

## Tyrosine Metabolism for Cuticle Tanning in the Tobacco Hornworm, *Manduca sexta* (L.) and Other Lepidoptera: Identification of $\beta$ -D-Glucopyranosyl-O-L-Tyrosine and Other Metabolites<sup>1</sup>

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When [<sup>14</sup>C]tyrosine and [<sup>14</sup>C]glucose were fed or injected into feeding fifth-instar larvae of the tobacco hornworm, *Manduca sexta* (L.), they were incorporated into a conjugate identified in hemolymph and carcass extracts as  $\beta$ -D-glucopyranosyl-O-L-tyrosine. In wandering larvae and pupae, the conjugate was hydrolyzed, and tyrosine was hydroxylated and decarboxylated to dihydroxyphenylalanine and 2-(dihydroxyphenyl)ethylamine. None of these metabolites were formed in fourth-instar larvae or in adults. [<sup>14</sup>C]Phenylalanine was hydroxylated to tyrosine in all stages of insect development.  $\beta$ -D-Glucopyranosyl-O-L-tyrosine was also detected in 18 other species of Lepidoptera but not in species from other insect orders. This conjugate appears to be the major tyrosine storage metabolite for production of tanning diphenol substrates in Lepidoptera.

One of the first steps leading to production of tanning diphenols in insects is the mobilization of tyrosine reserves shortly before ecdysis. Tyrosine or its precursor phenylalanine may be sequestered as dipeptides during larval feeding stages of Diptera (1). Little is known about peptide storage forms in other insect orders except that certain Lepidoptera have also been reported to utilize peptides (2, 3). Glucosides and phosphate or sulfate esters of substrates may act as protecting groups for regulating substrate availability and therefore production of tanning compounds. The tanning agent of cockroach oothecal protein, 3,4-dihydroxybenzoic acid, is stored as a glucoside to prevent premature tanning (4). Glucosides of

tyrosine or *N*-acetyldopamine have been found in several insects and may be important in storage and transport roles for tanning substrates (5, 6). Dopamine 3-*O*-sulfate has been isolated in relatively high concentrations from untanned *Periplaneta americana* (L.), and, as the sequestered form of dopamine, is protected from indole formation by the sulfate group (7). Tyrosine-*O*-phosphate accumulates in mature larvae and decreases during pupariation in *Drosophila melanogaster* Meigen (8). The 3-*O*-phosphate and sulfate esters of *N*-acetyldopamine are also rapidly formed in *P. americana* after ecdysis (9). In all cases the catechol was incorporated into the cuticle during sclerotization but not the phosphate or sulfate moiety. Protective and possible transport roles have been suggested by these authors for the conjugated substrates, but as yet experimental evidence is lacking to support this hypothesis.

We have undertaken a detailed study of tanning diphenol production in the tobacco hornworm, *Manduca sexta* L. We have developed a screening procedure for identifying compounds that are likely candidates

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for tanning metabolites in a particular tissue. Using radiolabeled tracers and two-dimensional mapping techniques, we have identified three major tyrosine metabolites:  $\beta$ -D-glucopyranosyl-O-L-tyrosine, dihydroxyphenylalanine (dopa), and 2-(dihydroxyphenyl)ethylamine (dopamine). The first compound is the primary metabolite detected in fifth-instar feeding and wandering larvae and pupae, while the latter two occur only in wandering larvae and pupae.

#### MATERIALS AND METHODS

**Insects.** *M. sexta* eggs were a gift from Dr. J. Reinecke (Science and Education Administration, U. S. Department of Agriculture, Fargo, N. Dak.) and larvae were reared on a semiartificial diet (10) at 28°C under a nondiapausing photoperiod of 16L:8D. Other insects were collected from the field or were from stock colonies maintained at the U. S. Grain Marketing Research Laboratory or in the Department of Entomology, Kansas State University as denoted in Table I.

**Chemicals.** Tyrosine-O-phosphate was obtained from Research Plus Laboratories (Bayonne, N. J.). Other compounds were purchased from Sigma Chemical Company (St. Louis, Mo.).

**Administration of radiolabeled compounds.** L-[U-<sup>14</sup>C]Tyrosine, L-[U-<sup>14</sup>C]phenylalanine, and D-[U-<sup>14</sup>C]glucose, (Amersham, Arlington Heights, Ill. or Research Products International Corp., Elk Grove, Ill.) were injected into various stages of *M. sexta* for determination of metabolic pathways. Larvae were immobilized on chipped ice and then injected through an abdominal proleg followed by ligation with thread to prevent bleeding. Approximately 5–10  $\mu$ Ci was administered in 50  $\mu$ l of saline with a microsyringe. In some experiments, fifth-instar larvae were fed [<sup>14</sup>C]-tyrosine (0.5  $\mu$ Ci) in small cubes of diet. Larvae starved overnight readily consumed the entire cube within 1 h.

**Extraction.** After 24 h, *M. sexta* larvae or pupae were clipped at the base of the anal horn or proboscis and hemolymph was collected in 5 ml of ice-cold extraction solvent (80% aqueous methanol with 5 mM ascorbic acid). Precipitate was dispersed with a glass rod, centrifuged, and washed four times with solvent. Pooled extracts were concentrated on a rotary evaporator. Larval carcasses, whole larvae, pupae, or adults were homogenized in 15 ml of ice-cold solvent per animal, and the extracts were pooled and concentrated as described above.

**Metabolite two-dimensional mapping.** Fingerprint maps of amino acids, peptides, and other ninhydrin-positive substances in methanolic extracts of hemolymph, carcass, or whole insects were prepared by electrophoresis on thin-layer cellulose sheets (Eastman, 20  $\times$  20 cm) at pH 1.9 (8% acetic acid, 2% formic

acid) at 200 V per plate for 2–2.5 h, followed by chromatography with butanol: pyridine: acetic acid: water (15:10:3:12). Amino compounds were located by staining with ninhydrin-cadmium acetate (11) and phosphorus compounds by spraying with the molybdenum blue visualization reagent (12). Radioactive zones were located by autoradiography. Unsprayed plates were overlaid with Kodak No Screen X-ray film and clamped between glass plates in a light tight box for 2 weeks. The films were then developed and the plates sprayed with ninhydrin for correlation of radioactivity with colored spots. We quantitated radioactivity by scraping the cellulose from the labeled spot area and counting in a Searle Isocap 300 liquid scintillation instrument with a scintillation cocktail composed of 500 ml toluene, 500 ml methyl Cellosolve, and 4 g 98% 2,2'-(1,4-phenylene)bis(5-phenyloxazole) and 2% 1,4-bis[2-(2-methylphenyl)ethenyl]benzene (Research Products International Corp.).

**High-pressure liquid chromatography.** Analyses were performed with a Varian Model 5020 pump equipped with a 20- $\mu$ l sample loop on a Rheodyne 7120 injection valve and a Tracor Model 970 UV-VIS variable wavelength detector (wavelength = 270 nm). A DuPont 5- $\mu$ m particle Zorbax-ODS column (25 cm  $\times$  4.6 mm) was used in the reverse phase mode at 60°C. The detector output was plotted and peaks were integrated by a Hewlett-Packard Model 3385A printer-plotter. The eluting solvent for the separation was 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 2.5. At a flow rate of 1 ml/min, tyrosine, tyrosine-O-phosphate, and tryptophan eluted at 4.1, 5.2, and 16.2 min, respectively.

**Purification of  $\beta$ -D-glucopyranosyl-O-L-tyrosine.** Methanolic extracts from about 30 insects were evaporated to dryness, redissolved in 10 ml of a solution of 10% acetic acid–5% formic acid, and subjected to ion-exchange chromatography on a Dowex 50  $\times$  2 column (1.5  $\times$  40 cm) equilibrated with 0.1 M ammonium formate, pH 2.9 (buffer A).<sup>2</sup> Fractions of 3 ml were collected and the effluent was monitored at 270 nm with a Cary 118C spectrophotometer. A gradient of 100 ml each of buffer A and 0.2 M ammonium formate pH 3.8 was initiated at fraction 20. Combined fractions from the Dowex 50 column were lyophilized and subjected to gel permeation chromatography on a Bio-Gel P-2 column (0.9  $\times$  140 cm) at a flow rate of 5 ml/h in 2% acetic acid. Two-milliliter fractions were collected.

**Amino acid and carbohydrate analyses.** Samples were analyzed on a Beckman 121 amino acid analyzer with a PA-35 sulfonated polystyrene copolymer resin before and after hydrolysis for 22 h *in vacuo* at 110°C in 6 N HCl containing 0.1% phenol. Total carbohydrate was measured by the anthrone reaction (13) or by

<sup>2</sup> Abbreviations used: buffer A, 0.1 M ammonium formate, pH 2.9; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate; hplc, high-performance liquid chromatography.

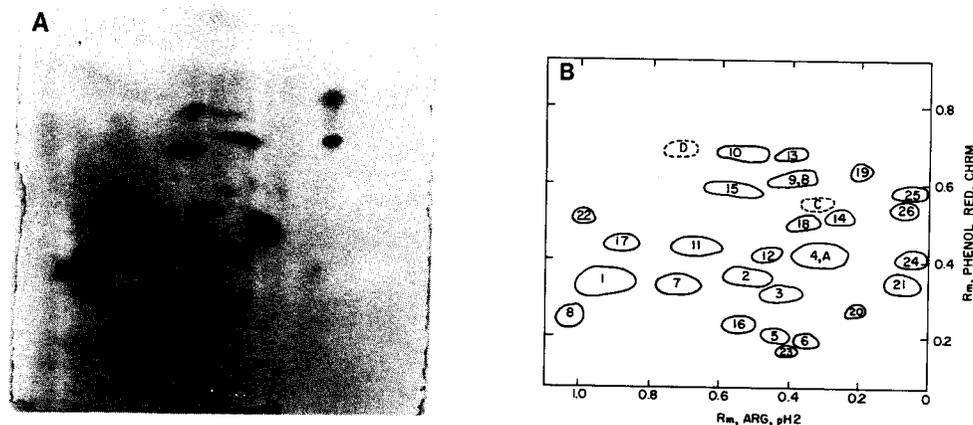


FIG. 1. Original (A) and reconstructed (B) two-dimensional fingerprint map of ninhydrin-positive components from hemolymph of fifth-instar larvae of *M. sexta*. The mobilities are located in relation to the migration of arginine and phenol red in electrophoresis and chromatography, respectively, as indicated by the coordinates. Numbering indicates relative order of ninhydrin spot development; letters indicate relative intensity of zones from autoradiography.

chromatography on silica gel high-performance thin-layer plates. The purified metabolite was dissolved in 2 N HCl and hydrolyzed *in vacuo* at 100°C for 120 min or incubated in 0.2 M acetate buffer pH 5.5 at 30°C for 60 min with either  $\alpha$ -glucosidase (Sigma) or  $\beta$ -glucosidase (Worthington Biochemical Corp., Freehold, N. J.). The hydrolysates and reference compounds were spotted on 60- $\mu$ m particle size silica plates (E. Merck) and developed with the solvent system described above for two-dimensional mapping, or with butanol: dioxane: water (2.5:6:1.5).  $\alpha$ -Naphthol reagent was used to detect sugar (14).

**Nuclear magnetic resonance spectroscopy.** The purified tyrosine glucoside was lyophilized twice from 99.8% and once from 100% D<sub>2</sub>O. After essentially saturating 0.4 ml of 100% D<sub>2</sub>O with the dried product, its <sup>1</sup>H FT NMR was recorded with a Varian XL-100 spectrometer with sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) as an internal standard.

## RESULTS

### Mapping of Tyrosine Metabolites

To determine the low-molecular-weight tyrosine metabolites in *M. sexta*, we fed or injected larvae with [<sup>14</sup>C]tyrosine and prepared 80% methanolic extracts from both hemolymph and carcass. After 24 h, approximately 30% of the total radioactivity injected was extracted in the hemolymph (5–10%) and the carcass (20–25%). These extracts were then subjected to a two-dimensional fingerprinting procedure and autoradiography. As shown in Fig. 1A,

approximately 25 ninhydrin-positive components from hemolymph were detected in feeding fifth-instar larvae. The numbering system (Fig. 1B) denotes relative concentrations as estimated from the rate of appearance of the ninhydrin color. Most of the components were amino acids, and some of these were identified by comparison of mobility to reference compounds and amino acid analysis (Fig. 1B): 1 = Arg, 8 = Lys, 9 = Tyr, 10 = Leu, 13 = Phe, 14 = Pro, 15 = Val. The fingerprint from the carcass extract was very similar in composition to that of the hemolymph extract; however, the components were present at much lower concentrations in the carcass extract except for Arg, Lys, and metabolite 4.

Radioactive zones are denoted in Fig. 1B by the letters A–D in the fingerprint maps. Autoradiography of the map from hemolymph or carcass from feeding fifth-instar larvae revealed that only metabolites 4 (unknown) and 9 (Tyr) were radioactive (denoted by A and B in Fig. 1B, respectively). Scintillation counting showed that metabolite 4 was approximately 2 orders of magnitude more radioactive than tyrosine. This result indicated that component 4 was the major tyrosine metabolite in feeding fifth-instar larvae.

Prior to pupation, larvae cease feeding and enter a wandering stage to search for

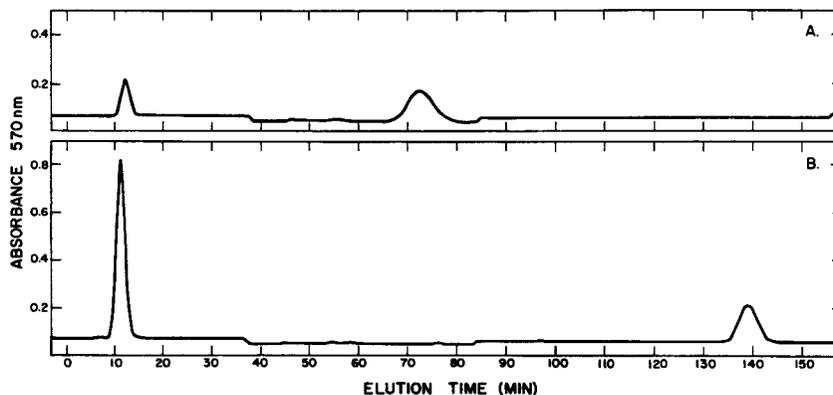


FIG. 2. Ion-exchange chromatography on amino acid analyzer of major tyrosine metabolite from *M. sexta* hemolymph. (A) Before hydrolysis in 6 N HCl. (B) After hydrolysis.

a pupation site. The maps derived from extracts of wandering larvae were nearly identical to those from feeding larvae, except that tyrosine and unknown metabolite 4 were present in relatively greater and lesser amounts, respectively. In addition to the presence of radioactivity in tyrosine and metabolite 4, autoradiographic analysis revealed radioactivity in two newly appearing ninhydrin-positive compounds, denoted C and D in Fig. 1B. Compounds C and D comigrated with 3,4-dihydroxyphenylalanine (dopa) and 2-(3,4-dihydroxyphenyl)ethylamine (dopamine), respectively. The ratio of radioactivity was approximately 1(A):10(B = Tyr):0.1(C):0.1(D) in wandering larvae. These results suggested that tyrosine and the unknown metabolite 4 were converted in wandering larvae to hydroxylated and decarboxylated metabolites.

#### Identification of Major Tyrosine Metabolite

To determine the chemical identity of tyrosine metabolite 4, we first considered the possibility of a peptide component. Several fingerprints were conducted and exposed to X-ray film in our isolation of a sufficient quantity for peptide analysis. After elution with 80% methanol, a portion of this preparation was fingerprinted a second time, and homogeneity was verified when a single ninhydrin spot was detected. A second portion of the sample was chromatographed on the amino acid analyzer. The metabolite eluted at 71 min as a rather broad peak

between Ala and Val (Fig. 2A). After hydrolysis, the component was converted to tyrosine (elution time = 139 min, Fig. 2B). Fingerprint analysis of the hydrolytic product yielded a single ninhydrin spot that aligned with Tyr.

Since Tyr was the only amino acid present in the major metabolite, we next investigated whether the compound was a carbohydrate conjugate of tyrosine. First, the purified metabolite yielded a positive anthrone reaction, an indication of the presence of neutral hexose. Second, after acid hydrolysis and chromatography on silica gel, an  $\alpha$ -naphthol-positive spot that comigrated with glucose was detected. Third, when extracts from larvae that had been injected with [ $^{14}$ C]glucose were analyzed by the fingerprinting procedure and autoradiography, several radioactive zones were observed, the second most intense spot aligning with the unknown tyrosine metabolite 4 (fingerprint not shown). Fourth, incubation of the unknown metabolite with  $\beta$ -glucosidase, but not  $\alpha$ -glucosidase, resulted in the release of tyrosine and glucose in about equimolar amounts as measured by thin-layer chromatography on silica gel. These results strongly suggested that metabolite 4 was composed of tyrosine and glucose bonded by a  $\beta$ -glucosidic linkage.

#### Isolation of $\beta$ -D-Glucopyranosyl-O-L-Tyrosine

To conduct a more complete chemical characterization of the tyrosine glucoside, we

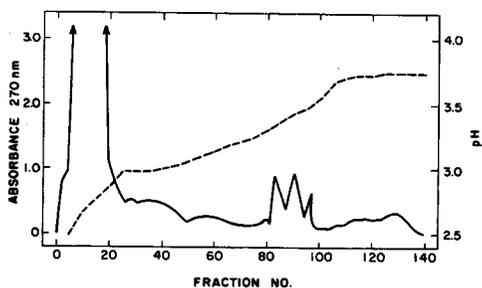


FIG. 3. Preparative ion-exchange chromatography on Dowex 50-X2 of tyrosine metabolite from 30 larvae of *M. sexta* with pH gradient from 2.9 to 3.8 in ammonium formate buffer.

first purified a larger quantity of material. An extract from 30 larvae was chromatographed on Dowex-50 with a pH gradient (Fig. 3). The conjugate was eluted at pH 3.4 as a relatively symmetrical peak in fractions 89–92 where the conjugate apparently existed in its zwitterionic form. This chromatographic behavior was good evidence for the presence of free amino and carboxyl groups instead of ester and amide functions. Also, the lack of electrophoretic mobility at pH 5.5 in 25 mM triethylamine acetate on cellulose indicated that the tyrosine metabolite contained ionized carboxyl and amino groups and was linked to carbohydrate via the phenolic oxygen atom.

After ion-exchange chromatography, the metabolite appeared to be 80% homogeneous. The final step in the preparative procedure for isolation was gel filtration on polyacrylamide. Fractions 88–92 from the Dowex column were pooled, lyophilized, and chromatographed on Bio-Gel P-2 (Fig. 4). Tyrosine glucoside eluted in fractions 55–60. When compared to the elution behavior of peptide standards, the elution volume of the conjugate corresponded to that of a peptide of apparent molecular weight =  $400 \pm 50$ , which compared quite favorably with the calculated molecular weight of 401. This product exhibited a single ninhydrin-positive spot in the fingerprint map and hplc analysis showed the conjugate to be >99% homogeneous (Fig. 5, elution time = 4.3 min).

#### Spectral Properties

The ultraviolet absorption spectrum of the tyrosine glucoside at pH 3 is shown in

Fig. 6 with maxima at 269 and 275 nm and minima at 240 and 273 nm. The 269 nm: 275 nm absorbance ratio was 1.20. The same spectrum was observed over the pH range 3 to 11, an indication that the phenolic group did not ionize. This result also suggested that the conjugate was covalently linked via the phenolic oxygen.

Characterization of the structural geometry of the tryosine metabolite was determined by proton magnetic resonance spectroscopy (Fig 7A). The four (actually eight or more) peaks centered at 7.18 ppm were characteristic of a 1,4-disubstituted phenyl ring and the doublet at 5.1 ppm was suggestive of an anomeric carbohydrate proton. Accordingly, comparison of this spectrum with that of phenyl  $\beta$ -D-glucopyranoside in the same solvent matched several of the methyne and methylene resonances between 3.5 and 4.0 ppm as well as coinciding exactly with the doublet at 5.1 ppm. Consequently, we determined that the metabolite was most likely a 4-substituted *O*-phenyl- $\beta$ -glucosyl derivative, since the  $\alpha$ -anomeric resonance (determined using phenyl- $\alpha$ -D-glucopyranoside) occurred significantly farther upfield.

Similar comparisons of the aromatic and remaining methyne and methylene proton signals with those of DL-tyrosine (Statler Spectrum No. 27545M) indicated that the *O*-phenyl substituent was tyrosine bonded through the hydroxyl oxygen, since  $\alpha$ - and

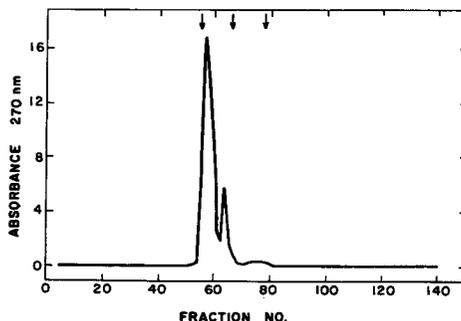


FIG. 4. Gel filtration of tyrosine metabolite from *M. sexta* on a Bio-Gel P-2 column (0.9  $\times$  140 cm). Flow rate: 5 ml 2% acetic acid/h. Two-milliliter fractions were collected. The arrows represent elution volumes of peptide standards: Glu-Tyr-Glu, Tyr-Gly, and Tyr.

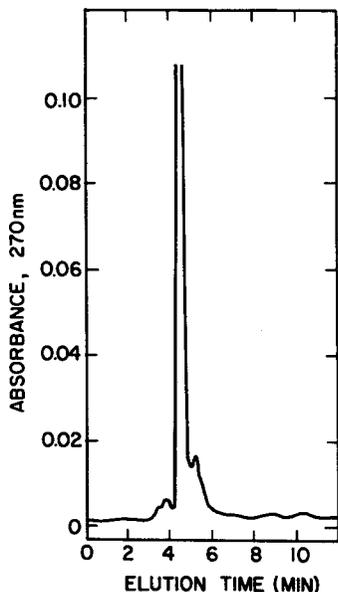


FIG. 5. High-pressure liquid chromatographic analyses of purified tyrosine metabolite from *M. sexta* with isocratic elution with 0.1 M  $\text{NaH}_2\text{PO}_4$ , pH 2.5.

$\beta$ -proton coupling patterns were identical in the two samples. The chemical shifts, however, did not match exactly perhaps because the Statler spectrum was obtained in a basic and ours in an acidic medium. In fact, the small peak at 1.9 ppm probably resulted from residual acetic acid. The observed splitting pattern in the upfield region arose from the nonequivalence of the  $\beta$ -protons of tyrosine, which were also coupled to the  $\alpha$ -hydrogen. The resulting pattern was characteristic of an ABX spectrum (15), where X represented the  $\alpha$ -proton and AB the  $\beta$ -protons in which the middle two resonances coincided (peaks 8 and 9, Fig. 7B). Therefore, peaks 1-4 in Fig. 7B arose from the  $\alpha$ -proton of tyrosine and peaks 5-12 from the nonequivalent  $\beta$ -hydrogens with  $J_{AB} = 15$  Hz and  $J_{AX} + J_{BX} = 13$  Hz. All the remaining peaks in Fig. 7B were from the glucosyl moiety of the compound, including one under peak 4. In summary, the  $^1\text{H}$  NMR spectrum of the metabolite is completely compatible with that of  $\beta$ -D-glucopyranosyl-*O*-L-tyrosine.

#### Other Tyrosine Metabolites

In addition to the presence of  $\beta$ -glucopyranosyl-*O*-L-tyrosine, dopa, and dopa-

mine, one other metabolite (spot 2, Fig. 1B) found in carcass extracts became labeled in wandering larvae when either [ $^{14}\text{C}$ ]tyrosine or [ $^{14}\text{C}$ ]glucose was injected. This component has not been identified but is probably a metabolite of  $\beta$ -D-glucopyranosyl-*O*-L-tyrosine. Since it was not present in hemolymph, it may be an intermediate produced by and incorporated directly into other tissues.

Another metabolite containing tyrosine (spot 21, Fig. 1B) was also present in the fingerprints of both hemolymph and carcass of larvae, but no significant radioactivity was associated with it when [ $^{14}\text{C}$ ]tyrosine was injected. This component yielded only tyrosine upon acid hydrolysis and subsequent analysis on the amino acid analyzer. Treatment of the fingerprint with a modified Hanes and Isherwood reagent (12) yielded a positive test for phosphate in zone 21 (fingerprint not shown). In addition, tyrosine-*O*-phosphate comigrated to the same area of the fingerprint. These results suggested that tyrosine-*O*-phosphate is also present in *M. sexta*, but that it is only a minor metabolite.

With regard to tyrosine production, insects obtain the amino acid from diet pro-

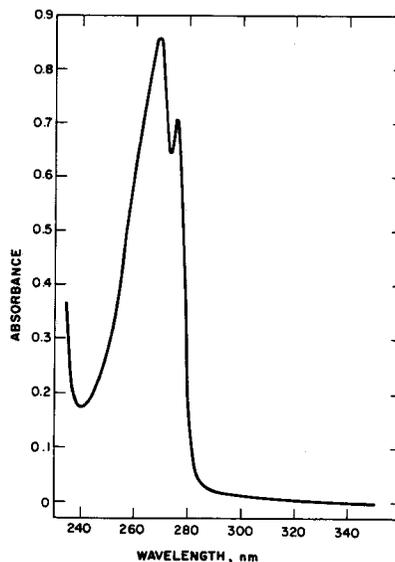


FIG. 6. Ultraviolet absorption spectrum of  $\beta$ -D-glucopyranosyl-*O*-L-tyrosine in 2% acetic acid.

tein or they synthesize it from phenylalanine (16–19). The tobacco hornworm converted [ $^{14}\text{C}$ ]phenylalanine to tyrosine in all stages examined, including the pupa and the fourth- and fifth-instar larvae.

#### *$\beta$ -D-Glucopyranosyl-O-L-Tyrosine in Developmental Stages*

$\beta$ -D-Glucopyranosyl-O-L-tyrosine was not detected by fingerprint analysis and autoradiography in fourth-instar larvae of *M. sexta*. Synthesis of the glucoside and sequestration in tissues began during the fifth larval feeding period. This metabolite accumulated to relatively high levels in hemolymph (>10 mM) such that it became the predominant ninhydrin-positive substance present and therefore was the major storage form of tyrosine. The glucoside was also present in wandering larvae and in pupae but not in adult moths (Table I). No major tyrosine metabolites were observed in extracts from fourth-instar larvae or adults.

#### *Frequency of $\beta$ -D-Glucopyranosyl-O-L-Tyrosine in Other Insects*

An examination for  $\beta$ -D-glucopyranosyl-O-L-tyrosine in other insects with the fingerprint mapping technique revealed that it was present in eight other species of Lepidoptera but not in species of Coleoptera or Orthoptera (Table I). Concentrations of tyrosine glucoside were similar to that found in *M. sexta* (~10 mM). Only the lepidopteran, *Thyridopteryx ephemeraeformis* (Haworth) (bagworm), showed no  $\beta$ -D-glucopyranosyl-O-L-tyrosine in field-collected larvae. These samples were mixed larger instars and may not have included the last larval instar in which the glucoside is synthesized in those lepidopteran species studied so far. Two species of cockroaches and two species of stored grain beetles did not contain the glucoside. We did not investigate whether other major tyrosine metabolites were present in the latter insects.

#### DISCUSSION

Storage of tyrosine and its release during the molting cycle for tanning of cuticle

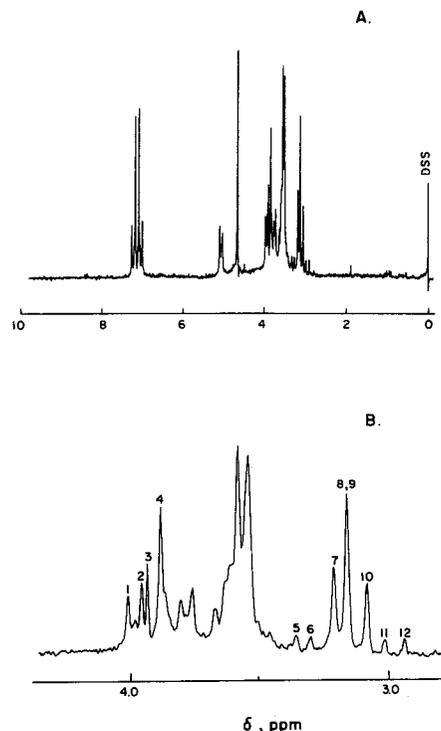


FIG. 7. (A)  $^1\text{H}$  NMR spectrum of  $\beta$ -D-glucopyranosyl-O-L-tyrosine in  $\text{D}_2\text{O}$  after extraction and purification from *M. sexta* hemolymph. The spectrum was obtained from 1024 pulses with a 2000-Hz spectral width, 4096 data points, and a 1-s delay between acquisitions to give a 2-point/Hz resolution after zero filling the time domain spectrum. (B) Expansion of the methyne and methylene proton regions between 2.8 and 4.3 ppm. The numbered peaks correspond to tyrosine  $\alpha$  (1–4) and  $\beta$  (5–12) resonances.

appears to be a common phenomenon in insects. Tyrosine or phenylalanine is sequestered during larval feeding stages as peptides or conjugates that are more water soluble than the free amino acid. These circulate primarily in hemolymph and serve as readily available substrates for tanning of new cuticle (1). Tyrosine and phenylalanine storage molecules have been identified mostly from Diptera in which they supply the substrates for puparial tanning. Peptides have also been reported as tyrosine storage forms in Lepidoptera. Sienkiewicz and Piechowska (2) isolated "celerin" from *Hyles euphorbiae* (L.); they identified this substance as L-tyrosyl-O-acetyldopamine. This "peptide" appeared at the beginning of

TABLE I  
OCCURRENCE OF  $\beta$ -D-GLUCOPYRANOSYL-O-L-TYROSINE IN LABORATORY AND FIELD-COLLECTED INSECTS

Classification	Stage	Source <sup>a</sup>	Compound present <sup>b</sup>
Lepidoptera			
Noctuidae			
<i>Anavitrinella pampinaria</i> (Guenee)	Larva (last)	F	+
<i>Spodoptera ornithogalli</i> (Guenee)	Larva (last)	F	+
<i>Trichoplusia ni</i> (Hubner)	Larva (last)	L	+
<i>Peridroma saucia</i> (Hubner)	Larva (last)	L	+
Psychidae			
<i>Thyridopteryx ephemeraeformis</i> (Haworth)	Larva (large)	F	-
Pyrilidae			
<i>Diatraea grandiosella</i> (Dyar)	Larva (last)	L	+
<i>Ostrinia nubilalis</i> (Hubner)	Larva (last)	L	+
<i>Plodia interpunctella</i> (Hubner)	Larva (last)	L	+
Sphingidae			
<i>Ceratomia catalpae</i> (Boisduval)	Larva (last)	F	+
<i>Manduca sexta</i> L.	Larva (penultimate)	L	-
	Larva (last)	L	+
	Pupa	L	+
	Adult	L	-
Orthoptera			
Blaberidae			
<i>Leucophaea maderae</i> (Fabricius)	Nymph (last)	L	-
Blattidea			
<i>Periplaneta americana</i> L.	Nymph (last)	L	-
Coleoptera			
Dermestidae			
<i>Trogoderma variabile</i> (Ballion)	Larva (last)	L	-
Tenebrionidae			
<i>Tenebrio molitor</i> L.	Larva (last)	L	-

<sup>a</sup> F = field collected, L = laboratory reared.

<sup>b</sup> (+) = glucoside was detected in extract at approximately 1 to 10 mM using two-dimensional mapping procedure with ninhydrin visualization; (-) = glucoside was not detected (<0.1 mM).

the fifth larval instar, reached maximal concentration in wandering larvae, and decreased during pupation. The chemical identity of "celerin" has been questioned by Junnikkala (3) and Bodnaryk (1). Junnikkala (3) also detected a "peptide" rich in tyrosine in *Pieris brassicae* L., *Smerinthus ocellata* (L.), *Deilephila elpenor* (L.), *Hyles galii* (Rottenberg), and *Cerura vinula* (L.) with properties very similar to "celerin." This storage molecule was also synthesized during the feeding period of the fifth instar and increased to a peak concentration before pupal ecdysis. Based on the spectral and chemical properties of these "peptides," we

conclude that they are most likely  $\beta$ -D-glucopyranosyl-O-L-tyrosine as we have identified in *M. sexta* and eight other species of Lepidoptera. Their ultraviolet spectra show absorption maxima identical to the tyrosine conjugate isolated from the hornworm, and products from acid hydrolysis exhibit spectra identical to tyrosine. No carbohydrate analyses were performed in the earlier studies.

We therefore conclude that  $\beta$ -D-glucopyranosyl-O-L-tyrosine is the predominant tyrosine storage form in 15 species of Lepidoptera with synthesis taking place in the last larval instar. Free tyrosine is released

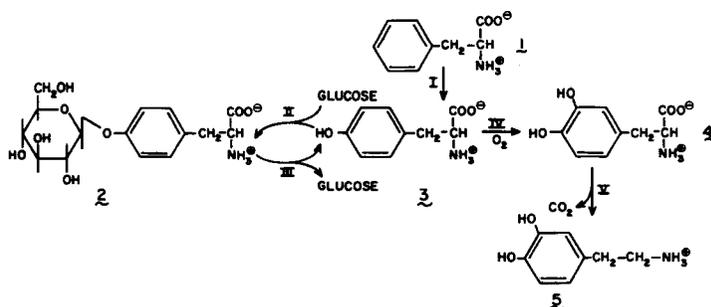


FIG. 8. Scheme of tyrosine metabolism for cuticle tanning in *M. sexta*. The substrates and enzymes include 1, phenylalanine; I, phenylalanine hydroxylase; 2,  $\beta$ -D-glucopyranosyl-O-L-tyrosine; II,  $\beta$ -D-glucopyranosyl-O-L-tyrosine synthase; III,  $\beta$ -D-glucopyranosyl-O-L-tyrosine hydrolase; 3, tyrosine; IV, tyrosine hydroxylase; 4, dihydroxyphenylalanine; V, dihydroxyphenylalanine decarboxylase; 5, 2-(dihydroxyphenyl)ethylamine.

in the wandering larva of *M. sexta*, and titers reach a peak by the time of pupal ecdysis (Hopkins, unpublished data), when it is hydroxylated and decarboxylated to produce the diphenol substrates used in cuticular tanning reactions. The two-dimensional fingerprints also showed that the amount of free tyrosine increases during the wandering stage and that an appreciable quantity of glucoside carries over into the pupal stage. This may represent the tyrosine reserve utilized for tanning and melanization of adult cuticle. A second large release of free tyrosine occurs during the pupal-pharate adult period (Hopkins, unpublished data).

$\beta$ -D-Glucopyranosyl-O-L-tyrosine was first tentatively identified in *Drosophila busckii* Coquillett (5). This appears to be an unusual example of the genus, since the other species of *Drosophila* were found to synthesize tyrosine-O-phosphate (20-22). Nevertheless, the patterns of synthesis and utilization of  $\beta$ -D-glucopyranosyl-O-L-tyrosine and tyrosine-O-phosphate during development appear to be similar in the dipteran and lepidopteran species. Tanning metabolism in *M. sexta* is also modified from that in *Sarcophaga* and *Musca* species (22, 23). The former contained tyrosine conjugated to  $\beta$ -alanine, while the latter sequestered phenylalanine in the form of a glutamyl peptide.

The identification of  $\beta$ -D-glucopyranosyl-O-L-tyrosine, dopa, and dopamine as metabolites of tyrosine in stages of *M. sexta*

undergoing cuticular tanning has directed our attention for future study to four enzymes as regulatory sites for control of diphenol production (Fig. 8). These are tyrosine glucoside synthase (enzyme II), tyrosine glucoside hydrolase (III), tyrosine hydroxylase (IV), and dopa decarboxylase (V). All of the reactions catalyzed by these enzymes occur just prior to or during metamorphosis and therefore are probably subject to control by morphogenetic hormones. Phenylalanine hydroxylase (I), on the other hand, was present in all developmental stages examined and apparently is not regulated in a similar manner.

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