

Incorporation of Radiolabeled Tyrosine, N-Acetyldopamine, N- β -Alanyldopamine, and the Arylphorin Manducin into the Sclerotized Cuticle of Tobacco Hornworm (*Manduca sexta*) Pupae

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Z. Naturforsch. **39c**, 1066–1074 (1984); received June 1/August 27, 1984

Arylphorins, Copolymerization, Insect Cuticle, Sclerotization, Tanning Substrates

Radiolabeled manducin, which is the arylphorin (*i.e.* tyrosine-rich larval serum protein) of the tobacco hornworm *Manduca sexta* was prepared by *in vivo* biosynthesis from [U- 14 C]-L-tyrosine. In order to see whether manducin is incorporated into the cuticle of developing pupae and participates in cuticle sclerotization, the radiolabeled protein was injected into late last larval instar larvae. Fractionation of the sclerotized pupal cuticle into a buffer soluble, acid soluble and acid insoluble fraction shows that up to 2.6% of peptidic tyrosine residues end up in the acid soluble portion. Another 0.5% are recovered from the acid insoluble fraction by combustion. Only 30% of peptidic tyrosine residues of manducin incorporated into the acid soluble fraction are recovered as tyrosine. The presence of radioactivity in the acid insoluble fraction suggests that peptidic tyrosine residues are transformed partly into melanin-like material. The incorporation of manducin into the cuticle of pupae is also evident from immunological studies.

Relative large quantities of radiolabeled acid insoluble melanine like material is also recovered from sclerotized cuticle after incorporation of radiolabeled tyrosine as well as tanning substrates N-acetyldopamine and N- β -alanyldopamine. Application of doubly labeled [7- 3 H,8- 14 C]-N-acetyldopamine shows a high loss of 3 H in the acid insoluble fraction. It is suggested that tanning agents form lignine-like polymers and that sclerotization results from copolymerization with peptidic tyrosine residues in the cuticle. Thus, the arylphorin manducin appears to be an important constituent of the sclerotization system in *Manduca sexta*.

Sclerotization of the cuticle depends in many insects on the oxidation of 1,2-diphenolic substrates in the outer parts of the integument. Reactive intermediates such as *o*-quinones, *p*-quinonemethides or free radicals, which may be generated during the oxidative transformations are thought to effect covalent crosslinking of structural proteins by Michael-type additions and/or free radical polymerizations (for a recent review see [1]). Chemical models are apparently consistent with such theories. However, structures of crosslinks in sclerotized cuticle are essentially unknown due to the difficulties encountered in attempts to isolate soluble derivatives of native structures from the exoskeleton. Thus, an unambiguous chemical identification of a crosslink partial structure has not been reported with full details. Likewise, very little is known on the identity of structural polypeptides participating in the formation of crosslinks.

Applications of immunological [2], radiochemical [2, 3], and histochemical (K. Scheller, personal

communication) techniques have yielded evidence that calliphorin which is the arylphorin [4] of *Calliphora* species is incorporated into the cuticle of the developing fly. We have reported previously [5] that the arylphorins drosophilin, calliphorin, and manducin are crosslinked *in vitro* in a reaction mixture with the sclerotizing agent N-acetyldopamin (NADA) [6] or N- β -alanyldopamin (NBAD) [7] and monophenol monooxygenase. It thus appeared that arylphorins eventually may play a role in cuticle sclerotization, in addition to their function as storage proteins (see [3] and references cited therein). However, before the biological relevance of arylphorins for cuticle sclerotization can be evaluated in detail, it is necessary to prove a participation in this process by an experimental approach. The aim of this study therefore was to gain insight into the metabolism of an arylphorin in a lepidopteran species. We have studied the incorporation of radiolabeled manducin into the sclerotized cuticle of *Manduca sexta* pupae. Furthermore, incorporation of free [U- 14 C]-L-tyrosine as well as radiolabeled NADA and NBAD was investigated in a comparative way.

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0341-0382/84/1100-1066 \$ 01.30/0



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Materials and Methods

Insects

Manduca sexta larvae were reared at 26 °C and 60–80% relative humidity on an artificial diet according to Bell and Joachim [8]. The photoperiod was 16 h light and 8 h dark. Pupal ecdysis occurred 10 days after the last larval moult.

Analytical procedures

HPLC separations were performed with an apparatus consisting of components from Waters (Königstein): pumps M45 and M6000; gradient programmer M660; injector U6K; UV-detectors M440 and M481, refractometer R401. Columns: μ -C₁₈-Bondapak: 4 × 300 mm (Waters); 5 μ m Nucleosil C-18: 4 × 250 mm (Knauer); 37–44 μ m Biogel-P-2: 7 × 1200 mm, slurry packed in water; TSK-G-3000-SW: 7 × 600 mm (LKB). — Manducin was quantified photometrically, using a specific absorption $A_{280}^{1\%} = 18.6$ [9]. — Radioactivity was determined in aqueous samples after mixing with 4 ml Aquasol cocktail (Baker) by counting in a BF 820 liquid scintillation counter (Berthold, Wildbad). Samples were counted with a statistical error $2\sigma \leq 4\%$. Quench corrections were applied by the external standard method via an on line HP 9815 calculator. Radioactivity in insoluble samples was measured after combustion as described by Frohofer [10].

Radiolabeled substrates

[U-¹⁴C]-L-tyrosine ($370 \text{ KBq} \cdot \text{mmol}^{-1} = 10 \text{ mCi} \cdot \text{mmol}^{-1}$) was purchased as a 5 mM solution in H₂O/2% ethanol from Amersham (Braunschweig). [7-³H,8-¹⁴C]NADA (³H/¹⁴C = 4.29; ¹⁴C: $0.72 \text{ mCi} \cdot \text{mmol}^{-1}$) was synthesized as described previously [11] by peracetylation of radiolabeled dopamine and subsequent cleavage of the O-acetyl groups by means of sodium in dry methanol. [8-¹⁴C]NBAD ($3.29 \text{ KBq} \cdot \text{mmol}^{-1} = 0.089 \text{ mCi} \cdot \text{mmol}^{-1}$) was prepared by standard methods [5, 12] and purified by chromatography on a $1.5 \times 110 \text{ cm}$ Sephadex-G-10 column, eluted with 0.2 N acetic acid.

Incorporation experiments

Aliquots ($\leq 400 \mu\text{l}$) of radiolabeled stock solutions were injected laterally into the abdomens of

larvae that had been immobilized before by exposure to an atmosphere of CO₂. After the time intervals of the particular experiments, the larvae were immobilized by submersion into ice water and then blotted dry. A small incision was made into the head capsula and the haemolymph collected in 0.2 ml ice cold 50 mM Tris-HCl buffer pH 7.2, containing 7.3 mM phenylthiourea. Mixing was followed by centrifugation (15 min; $6000 \times g$; 4 °C) and liquid scintillation counting of an aliquot of the supernatant.

The bled larvae or pupae were cut open laterally along the stigmata, adhering tissue was scraped off with a spatula and the remaining cuticle was washed extensively in 0.1 mM NaCN solution. Remaining patches of epidermis were rubbed off with a paper wiper. Pupal procuticle was isolated by careful separation from the overlapping larval cuticle (L5d10) by means of a scalpell.

Cuticle preparations were ground in an all glass Potter-Elvehjem-homogenizer in ice cold 5 mM Tris-HCl buffer pH 8.2, containing 38 mM glycine and 0.1 mM NaCN. After centrifugation (10 min; $6000 \times g$; 4 °C), the supernatant was taken as buffer soluble fraction and an aliquot counted. The sediment was dried *in vacuo* over P₄O₁₀ and its weight determined. Hydrolysis in 6 N HCl, containing 1% phenol, for 24 h at 115 °C under an atmosphere of N₂ was followed by centrifugation (15 min; $4000 \times g$; 20 °C) and liquid scintillation counting of an aliquot of the supernatant after neutralization with 10% aqueous NaHCO₃ solution. Radioactivity incorporated into the acid insoluble fraction was determined by combustion analysis according to [10]. The weight of the ash remaining after combustion was determined.

Preparation of [(U-¹⁴C)-L-tyrosine]manducin

Each of five larvae L5d2 was injected with 20 μl of [U-¹⁴C]-L-tyrosine solution. A second injection (40 μl) followed two days later. Thus, the total amount of radioactivity applied was $555 \text{ KBq} = 15.0 \mu\text{Ci}$. Three days after the second injection (i.e. L5d7) a total of 2.5 ml haemolymph was collected from the surviving four larvae. The procedure for the isolation of manducin was with minor modifications the same as described previously [5] (see also [13]): chromatography on Sepharose 6-B and combining the protein containing fractions of

M_r range 200 000–500 000; freezing at -20°C for 24 h; thawing and centrifugation (15 min; $3600 \times g$; 4°C). The supernatant was 50% saturated with ammonium sulfate and stirred magnetically for 6 h at 4°C . Another centrifugation (15 min; $3600 \times g$; 4°C) was followed by 48 h dialysis of the supernatant against 20 mM sodium phosphate buffer pH 7.0 and lyophilization of the contents of the dialysis tube. The residue was dissolved in 4 ml of a solution of 0.27 g KCl, 3.74 g NaCl, 0.14 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.13 g NaHCO_3 in 1000 ml H_2O . The protein concentration was $15.8 \text{ mg} \cdot \text{ml}^{-1}$; the radioactivity recovered was 14.13 KBq (3.18% of the injected radioactivity). This corresponds to a specific radioactivity of $13\,420 \text{ dpm} \cdot \text{mg}^{-1}$. The radio-labeled manducin was pure according to the following analytical procedures: UV-spectrum: $E_{280/250} = 2.51$ (cf. [9]); HPLC on a TSK-G-3000-SW column: see Fig. 1a; hydrolysis in 6 N HCl followed by HPLC analysis on $\mu\text{-C}_{18}$ -bondapak (25 mM ammonium trifluoroacetate buffer pH 3.0 isocratic for 8 min, then linear gradient up to 100% methanol within 20 min; constant flow $0.8 \text{ ml} \cdot \text{min}^{-1}$) showed radioactivity cochromatographing with tyrosine only; amino acid analysis yielded values consistent with those given in [13].

Determinations of endogenous substrate concentrations

Tyrosine (cf. [14]): Haemolymph (not centrifuged) of L5d8 was mixed with a 10-fold volume of a 2 mM solution of ascorbic acid in 80% methanolic 0.1 N HCl and homogenized in a Potter-Elvehjem-homogenizer which was cooled in an ice bath. Stirring of the homogenate for 2 h at 4°C was followed by centrifugation (20 min; $6000 \times g$; 4°C) and rotary evaporation of the supernatant (bath temperature 40°C). The residue was dissolved in H_2O , the volume of which was equal to the original haemolymph volume. Tyrosine was determined by HPLC on Biogel-P-2 as described elsewhere [15].

Manducin: The protein was quantified in haemolymph by an isotope dilution procedure. Fixed amounts (0.198 mg) [(U- ^{14}C)-L-tyrosine]manducin were mixed with 12.5 or 25 μl L5d8 haemolymph and chromatographed on TSK-G-3000-SW. The peak areas detected at 280 nm at M_r 450 000 were compared with that of a sample of 0.395 mg [(U- ^{14}C)-L-tyrosine]manducin.

NADA and NBAD (cf. [7]): Five ml haemolymph (not centrifuged) of L5d10 was mixed with 4.5 ml

of a 4.8 mM NaHSO_3 solution in 1.2 N HCl and homogenized as described above. Centrifugation (20 min; $6000 \times g$; 4°C) was followed by heating of the supernatant at 100°C for 10 min in order to hydrolyze glycosides. After lyophilization, a residue was obtained which was dissolved in 0.5 ml 25 mM ammonium trifluoroacetate buffer pH 3.0 and analyzed by HPLC on a $\mu\text{-C}_{18}$ -Bondapak column (25 mM ammonium trifluoroacetate buffer pH 3.0 isocratic for 8 min, then linear gradient up to 50% methanol within 20 min; constant flow $0.8 \text{ ml} \cdot \text{min}^{-1}$). NADA and NBAD were detected at 280 nm. The identification and quantification was confirmed by adding known amounts of radio-labeled NADA and NBAD to haemolymph prior to homogenization.

Chromatographic analysis of cuticle hydrolysates

Solutions obtained after hydrolysis of cuticle or manducin (see above) in 6 N HCl were neutralized with 10% NaHCO_3 and then subjected to HPLC analysis on $\mu\text{-C}_{18}$ -Bondapak (25 mM ammonium trifluoroacetate buffer pH 3.0 isocratic for 8 min, then linear gradient up to 100% methanol within 20 min; constant flow $0.8 \text{ ml} \cdot \text{min}^{-1}$). Fractions containing radioactivity were evaporated, the residue dissolved in saturated NaHCO_3 and stirred for 1 h at 20°C with excess acetic anhydride. The resulting mixture was extracted with ethyl acetate and analyzed on a $5 \mu\text{m C-18-Nucleosil}$ column (25 mM ammonium trifluoroacetate buffer pH 3.0, linear gradient up to 100% methanol within 30 min; constant flow $0.8 \text{ ml} \cdot \text{min}^{-1}$). The presence of tyrosine in cuticle hydrolysates was further verified by mass spectrometry.

Immunological procedures

Proteins with a $M_r \geq 18\,000$ were separated from L5d6 haemolymph by chromatography on a Sepharose 4-B column with 10 mM sodium/potassium phosphate buffer pH 6.5. The protein concentration was adjusted to ca. $0.5 \text{ mg} \cdot \text{ml}^{-1}$ and the resulting protein solution was mixed with an equal volume of complete Freund's adjuvans and injected in 0.3 ml aliquots subcutaneous into two rabbits. In 2–3 weeks intervals, two booster injections were made with complete and another one with incomplete

Freund's adjuvans. A fifth injection followed with protein solution only. One week after the last injection, 80 ml blood were collected and the immunoglobulins obtained from serum by precipitation with 50% saturated ammonium sulfate. The ammonium salts were removed from the precipitate by dialysis against 0.1 M sodium phosphate buffer pH 8.0, containing 0.5 M NaCl.

The double immunodiffusion assays were performed according to Ouchterlony [16]. Immunofluorescence studies were carried out with FITC-anti-rabbit IgG (Sigma) on 7 µm tissue slices according to Weller and Coons [17].

Results

Estimations of the minimum amounts of substrates incorporated into the cuticle of *Manduca sexta* pupae are based on the following assumptions: 1. Radiolabeled precursors injected into the haemolymph of the larvae are diluted by endogenous substrates. The incorporation is then calculated from the lower specific activity which yields the total amount of the precursors, and from the total amount of radioactivity found in the cuticle. This assumes that there is no further dilution of the substrates due to *de novo* biosynthesis during the time of incubation. The actual values of incorporation therefore may be higher than reported below. — 2. Calculation of the total amounts of precursors are based on an estimated haemolymph volume of 1.5 ml for the larvae and 1.0 ml for the pupae. — 3. The total amounts of radioactivity incorporated into individual cuticle fractions are calculated from their medium weight (Table I) and the radioactivity found in the aliquots analyzed. Despite the uncertainties resulting from these limitations, the incorporation data shown in Tables II and III yield useful informations with respect to the metabolic

fate of tyrosine, manducin, NADA and NBAD in context with cuticle sclerotization.

Incorporation of [U - ^{14}C]-L-tyrosine

The titer of free tyrosine in haemolymph of L5d8 was found to be $3.78 \pm 0.28 \mu\text{mol} \cdot \text{ml}^{-1}$ ($\pm$ S.D.; analysis of three haemolymph samples) (*cf.* [14]). Thus, after injection of $0.25 \mu\text{mol}$ [U - ^{14}C]-L-tyrosine, the total amount available for incorporation in one larva is $5.92 \mu\text{mol}$. The data in Table II show that most of the tyrosine is incorporated within the first 24 h after pupal ecdysis: $1.13 \mu\text{mol}$ are found in the cuticle of Pd1 (24 h), whereas the cuticle of Pd4 contains radioactivity corresponding to $1.36 \mu\text{mol}$ tyrosine. The major portion is found always in the acid soluble fraction. Calculation of the specific incorporation (*i.e.* nmol precursor per mg cuticle fraction) reveals similar values for the acid soluble and the combustible part of the acid insoluble fraction (Table III). The specific incorporation decreases during further development due to the gain in cuticle weight up to Pd4. Thus the acid soluble fraction has at this stage incorporated $15 \text{ nmol} \cdot \text{mg}^{-1}$ and the combustible part of the acid insoluble fraction $26 \text{ nmol} \cdot \text{mg}^{-1}$.

Analysis of the acid soluble fraction of Pd4 cuticle by HPLC shows that 16.2% of the radioactivity are obtained as an unidentified polar product which cochromatographs with noradrenaline, 54.5% are present as tyrosine, 3% as an unidentified metabolite which elutes between noradrenaline and tyrosine, and the rest of the radioactivity appears at high methanol concentrations together with unipolar products.

Incorporation of [U - ^{14}C]-L-tyrosine]manducin

The concentration of manducin in haemolymph of L5d8 was found to be $80.5 \pm 4.2 \text{ mg} \cdot \text{ml}^{-1}$

Table I. Dry weight of *Manduca sexta* cuticle and cuticle fractions.

Age	Cuticle	Acid soluble		Combustible		Ash	
	[mg]	[mg]	[%]	[mg]	[%]	[mg]	[%]
L5d10	7.9 ± 3.6	5.9 ± 3.4	71.4	1.1 ± 0.6	14.7	< 1	—
Pd1 (24 h)	25.5 ± 6.7	20.2 ± 6.0	78.1	3.3 ± 0.8	15.7	1.5 ± 0.5	6.2
Pd4	84.0 ± 13.1	69.8 ± 10.4	83.2	6.2 ± 0.7	7.5	6.5 ± 1.0	8.2

Dry weight of cuticle was determined after extraction with Tris-HCl-glycine buffer; see materials and methods. Weights $\pm$ S.D.; $n = 6$.

Table II. Incorporation of radiolabeled substrates into the cuticle of *Manduca sexta* pupae.

Substrate	dpm per larva injected	Number and age of larvae	Age at workup ^a	% Of injected radioactivity found in				% Radioactivity recovered	
				Haemolymph	Buffer soluble fraction of cuticle	Acid soluble fraction of cuticle	Combustible	Total	From cuticle
[U- ¹⁴ C]-L-tyrosine	5.55 × 10 ⁶	2 L5d8	L5d10	58.2	0.30	n.d.	n.d.	—	—
	5.55 × 10 ⁶	1 L5d8	Pd1 (12 h)	n.d.	0.31	6.7	0.51	—	7.52
	2.22 × 10 ⁶	3 L5d8	Pd1 (24 h)	27.9 ± 13.2	1.81 ± 0.77	15.4 ± 4.3	2.05 ± 0.66	47.2	19.3
[(U- ¹⁴ C)-L-tyrosine]manducine	7.15 × 10 ⁴	3 L5d8	Pd4	4.8 ± 1.0	3.08 ± 0.91	17.3 ± 2.7	2.73 ± 0.88	27.9	23.1
	7.15 × 10 ⁴	3 L5d4/8/9 ^b	L5d10	54.5 ± 3.9	0.033 ± 0.03	0.12 ± 0.1	< 0.02	54.7	0.15
	7.15 × 10 ⁴	3 L5d4/8/9	Pd4	33.9 ± 7.1	0.29 ± 0.13	2.01 ± 0.65	0.35 ± 0.11	36.6	2.65
[(U- ¹⁴ C)-L-tyrosine]manducine plus 1.1 mg/larva L-tyrosine	7.15 × 10 ⁴	3 L5d4/8/9	L5d10	52.9 ± 3.5	0.053 ± 0.02	0.22 ± 0.15	< 0.02	53.2	0.27
	7.15 × 10 ⁴	3 L5d4/8/9	Pd4	24.6 ± 10.9	0.28 ± 0.04	2.59 ± 0.35	0.50 ± 0.20	28.0	3.37
[7- ³ H,8- ¹⁴ C]NADA	1.04 × 10 ⁶	3 L5d10	Pd1 (24 h)	³ H: 31.8 ± 8.0 ¹⁴ C: 26.1 ± 5.8	0.27 ± 0.08 0.23 ± 0.03	14.8 ± 6.4 12.3 ± 5.0	2.2 ± 1.1 8.6 ± 4.2	49.1 47.2	17.3 21.1
[8- ¹⁴ C]NBAD	0.89 × 10 ⁶	3 L5d10	Pd1 (24 h)	38.9 ± 12.3	0.14 ± 0.01	9.94 ± 3.1	7.9 ± 2.2	56.9	18.0

^a All larvae were worked up individually.^b Injection of manducine was made in approximately equal aliquots on days 4, 8, and 9.

(± range; two determinations) (cf. [13]). After injection of 5.33 mg radiolabeled manducine into one larva, the total amount available for incorporation is 126.1 mg. The results shown in Table II reveal that a major portion of the label is incorporated after pupal ecdysis. The acid soluble fraction of Pd4 cuticle has incorporated up to 2.59% of the radioactivity, corresponding to 3.27 mg manducine or 47 µg manducine per mg cuticle. From the known amino acid composition of the protein which contains 0.152 mg tyrosine per mg manducine [13], the amount of peptide bound tyrosine incorporated into the acid soluble fraction is calculated to be 39 nmol · mg⁻¹. Similarly, the acid insoluble fraction yields after combustion analysis the following data: incorporation 0.5%, corresponding to 0.63 mg manducine or 102 µg manducine per mg cuticle fraction which again corresponds to 85 nmol peptide bound tyrosine per mg cuticle fraction.

Coinjection of radioinactive tyrosine together with radiolabeled manducine does not result in a decrease of the amounts of label incorporated into Pd4 cuticle. This result presents evidence that the transfer of radiolabel from haemolymph into cuticle does occur in form of peptide bound tyrosine and not *via* free tyrosine that eventually could have been generated by a proteolytic degradation of manducine. HPLC analysis of haemolymph from L5d10 and from Pd4 shows the presence of radiolabeled manducine only (Fig. 1b). Low molecular weight degradation products cannot be detected in haemolymph after injection of radiolabeled manducine at L5d8.

Analysis of the acid hydrolysate from Pd4 cuticle by HPLC shows that 30% of the soluble radioactivity are present as tyrosine. The rest elutes with low polarity products at a high methanol concentration. No radiolabeled material cochromatographing with noradrenaline is detected in the cuticle hydrolysate after incorporation of [(U-¹⁴C)-L-tyrosine]manducine.

Incorporation of [7-³H,8-¹⁴C]-NADA

The titer of NADA in haemolymph of L5d10 was found to be ca. 5 nmol · ml⁻¹ after acid catalyzed hydrolysis (cf. [7]). Since 650 nmol radiolabeled NADA were injected into one larva, the total amount available for incorporation is 658 nmol. The ¹⁴C-radioactivity recovered from the cuticle of

Table III. Specific incorporation ($\text{nmol} \cdot \text{mg}^{-1}$) of precursors into cuticle fractions.

Precursor	Age of pupae	Acid soluble fraction	Acid insoluble fraction	Acid insoluble
				Acid soluble
free tyrosine	Pd1	45	37	0.8
	Pd4	15	26	1.7
tyrosine <i>ex</i> manducin	Pd4	39	85	2.2
NADA	Pd1	4	17	4.3
NBAD	Pd1	38	186	4.9

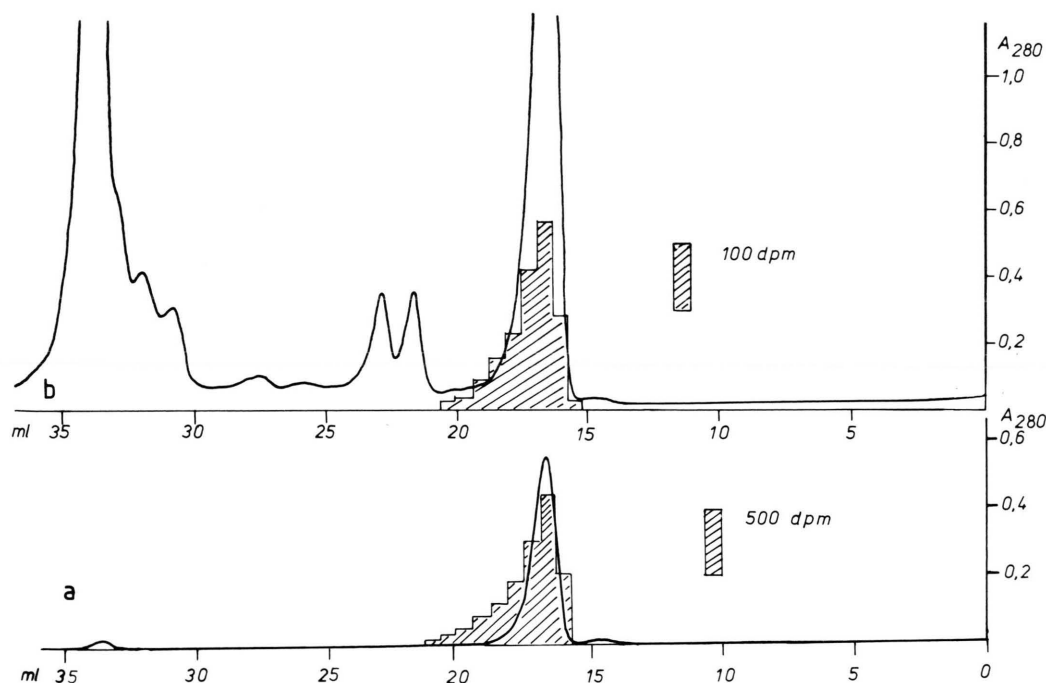


Fig. 1. HPLC of a) 50 μl [(U- ^{14}C)-L-tyrosine]manducin solution, b) 50 μl Pd4 haemolymph after injection of 5.33 mg [(U- ^{14}C)-L-tyrosine]manducin at L5d8. Conditions: 0.7×60 cm TSK-G-3000-SW column eluted with 0.1 M potassium phosphate buffer pH 6.8 at $0.2 \text{ ml} \cdot \text{min}^{-1}$.

Pd1 (24 h) reveals that 22.1%, corresponding to 145.4 nmol NADA were incorporated (Table II). The specific incorporation into the combustible part of the acid insoluble fraction (Table III) is 4.3 times higher than into the acid soluble. The relative amounts of ^3H -radioactivity are much lower in the combustible part of the acid insoluble fraction than in the acid soluble. This difference may reflect a side chain oxidation of NADA and will be considered in more detail in the discussion section below.

Incorporation of [8- ^{14}C]NBAD

The titer of NBAD (free plus conjugates) in haemolymph of L5d10 was found to be $2.20 \pm 0.15 \mu\text{mol} \cdot \text{ml}^{-1}$ ($\pm$ S.D.; three haemolymph samples were analyzed) (*cf.* [7]). After injection of 4.48 μmol [8- ^{14}C]NBAD, 18% of the totally available 7.78 μmol are incorporated into the cuticle of Pd1 (24 h). The specific incorporation into the combustible part of the acid insoluble cuticle fraction is 4.9 times higher than into the acid soluble fraction (Table III).

Immunological experiments

When pupal procuticle from L5d10 is extracted with cyanide containing buffer and the extract subsequently tested in the Ouchterlony double diffusion assay with antibodies against L5d6 haemolymph proteins, a faint diffuse zone of precipitation is observed (Fig. 2). A comparison with the appearance of a precipitation line from purified manducin which had been applied in an adjacent well, shows that the cuticle extract contains some but not all of the antigenic determinants of manducin. No precipitation with antibody was observed at all when the cuticle was extracted with buffer containing no cyanide. In those cases a lightly tan coloured ring formed at the edges of the well containing the cuticle extract.

The presence of manducin antigenic determinants in procuticle of pharate pupae was also detected by immunofluorescence. Procuticle itself shows a bright yellow fluorescence arising from the already sclerotized pockmarks. Treatment with antibodies against L5d6 haemolymph proteins followed by FITC labeled anti-rabbit-IgG from goats lead to a green fluorescence in addition to the yellow pockmarks. When antibodies against L5d6 haemolymph proteins are titrated with manducin before application to L5d10 pupal procuticle, there is no development of a green immunofluorescence*. Analogous studies performed with L5d5 larval cuticle did not show notable differences between preparations treated with full antibodies or with a titrated sample. The cuticle of Pd1 (24 h) shows a very bright "eigen"-fluorescence of the sclerotized parts, and no manducin antigenic determinants can be

detected in the endocuticle which apparently does not contain immunoreactive material at this stage.

Discussion

The results of this study provide experimental support for an earlier hypothesis [18, 19] according to which arylphorins are participating in cuticle tanning during sclerotization. Scheller *et al.* [2] have shown that calliphorin antigen is present in the unsclerotized parts of the cuticle of *Calliphora vicina* adults. Incorporation of radioactivity from [¹²⁵I]calliphorin [2] as well as [³H]calliphorin, coupled with histochemical and autoradiographic techniques have provided further evidence for the incorporation of calliphorin into the cuticle (K. Scheller, personal communication). Levenbook and Bauer reported [3] that 10.8% of the radioactivity applied as [(U-¹⁴C)-L-phenylalanine]calliphorin to larvae was incorporated into as SDS insoluble cuticle fraction of the puparium in *Calliphora*.

Following the observation of Scheller *et al.* [2], we have investigated some further questions:

1) Is the arylphorin of a lepidopteran species also incorporated into the cuticle? Radiolabeled manducin was prepared from [U-¹⁴C]-L-tyrosine by *in vivo* biosynthesis and injected into late last larval instar of *Manduca sexta*. Fractionation of the cuticle of pupae by extraction with buffer, acid hydrolysis and combustion analysis reveals that radioactivity is incorporated indeed into sclerotized structures. The degree of incorporation is lower than that reported by Levenbook and Bauer [3] for the puparium of *Calliphora*. Since different insects were used, a direct comparison of the incorporation values will not be possible. The results of the analytical procedures show that manducin is involved in cuticle sclerotization.

The presence of manducin immunoreactive material in the procuticle of the pharate pupae is evident from corresponding studies, using immunofluorescence labelling. Immunoreactive material can also be extracted from the procuticle under reducing conditions with sodium dithionite containing buffer or with cyanide containing buffer which will inhibit phenol oxidation. Since, however, not all of the antigenic determinants present in manducin

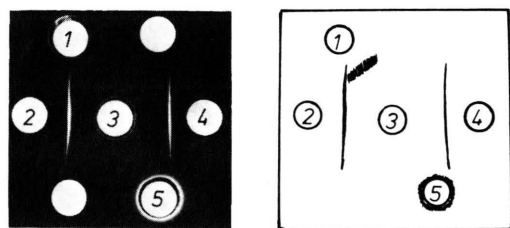


Fig. 2. Ouchterlony double diffusion assay of manducin and cuticle extracts. Left: original photograph; right: ink drawing of precipitation zones in order to show precipitation zones more clearly. The following samples were applied into the wells: 1. Extract of L5d10 pharate pupal procuticle with cyanide containing buffer; 2. purified manducin; 3. antibody against L5d6 haemolymph proteins; 4. purified manducin; 5. extract of L5d10 pharate pupal procuticle with cyanide-free buffer.

* Colour photographs are available from the authors upon request.

are detected in cuticle extracts, it appears that the protein is changed metabolically to a certain extent.

2) Is there a partial proteolytic degradation of manducin in the haemolymph which might result in an incorporation of free tyrosine? The results of the immunological experiments show that, if degradation should occur, it cannot be complete. Simultaneous injection of unlabeled free tyrosine and radiolabeled manducin leads to an incorporation which is similar to that of radiolabeled manducin alone. Finally, low molecular weight degradation products of manducin are not detectable in haemolymph during late larval or early pupal development. Another strong argument against incorporation *via* free tyrosine is derived also from the fact that [(U- ^{14}C)-L-tyrosine]manducin is not metabolized to a product which cochromatographs after acid hydrolysis with noradrenaline. Thus the metabolic fate of free tyrosine is at least with respect to this product clearly different from that of peptidic tyrosine.

3) Does manducin actually participate in sclerotization? In order to answer this quite difficult but most intriguing question it would have to be demonstrated that reaction products of manducin and diphenolic sclerotization agents are actually present in the cuticle. So far we have only shown by chromatographic procedures that ca. 70% of the tyrosine residues incorporated into the acid soluble cuticle fraction *via* manducin are chemically modified, appearing as low polarity products in HPLC. Similar low polarity products are also observed after incorporation of free tyrosine. Extraction of procuticle from pharate pupae that were injected with [U- ^{14}C]-L-tyrosine at L5d8 reveals the presence of peptides with M_r $40\,000 \pm 20\,000$ which have incorporated ^{14}C -label (unpublished observations). Another metabolic pathway of free tyrosine will be its conversion into eumelanin *via* DOPA [20] (see also [21]) and into tanning agents. Since, in addition to the polar product cochromatographing with noradrenaline on $\mu\text{-C}_{18}$ -Bondapak, a mixture of low polarity products results from both, free and peptidic tyrosine, it appears possible that this may actually reflect a copolymerization of peptide bound and free tyrosine metabolites.

Melanin-type polymeric material is present in the acid insoluble cuticle fraction not only after incorporation of free tyrosine and peptidic tyrosine, but also after incorporation of NADA and NBAD

(Table III). The relative incorporation of NADA and NBAD is comparable to that of free tyrosine (Table II). The specific incorporation appears to be remarkably high in the case of NBAD. Compared with NADA, the tenfold lower incorporation of this substrate results eventually from the much lower concentration of NADA in the haemolymph. The ratio of acid soluble versus acid insoluble products (Table III) is ca. twice as high with the diphenolic substrates than with tyrosine or with manducin. It appears from this that the diphenolic substrates are more efficiently incorporated into products that are detected as melanin after acid hydrolysis.

The mechanism of melanin formation is not yet clear. It is possible that acid catalyzed hydrolysis of N-acyl groups generates amines which will cyclize with quinonoid structures [22]. However, we found recently by application of solid state CP-MAS- ^{13}C -NMR-spectroscopy [23] that substantial amounts of diphenolic compounds are present in fully sclerotized native cuticle of *Manduca sexta* pupae. This proves that oxidation products of tanning substrates are present in the cuticle finally as diphenolic derivatives. Since the incorporation of ^3H from [7- ^3H , 8- ^{14}C]NADA is much lower than that of ^{14}C in the acid insoluble fraction (Table II), it is likely that the polymerization of NADA occurs after side chain oxidation [24] *via* free radicals or quinone methides [11]. Thus the polymerization of tanning substrates resembles the formation of lignine in plants [25] and sclerotization may be described as a unique sort of lignification in the animal kingdom, in which the aromatic polymer is not attached to cellulose but to structural proteins.

It is tempting to speculate that sclerotization of the insect cuticle not only results from the formation of lignine-like polymers, but that actually copolymerization of tanning agents with peptide bound tyrosine residues occurs. Such a type of copolymerization has been observed before in studies on the simultaneous oxidation of NADA and tyrosine *in vitro* [26]. Furthermore, it has been shown that graft polymers can be prepared by oxidation of NADA in the presence of tyrosine melanin or *vice versa* by oxidation of tyrosine in the presence of NADA polymeric oxidation products [26]. Lipke *et al.* [27] have presented evidence, without reporting quantitative data, that covalent complexes of tanning agents and tyrosine can be recovered from sarcophagid puparial cuticle. Taking all the evidences

together, the idea that arylphorins participate in sclerotization by copolymerization with diphenolic substrates appears to be very attractive. Further work will have to examine the validity of such a proposal.

Arylphorins are apparently not the only polypeptides transported into the cuticle, since immunological studies have revealed similarities of haemolymph and cuticle polypeptides in several insects [28–31] and in a crustacean species [32]. The question arises whether copolymerization of sclerotization agents with tyrosine residues is eventually also observed with proteins that may not be classified as arylphorins. Our previous studies on the selectivity of *in vitro* crosslinking of arylphorins support the view [5] that primarily these are responsible for the formation of a sclerotized macro-

molecular network in the cuticle. Since we know now that arylphorins not only can be crosslinked *in vitro* but also that they are incorporated into the cuticle and chemically modified during sclerotization, we have a tool for the further investigation of the molecular principles of this important biological process.

Acknowledgements

We are very much indebted to Dr. K. Lehmann, Köln, for a generous gift of antibodies, and to Dr. R. Hartmann, Köln, for his help with the immunofluorescence studies. This work was supported by the Deutsche Forschungsgemeinschaft (Pe 264/2-3 and Pe 264/4-1).

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