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The Presence of Tyrosine Glucoside in the Haemolymph of Lepidopteran Insects

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Abstract A ninhydrin-positive substance from the haemolymph of *Papilio xuthus* was purified and identified as β-glucosyl-O-tyrosine by (1) color reactions, (2) incorporation of ^{14}C-tyrosine, (3) identification and estimation of hydrolysis products, (4) α-and β-glucosidase tests, and (5) UV-spectrum.

The concentration of the tyrosine glucoside in haemolymph reaches a maximum at the prepupal stage, then decreases, and is on a low level during the middle stage of pupa. At the late pupal stage, the level again rises and is kept high before emergence. After emergence, it rapidly decreases.

The same tyrosine glucoside has proved to be also present in the haemolymph of twelve other species of Lepidoptera.

Introduction

In Diptera, some tyrosine conjugates are said to serve as a tyrosine doner or tyrosine reservoir for the sclerotization of cuticle at the time of puparium formation (Bodnaryk, 1972). These tyrosine conjugates increase before puparium formation and then rapidly decrease. Among them, tyrosine-O-phosphate in *Drosophila* (Mitchell and Lunan, 1964 ; Lunan and Mitchell, 1969) and β-alanyl-tyrosine in *Sarcophaga* (Levenbook et al., 1969 ; Bodnaryk, 1970a, 1971a,b) are well known. Tyrosine-O-prosphate has been reported to be present also in the haemolymph of newly emerged adults of *Sarcophaga* (Seligman et al., 1969). Recently, in addition, β-glucosyl-O-tyrosine has been found in *Drosophila busckii* (Chen et al., 1978). Moreover, γ-glutamyl-phenylalanine was reported to play such a role in *Musca* (Bodnaryk, 1970b).

In the haemolymph of Lepidoptera also, two tyrosine-containing substances, tyrosine-O-acetylDOPAmine (Sienkiewicz and Piechovska, 1973) and an unidentified substance (Junnikkala, 1976) have been reported. These two tyrosine conjugates too increase before pupa formation and then decrease.

In the course of the investigation of haemolymph amino acids of the papilionid butterfly, *Papilio xuthus*, we found a large quantity of tyrosine conjugate in the

haemolymph. The present paper deals with (1) purification and identification of the tyrosine conjugate, (2) the change of concentration from the fifth inster of larva to adult, and (3) the distribution in Lepidoptera.

Materials and Methods

Materials

Papilio xuthus was raised from egg or early larval stage in our laboratory under the photoperiod of 15 hr light and 9 hr dark, at 25±1°C. Under this condition, the pupal period was 10 to 14 days.

In order to examine the distribution of tyrosine glucoside in Lepidoptera, the following twelve species other than *P. xuthus* were also raised in the laboratory: *Maruca testulalis* (Pyralididae), *Bombyx mori* (Bombycidae), *Phalera flavescens* (Notodontidae), *Theretra japonica* (Sphingidae), *Cephanodes hyalis* (Sphingidae), *Polygonia c-aureum* (Nymphalidae), *Lampides boeticus* (Lycaenidae), *Papilio protenor* (Papilionidae), *Papilio helenus* (Papilionidae), *Papilio bianor* (Papilionidae), *Pieris rapae* (Pieridae), and *Pieris melete* (Pieridae). The haemolymph of these species was taken during the larval and pupal stages and examined for tyrosine conjugate.

Extraction, separation, and crystallization of tyrosine glucoside

Haemolymph was withdrawn with a microcapillary (Drammond microcaps, 100 μl) and was immediately deprotenized with an equal volume of 10% trichloroacetic acid (TCA). After centrifugation, the supernatant was applied to a Bio-gel P-2 column (1×65cm), which had been equilibrated with 0.2 N acetic acid. Elution was performed with the same acetic acid at a rate of 5.3 ml per hr. Fractions of 2.5 g were collected, and the absorbance of each fraction was measured at 280 nm.

A typical elution pattern is shown in Fig.1. The tyrosine conjugate which was described in the present paper was removed in fractions 18 to 20. This tyrosine conjugate is called T-substance in the present paper. The exact fraction tubes which contained the T-substance were confirmed by taking the UV-absorption spectrum of each fraction. The T-substance-containing fractions were combined

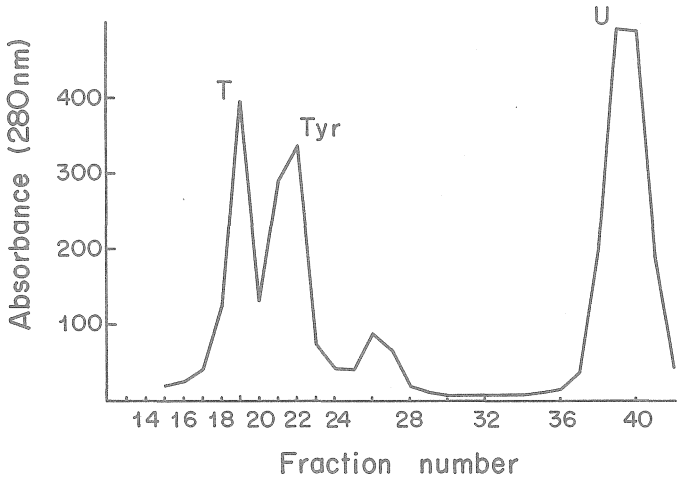

Fig. 1. Elution pattern of deprotenized haemolymph from Bio-gel P-2 column with 0.2 N acetic acid.
T, T-substance; Tyr, free tyrosine; U, uric acid.

and were applied to the Dowex 50×4 (H^+ type) column (1.2×16cm) The column was washed first with about 50 ml of water, by which TCA was removed. Then, the T-substance was eluted with 2 N ammonia water. Fifteen ml of the early part of ammonia effluent was taken and evaporated to dryness in a rotary evaporator at 40°C. The residue was dissolved in water, put in to a 10 ml test tube, and evaporated to dryness in a vacuum desiccator.

The final dry material was dissolved in a little water, and the insoluble part was removed by centrifugation. After the supernatant was warmed at 80°C, ethanol was added dropwise until the solution became slightly turbid. The solution was cooled to room temperature, and then kept at 4°C overnight. By this procedure, the T-substance crystllized in colorless rods (Fig. 2).

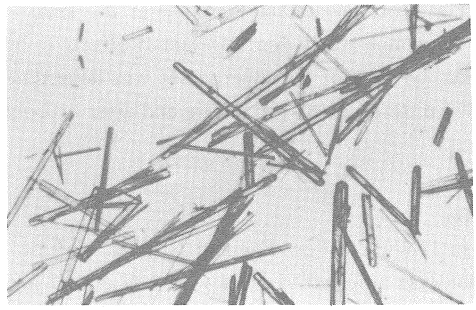

Fig. 2. Crystals of the T-substance.

Thin-layer chromatography

The presence or absence of the T-substance was examined by two-dimesional chromatographies. Pre-corted cellulose and silica gel thin-layer sheets (Merck, No.5552 and No.5553, respectively) were used. Solvents for cellulose sheet were 70% ethanol (70% EtOH) and a mixture of *n*-butanol-acetic acid-water (12 : 3 : 5) (BAW). For the sillica gel sheet, a mixture of methanol-chloroform-17% NH_3 *aq* (2 : 2 : 1) and phenol-water (5 : 1) (PhW) were used. For identifying the hydrolysate of T-substance also, the same two-dimensional chromatographies were performed.

For the identification of suger, one-dimensional thin-layer chromatography was also carried out with the cellulose sheet. The solvent was a mixture of pyridine-ethyl acetate-acetic acid-water (5 : 5 : 1 : 3), the upper layer of *n*-butanol-pyridine-acetic acid-water (5 : 1 : 3 : 3) or PhW.

The following detection reagents were used: (1) ninhydrin, (2) 2% phosphomolybdic acid-NH_3 for phenolic compounds (Riley, 1950), (3) aniline hydrogen phthalate for sugars, (4) naphthoresorcine-phosphoric acid for sugars (Prey et al., 1961), and (5) aniline-diphenylamine phosphate for sugars (Buchan and Savage, 1952).

Hydrolysis

In order to identify the hydrolysis products of T-substance, the purified T-substance was refluxed in 1 N HCI at 100°C for 30 min. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in water, and submitted to two-dimensional chromatography.

In order to decide the configuration of glycosidic bond between tyrosine and glucose, the T-substance was submitted to a slight modification of Burnet's method (Koeppe and Mills, 1974, 1975). The purified T-substance was dissolved in water and spotted on a cellulose thin-layer sheet (10×10cm) for two-dimensional chromatography. After being developed with BAW in the first direction, the sheet was dried, sprayed with a glucosidase solution, and incubated at 37°C. The incubation was performed

in a plastic bag, the inner surface of which was lined with some paper towels soaked with 0.1M ammonium acetate buffer. After 2 hr incubation, the cellulose thin-layer sheet was dried and again chromatographed with BAW in the second direction. The chromatogram thus obtained was submitted to one of the above-mentioned color tests. The glucosidase was either α-glucosidase (Sigma, No. G-5003, from yeast, Type I) or β-glucosidase (Sigma, No. G-8625, from almonds). The former enzyme was used at a concentration of 0.5% in 0.1M ammonium acetate buffer (pH 6.8) and the latter, at a concentration of 0.3% in 0.1M ammonium actate buffer (pH 5.2).

Estimation of tyrosine and glucose

Tyrosine was determined with 1-nitroso-2-naphthol (Udenfriend and Cooper, 1952), and glucose was estimated with the phenol-sulfuric acid method (Dubois et al., 1956). A bound form of tyrosine was estimated as follows: Haemolymph was withdrawn quantitatively (5-50 μl), and 2.5 ml of 5% TCA was added. After centrifugation, tyrosine of the supernatant was determined before and after hydrolysis (1N HCl, 100°C, 30 min). The difference between before and after hydrolysis was referred to as the bound tyrosine.

Incorporation of ^{14}C-tyrosine

L-[U-^{14}C] Tyrosine in 1 N HCl (New England Nuclear, specific activity, 527.0 mCi/m mol) was used. The original solution was adjusted to pH 4 with 1 N NaOH and injected into the haemocoel of pupa (50 μl, 2.3×10^{-1} μg tyrosine per pupa). Twenty or forty-two hr after injection, the haemolymph was withdrawn and deprotenized with 99.5% ethanol. After centrifugation, the supernatant was streaked on the cellulose thin-layer sheet and developed one-dimensionally with BAW. Radioactivity of the chromatogram was measured with a Berthold radioactive chromatogram scanner.

Results

Presence of tyrosine conjugate in haemolymph

The haemolymph from pupal stage of *P. xuthus* was deprotenized with TCA and applied to the Bio-gel P-2 column as described in the section of Materials and Methods, and the elution pattern as shown in Fig. 1 was obtained. Each fraction was hydrolyzed in 1 N HCl at 100°C for 30 min, and the hydrolysate was submitted to two-dimensional thin-layer chromatography. The hydrolysate of the fractions 18 to 20 of fig. 1 showed the presence of a large quantity of tyrosine. The position of free tyrosine in Fig. 1 was fraction 21 to 23. Therefore the fractions 18 to 20 were presumed to contain a kind of tyrosine conjugate, which was named T-substance.

The haemolymph of pharate adult (late pupal stage) was withdrawn and added to two volumes of 99.5% ethanol. After centrifugation, the supernatant was submitted to two-dimensional cellulose thin-layer chromatography. The chromatogram obtained with ninhydrin is shown in Fig. 3a. In addition to ordinary free amino acids, a spot which gave a dark brownish purple color with ninhydrin was present. The spot was named "T". Furthermore, the area corresponding to this spot in one-dimensional chromatogram developed with BAW was scraped and extracted with water. After centrifugation, the supernatant was hydrolyzed in 1 N HCl. After the hydrolysate was

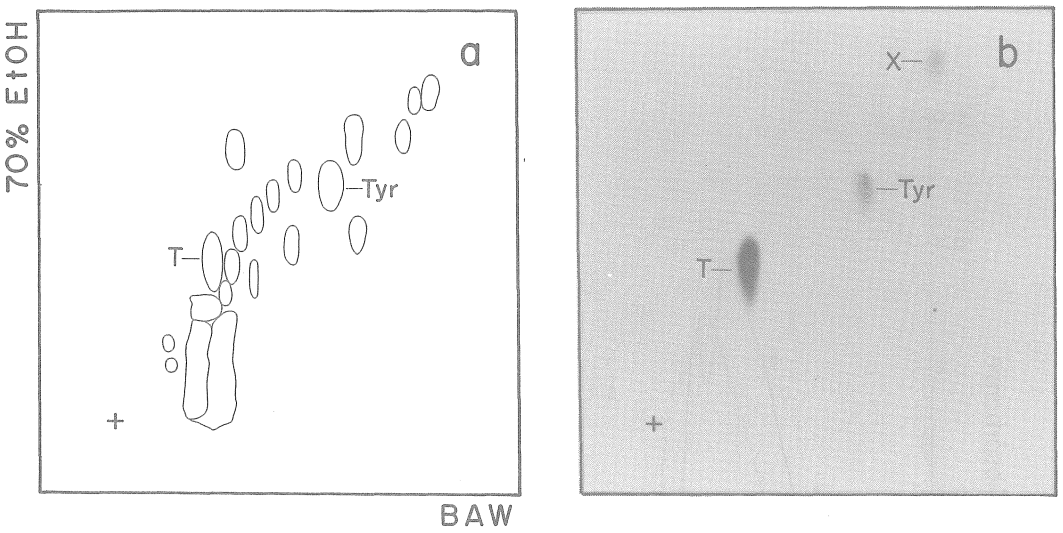

Fig. 3. (a) Two-dimensional chromatogram of the ninhydrin-positive substances of haemolymph. (b) Autoradiograph of the two-dimensional chromatogram of haemolymph from the pupa into which ^{14}C-tyrosine was injected. Sakura X-ray film, Medical QH. The time of exposure, one month.
T, T-substance; Try, free tyrosine; X, an unidentified substance.

evaporated to dryness under reduced pressure, the residue was dissolved in water and submitted to two-dimensional cellulose or silica gel thin-layer chromatography. The chromatogram obtained with ninhydrin always showed the presence of tyrosine.

^{14}C-Tyrosine was injected into a pupa 7 days after pupation. One day after injection, the haemolymph was withdrawn and deprotenized with 99.5% ethanol. After centrifugation, the supernatant was chromatographed two-dimensionally on cellulose thin-layer sheet. Autoradiograph of the chromatogram was taken as given in Fig. 3b, which showed that the spot T was radioactive.

Identification of the T-substance

With the T-substance which was purified and crystallized as described in the section of Matrials and Methods, spectral and some chemical properties were investigated.

The ultraviolet absorption spectrum is shown in Fig. 4 with that of tyrosine for the purpose of comparison. The absorption maxima of the T-substance were at 270.5 and 277nm, while those of tyrosine were at 275 and 280–285 (shoulder) nm.

The T-substance on thin-layer chromatogram was positive to the ninhydrin reaction and negative to the phosphomolybdic acid-NH_3 test. These results suggest the possibility that some group or compound may be substituted for the hydrogen atom of the aromatic OH-group of tyrosine.

The T-substance can be readily hydrolyzed on a mild hydrolysis (1 N HCl, 100°C, 30 min). When the hydrolysate was submitted to the cystein-sulfuric acid test (Dishe et

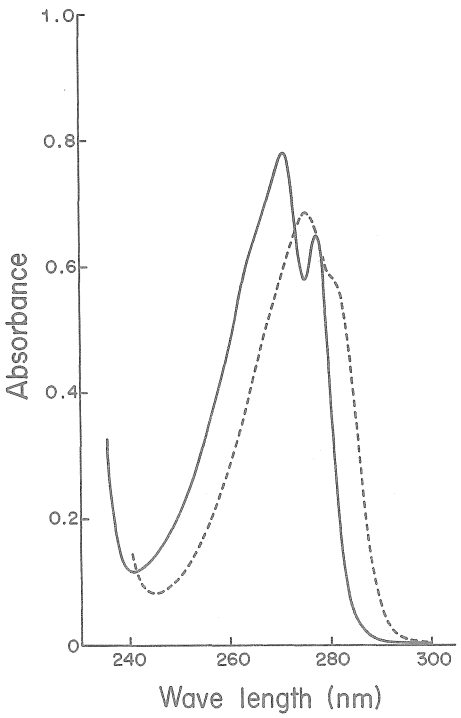

Fig. 4. Absorption spectra of the T-substance (sold line) and tyrosine (broken line) in 0.05 N HCl.

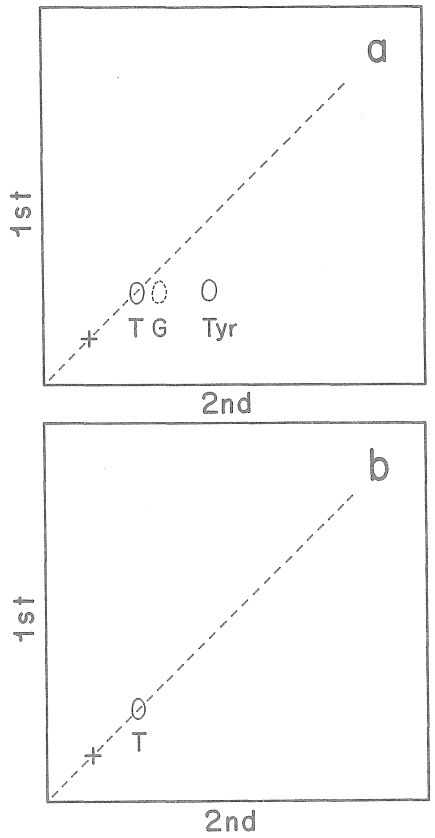

Fig. 5. Two-dimensional chromatograms of the T-substance by Brunet's method. The chromatogram was incubated with (a) β- or (b) α-glucosidase after development in the first direction. T, T-substance; Tyr, tyrosine; G, glucose. Circles by solid line, ninhydrin; a circle by dotted line, aniline hydrogen phthalate.

al., 1949), the solution became yellow. After standing overnight at room temperature, the color turned green. This suggested that the hydrolysate might contain glucose. The suger was further identified as glucose by co-chromatography with galactose, mannose, fructose, and glucose.

Thin-layer chromatogram of the hydrolysate of T-substance showed only tyrosine and glucose. And a molar ratio of tyrosine to glucose was 0.351 : 0.343. This shows that the T-substance consists of equimolar amounts of tyrosine and glucose.

When the T-substance was submitted to Brunet's method on thin-layer sheet, it was hydrolyzed to tyrosine and glucose with β-glucosidase but not with α-glucosidase (Fig. 5). This indicates that tyrosine and glucose are linked together by β-glycosidic bond.

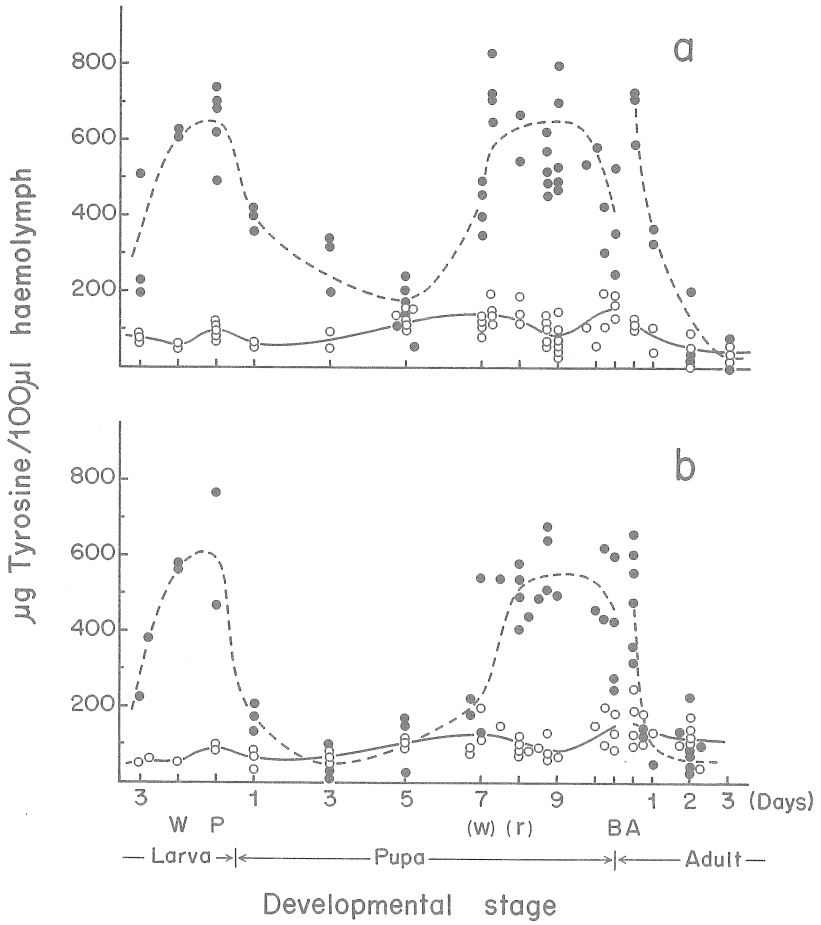

Fig. 6. Level changes of the bound (solid circle, broken line) and free (open circle, solid line) tyrosine from the fifth instar larvae to adults. (a) male, (b) female. W, wandering stage; P. prepupa; B, immediately before emergence; A, immediately after emergence; (w), the stage when white and thick wings are seen; (r), the stage when red spots appear in the hind-wings.

Changes in the concentration of the bound form of tyrosine in haemolymph from the fifth instar larva to adult

The level of the bound form of tyrosine in haemolymph is shown in Fig. 6. The general pattern of the level change was much the same between male and female. Both sexes showed two peaks of the bound form of tyrosine. The first peak is at the prepupal stage, and the second peak is seen before emergence. The middle pupal stage corresponds to a bottom between both peaks. Just after emergence, the volume of haemolymph decreases to about one-half, and the level of the bound form of tyrosine is rather high. At the time when wings become hard, however, haemolymph decreases to a very small volume, and yet the bound form of tyrosine is in a very low concentration.

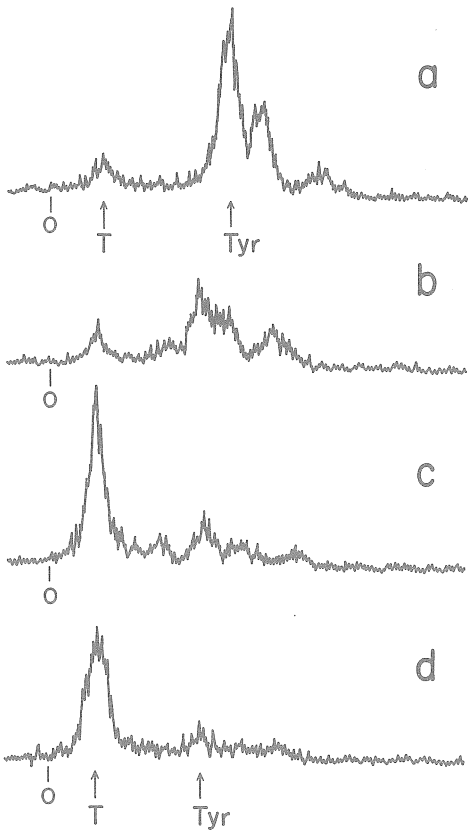

Fig. 7. Incorporation of ^{14}C-tyrosine into the haemolymph of pupa. (a) 5-day-old pupa, 20 hr after injection; (b) 5-day-old pupa, 42 hr after injection; (c) 7-day-old pupa, 20 hr after injection; (d) 7-day-old pupa, 42 hr after injection. T, T-substance; Tyr, tyrosine; O, the origin.

In vivo synthesis of tyrosine conjugate

^{14}C-Tyrosine was injected into 5-day-old pupae in which the level of bound tyrosine was low and into 7-day-old pupae in which the bound tyrosine was increasing. Twenty or forty two hours after the injection, the haemolymph was withdrawn, and after the removal of protain, was submitted to one-dimensional thin-layer chromatography on cellulose. After development with BAW, the radioactivity on chromatogram was measured with a radioactive TLC scanner (Fig. 7).

In the case of 5-day-old pupae, there were two major peaks of radioactivity 20 hr after injection. The higher one corresponded to free tyrosine. Besides, there were two small peaks, one of which corresponded to the T-substance. After 42 hr, the peak of free tyrosine became low, but the peak of the T-substance did not rise.

On the other hand, the 7-day-old pupae showed a major peak of radioactivity corresponding to the T-substance after 20 hr. The radioactivity of the position

corresponding to free tyrosine was low. Also after 42 hr, the pattern was much the same, though the radioactivity of free tyrosine was lower.

Distribution of the tyrosine glucoside in Lepidoptera

Haemolymph of twelve species other than *P. xuthus* as described in the section of Materials and Methods were examined for the presence or absence of tyrosine glucoside. Developmental stage was wandering, prepupal, early pupal, or late pupal stage. The haemolymph was withdrawn and mixed with an equal volume of 10% TCA. After centrifugation, the supernatant was applied to the Bio-gel P-2 column. The fractions corresponding to the T-substance were submitted to UV-spectrophotometry, thin-layer chromatography, and Brunet's method. It has proved that all the species examined contain the tyrosine glucoside, T-substance. In *P. rapae* and *P. melete,* in particular, the concentration of the tyrosine glucoside in haemolymph was determined from larval stage to adult. They showed the level change similar to that of *P. xuthus.*

Discussion

The T-substance in the haemolymph of *P. xuthus* decomposes to yield equimolar amounts of tyrosine and glucose on acid hydrolysis. This hydrolysis is also catalyzed by β-glucosidase but not by α-glucosidase. The T-substance on chromatogram is positive to the ninhydrin reaction. From these properties and the UV-absorption spectrum, the T-substance has been identified as β-glucosyl-O-tyrosine. And this substance seems to be widely distributed in the haemolymph of Lepidopteran insects.

As shown in Fig. 6, there are two peaks of the bound form of tyrosine from the fifth instar larva to adult. The first peak is at the prepupal stage, and the second peak is seen before emergence. Using the haemolymph from these two stages, it was proved that 85 to 105 per cent of the bound form of tyrosine in Fig. 6 was hydrolyzed by β-glucosidase. This shows that the two peaks of the bound form of tyrosine are due to tyrosine glucoside.

In some Dipteran insects, some tyrosine and phenylalanine derivatives are known to increase before puparium formation and to decrease after that. Among these compounds, are tyrosine-O-phosphate in *Drosophila* (Mitchell and Lunan, 1964; Lunan and Mitchell, 1969), β-alanyl-tyrosine in *Sarcophaga* (Levenbook et al., 1969; Bodnaryk and Levenbook, 1969; Bodnaryk, 1971a,b), and γ-glutamyl-phenylalanine in *Musca* (Bodnaryk, 1970b). These substances are said to be a kind of tyrosine reservoir or tyrosine doner. In *Drosophila busckii,* β-glucosyl-O-tyrosine was reported to play the same role (Chen et al., 1978). Now, the first peak of Fig. 6 in the present paper shows that β-glucosyl-O-tyrosine may play such a role in Lepidoptera. Junnikkala (1976) reported the wide distribution of some tyrosine conjugate in butteuflies, though he did not identified it. Anyway, it is interesting that β-glucosyl-O-tyrosine in the present paper, the tyrosine conjugate reported by Junnikkala (1976), and tyrosine-O-acetyl

DOPAmine reported by Sienkiewicz and Piechowska (1973) all show the similar level change at prepupal stage.

On the other hand, what meaning does the second peak of Fig. 6 have? There has been almost no report that shows such a peak before emergence. But Seligman et al. (1969) found a high level of tyrosine-O-phosphate in the haemolymph of newly emerged adults of *Sarcophage bullata* and presumed that tyrosine from the substance would be used for the tanning of adult cuticle. The possibility exists that β-glucosyl-O-tyrosine might such a role in Lepidoptera.

The incorporation experiments with ^{14}C-tyrosine (Fig. 7) show that the low level of tyrosine glucoside in the middle stage of pupa is caused not by a low level of free tyrosine but by a low synthetic activity.

It is possible that the same level pattern as in Fig. 6 occurs widely in Lepidoptera, because the simillar pattern was observed also in the haemolymph of *P. rapae* and *P. melete*. But, in order to clarify the role of tyrosine glucoside for sclerotization of cuticle, further studies including tracer experiments with ^{14}C-tyrosine derivatives and the measurment of β-glucosidase activity are needed.

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