supernatant, two dimensional ascending paper chromatography was carried out on Toyo Roshi paper, No. 50 (Toyo Roshi Co., Tokyo). Two kinds of solvents were used: (1) saturated aqueous phenol, (2) saturated aqueous mixture of *n*-butanol and acetic acid (4: 1). When the former solvent was used, chromatograms were developed in a saturated ammonia vapor. Spots of free amino acids were detected with 0.2% ninhydrin in butanol. Further, paper electrophoresis was applied to the amino acids analysis. Toyo Roshi paper No. 51, pH 6 phtharic acid buffer solution and 400 volts (0.5mA/cm) gave a successful separation of glutamic acid from other amino acids.

Results

Since all the results showed no difference due to the sex, following descriptions are made regardless of the sex.

1. *Nitrogen Changes*

Normal pupae: Total nitrogen shows a high level in the mature larvae and decreases to the minimum level at pupation. It recovers to 1.38g percent of the haemolymph 2 days after pupation. Two days before emergence, total nitrogen decreases abruptly to 59% of that in Stage 6. As is shown in Table 2, protein nitrogen is always more than 60% of the total nitrogen, but then falls to 50% when the adult formation becomes nearly completed. On the contrary, non-protein nitrogen shows a low level in Stage 0, and then increases to 50% in Stage 6.

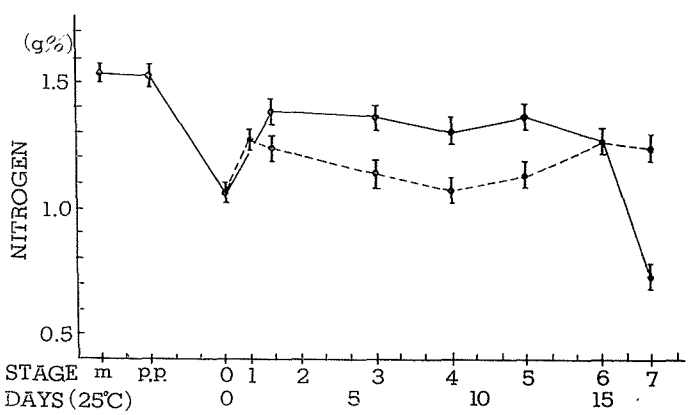

Fig. 1

Changes of total nitrogen of normal pupae (solid line) and brainless pupae (dotted line). Each value is the mean of two samples from males and two samples from females; no significant difference between sexes can be found. The range of each value shows a standard deviation.
m : mature larvae, p.p. : prepupae.

Table 2. Protein nitrogen and non-protein nitrogen in the haemolymph.

Stage	Total nitrogen (g%)	Protein nitrogen (g%)	Protein N per Total N	Non-protein nitrogen (g%)	Non-protein N per Total N
Normal					
Mature larvae	1.53	1.15	0.74	0.38	0.26
Middle prepupae	1.52	1.02	0.67	0.50	0.33
Stage 0	1.03	0.75	0.69	0.33	0.31
Stage 5	1.36	0.82	0.62	0.54	0.38
Stage 6	1.27	0.63	0.50	0.64	0.50
Brainless					
10-day pupae	1.28	0.56	0.44	0.72	0.56

Each value is the mean of two samples from males and two samples from females; no significant difference between sexes can be found.

Brainless diapausing pupae: Haemolymph of brainless pupae indicates a slight increase of total nitrogen one day after operation, though it is not so high as in the control pupae without receiving the operation. Total nitrogen keeps an approximately constant level of 1.2g percent for subsequent 100 days. Non-protein nitrogen is always nearly 60% of the total nitrogen, and this value is also stable so long as the pupae remain in diapausing state.

2. *Free Amino Acids*

Free amino acids identified in the haemolymph of the normal pupae are glycine, alanine, valine, methionine, leucine (and/or isoleucine), serine, threonine, aspartic acid, glutamic acid, lysine, arginine, tyrosine, proline, histidine and glutamine. In addition, there are 4 polypeptides.

In the haemolymph of the brainless pupae, there are glycine, alanine, valine, methionine, leucine (and/or isoleucine), serine, threonine, aspartic acid, lysine, arginine, tyrosine, proline, histidine, glutamine and 4 polypeptides.

A comparison of free amino acids between the normal and brainless pupae indicates that glutamic acid alone lacks in the latter haemolymph. Glutamic acid in the haemolymph of brainless pupae begins to decrease on the 4th day after the brain extirpation and can be hardly detectable 6 days later. In 30-day brainless pupae, glutamic acid can not be detectable at all.

3. *Recovery of Glutamic Acid due to Brain Implantation*

Five brains from the 5th instar larvae or 4 brains from pupae in Stage 5 were implanted into the brainless pupae through a hole made on the 4th abdominal segment (Table 3). Fifteen days after implantation, the haemolymph was collected. After blood-letting, the pupae were dissected to ascertain the developmental state towards adult. Autopsy indicates that the pupae develop at the same tempo as the normal pupae do after pupation. Recovery of glutamic acid is complete and to the same

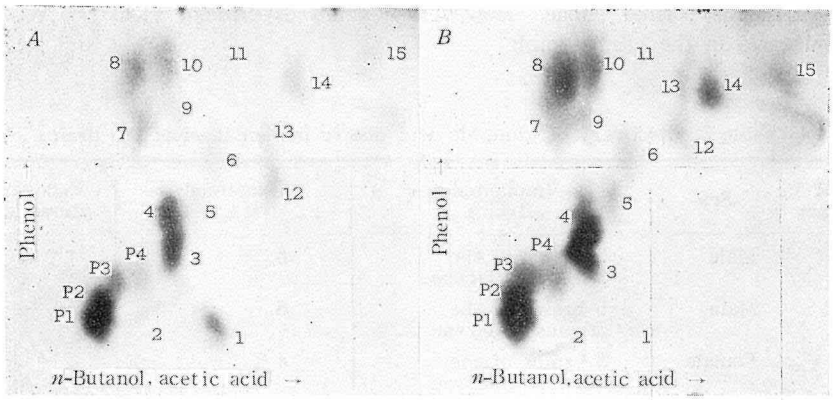

Fig. 2

Paper chromatograms of free amino acids and polypeptides in the haemolymph of normal pupae in Stage 3 (*A*) and seven-day brainless pupae (*B*).

1. glutamic acid, 2. aspartic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. histidine, 8. lysine, 9. glutamine, 10. argine, 11. proline, 12. tyrosine, 13. valine, 14. methionine, 15. leucine (and/or isoleucine), P1, P2, P3 and P4. polypeptides.

Spots of proline which turn out yellow after the ninhydrin reaction can not be recognized in these photographs. A labeling 11 indicates the location of the spots.

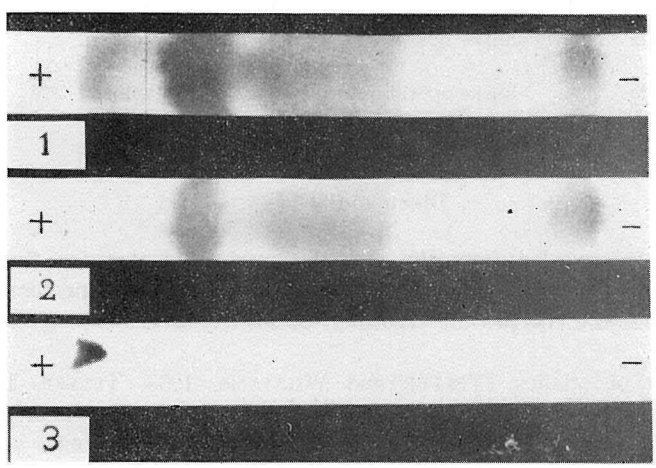

Fig. 3

Patterns of amino acids and polypeptides as revealed by paper electrophoresis.

1. Normal pupae in Stage 3. 2. Seven-day brainless pupae, 3. Glutamic acid.

level as in the normal pupae. However, a sham experiment yields no recovery of glutamic acid in the haemolymph.

Table 3. Recovery of glutamic acid due to implantation of the brains.

Test number	Sex	Implanted Brains	Examined stage	Recovery of glutamic acid
1	Male	5 brains of the 5th instar larvae	6	+
2	Male	5 brains of the 5th instar larvae	6	+
3	Female	5 brains of the 5th instar larvae	4–5	+
4	Female	5 brains of the 5th instar larvae	5–6	+
5	Female	5 brains of the 5th instar larvae	5	+
6	Male	4 brains of pupae in Stage 5	5	+
7	Male	4 brains of pupae in Stage 5	5	+
8	Female	4 brains of pupae in Stage 5	4	+
9	Female	(Sham operation)	Undeveloped	−
10	Female	(Sham operation)	Undeveloped	−
11	Male	(Sham operation)	Undeveloped	−
12	Male	(Sham operation)	Undeveloped	−

Discussions

It has been revealed from the present experiments that the deficiency of the brain hormone affects *Philosamia* to cause changes of nitrogen compounds in the haemolymph, but the changes are recovered completely by supplying the brains from outside.

A number of studies (TELFER and WILLIAMS, 1953, TELFER, 1954, LAUFER, 1959, 1960) have already shown that such changes of haemolymph proteins occur during the normal post-embryonic development of the silk moths. In the normal development, the decrease of total nitrogen before pupation may be due to the utilization for synthesis of new pupal tissues, and its increase after pupation may be due to the outflow of nitrogen compounds from dissociated larval tissues respectively (WIGGLESWORTH, 1950). That total nitrogen decreases again previous to adult emergence (Stage 6) seems to be a general phenomenon in holometabolic insects

(BUCK, 1953, LAUFER, 1960). This will be due to the utilization of the haemolymph components for adult histogenesis. Fifteen amino acids and 4 polypeptides are identified by means of paper chromatography in the haemolymph of the present *Philosamia*. It is interesting and important to follow more detailed changes of these amino acids and polypeptides. But, mention is limited on the behavior of the glutamic acid in the present study.

The disappearance of glutamic acid in the haemolymph of pupa deprived of the brain is a very surprising fact, because existence of this amino acid is a rule in the many kinds of insects including intact *Philosamia*. Now, various metabolic pathways have been established concerning the transformation of glutamic acid into other amino acids and *vice versa*. It is impossible, however, to surmise possible pathway responsible for the present fluctuation of glutamic acid.

Recently, SHIGEMATSU (1960) demonstrated that in *Bombyx*, globulins are synthesized in the fat body cell and released into the haemolymph. In addition, ISHIZAKI (1963) has observed that by means of electron microscopy marked changes occur in the ultrastructures of the *Philosamia* fat body cell when the insect enters the prepupal stage. These informations seem to suggest a possibility that the fat body cell is related intimately to other nitrogen compounds in the haemolymph. If it is the case, the deficiency of glutamic acid in the haemolymph may be a sequence of metabolic changes occurring in such cells or tissues as the fat body. The disappearance of glutamic acid in the case of brain extirpation and its recovery after the brain implantation may hint that the pathway of glutamic acid is governed by the brain hormone.

Summary

1. The brainless diapausing pupae of *Philosamia cynthia ricini* were compared with the normal pupae in terms of nitrogen content in the haemolymph.

2. Total nitrogen, protein nitrogen and non-protein nitrogen indicate marked differences from those of the normal pupae.

3. Fifteen amino acids and 4 polypeptides are identified in the haemolymph of the normal pupae by means of paper chromatography and paper electrophoresis. But, glutamic acid is absent in the haemolymph of the brainless diapausing pupae.

4. The brain implantation into the brainless pupae brings about the recovery of glutamic acid in the haemolymph and resumption of development of the recipient.

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