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PRODUCTS OF *IN VITRO* OXIDATION OF N-ACETYLDOPAMINE AS POSSIBLE COMPONENTS IN THE SCLEROTIZATION OF INSECT CUTICLE

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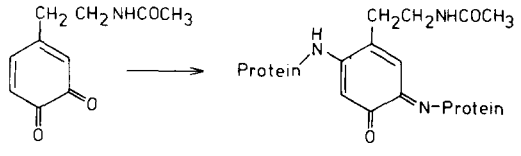
Abstract—[1-^{14}C]-N-Acetyldopamine (NADA) was oxidized in the presence of methyl [3-^{3}H]-β-alanate with mushroom tyrosinase. The complex mixture of reaction products was partly resolved by chromatographic procedures and analyzed by spectroscopic methods. Methyl-β-alanate is incorporated to only a small extent into oxidation products of NADA which *inter alia* are presumed to be oligomeric hydroxyquinones. After oxidation of [1-^{14}C, 2-^{3}H]-NADA with preparations from tanning *Manduca sexta* pupal cuticle, N-acetylnoradrenalin was identified as one of the products. Binding of radioactivity to melanin-like material was also observed. These results suggest that oxidation products different from those formulated usually for the crosslinkages between protein amino groups and N-acetyldopaquinone are deposited in darkly brown coloured insect cuticles during sclerotization.

Key Word Index—N-acetyldopamine, sclerotization, tyrosinase, *o*-quinones, tanning agents

INTRODUCTION

IT IS GENERALLY accepted that N-acetyldopamine (NADA) is the immediate precursor for compounds effecting sclerotization of the cuticle in a number of insect species (KARLSON and SEKERIS, 1962; KOEPPE and GILBERT, 1974; BODNARYK *et al.*, 1974; RAGHAVAN and NADKARNI, 1977). One principal mechanism for the molecular action of NADA in sclerotin formation is assumed to involve addition and condensation of amino groups from proteins to an *o*-quinone generated by a diphenoloxidase from the catechol type precursor (Scheme 1). Analogous reactions have indeed been observed to occur between a variety of *o*-quinones and amines; it should, however, be noted that reasonable yields of chemically defined products were obtained after conducting the reactions in non-aqueous solvents or with amines of an aromatic type, e.g. aniline (HACKMAN and TODD, 1953; reviews: MASON, 1955; WANZLICK, 1964; FINLEY, 1974). Isolation and structure elucidation of products generated from nascent *o*-quinones and an aliphatic amine in aqueous solution showed that the yields of compounds supporting the classical quinone tanning hypothesis are very small (PETER, 1978). Products resulting from the reaction of nascent N-acetyldopaquinone with an aliphatic amine have never been described. It was therefore of interest to investigate the enzyme-catalyzed oxidation of NADA in the presence of an aliphatic amine, utilizing either mushroom tyrinosinase or oxidative enzymes from pupal *Manduca sexta* cuticle, which presumably incorporates NADA during tanning (KOEPPE and GILBERT, 1974).

Scheme 1



MATERIALS AND METHODS

[1-^{14}C]-Dopamine (54.9 mCi/mmole), [2-^{3}H]-dopamine (14.8 Ci/mmole), and [3-^{3}H]-β-alanine (36.0 Ci/mmole) were purchased from NEN Chemicals (Dreieich). Unlabelled dopamine was from Merck (Darmstadt). N-Acetyldopamine (NADA) was prepared by the procedure of KOEPPE and MILLS (1975), purified by chromatography on a 1.5 cm i.d. × 110 cm Sephadex G-10 column (Pharmacia Fine Chemicals, Uppsala) with 0.2 M acetic acid, and identified by ^{1}H-NMR spectroscopy. Doubly labelled [1-^{14}C, 2-^{3}H]-NADA was obtained by mixing [1-^{14}C]-NADA (1.03 mCi/mmole) with [2-^{3}H]-NADA (1.12 mCi/mmole) as well as unlabelled NADA, and subsequent re-chromatography to ensure the removal of impurities that could have been formed during storage of the singly labelled precursors. Methyl [3-^{3}H]-β-alanate hydrochloride with a specific activity of 2.64 μCi/mmole was prepared by HCl-catalyzed esterification of [3-^{3}H]-β-alanine in dry methanol. After chromatography on a 2.2 cm i.d. × 70 cm Biogel P-2 column (10–37 μm; Bio-Rad Laboratories, München) with H_2O, the fraction used for the experiments contained 85 ± 5% (± S.D.; three integrations of the ^{1}H-NMR spectrum) methyl [3-^{3}H]-β-alanate, the remainder beeing [3-^{3}H]-β-alanine.

Mushroom tyrosinase (E.C. 1.14.18.1.) (500–1000 U/mg) was purchased from Serva (Heidelberg) or from Sigma (München). Enzyme catalyzed reactions were performed in Erlenmeyer flasks placed in a shaking water bath at 27 ± 2°C. Product analysis involved chromatography on a 2.2 cm i.d. × 100 cm Sephadex LH-20 column with glass distilled water

and continuous monitoring at 278 and 365 nm. HPLC was performed with equipment from Waters Associates (Königstein), using slurry packed 0.8 cm i.d. × 122 cm columns of Biogel P-2 (10–37 or 37–44 μm) with glass distilled water at a flow rate of 0.5 or 1.0 ml/min and monitoring of the eluate at 280 nm with a Model 440 u.v. detector. For the application of spectroscopic methods see PETER (1978).

Radioactivity was determined with a BF 8020 liquid scintillation spectrometer (Berthold Laboratories, Wildbad) using the external standard channels ratio for quench corrections. Aqueous samples of up to 0.5 ml were counted in 4 ml Aqualuma cocktail (Baker Chemicals, Gross Gerau). Counting efficiency was for unquenched samples in the dual channel mode 57.9% for ^{14}C in the upper channel, 6.7% for ^{14}C in the lower channel, and 34.9% for ^{3}H in the lower channel. Samples were counted with a statistical error smaller than $\pm 2\%$ (2σ) in the upper channel. The largest S.D. observed during determinations of $^{3}H/^{14}C$ ratios was $\pm 4\%$ with three replicate samples. Reproducibility of $^{3}H/^{14}C$ ratios is estimated to be within $\pm 5\%$.

Molar ratio (MR) values were calculated during analysis of reaction products resulting from oxidation of [1-^{14}C]-NADA in the presence of methyl [3-^{3}H]-β-alanate. They are a measure of the number of moles of NADA incorporated into the substances eluting with a distinguishable peak during chromatographic separations relative to one mole of methyl β-alanate in that same fraction. Their accuracy is estimated to be within $\pm 10\%$.

Last instar larvae of *Manduca sexta* were obtained through the courtesy of Dr. K. Lehmann, Universität Köln. They were kept at a photoperiod of 12 hr light/12 hr dark. Preparation of the cuticle was performed within 6 hr of pupal moulting by freezing the animals in liquid nitrogen and stripping of the integument during thawing; adhering tissues were not removed.

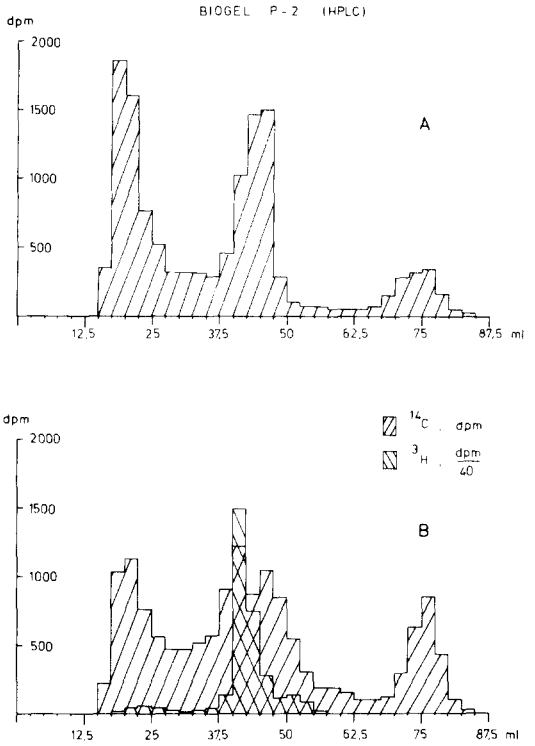


Fig. 1. Elution profiles of radioactivity from reactions with 10^{-2} M [1-^{14}C]-NADA and (a) no methyl β-alanate, (b) 10^{-2} M methyl [3-^{3}H]-β-alanate on a 0.8 cm i.d. × 122 cm Biogel P-2 column. Flow rate = 1.0 ml/min (H_2O).

RESULTS

Table 1 shows the colour changes that were observed during the mushroom tyrosinase-catalyzed oxidation of [1-^{14}C]-NADA in potassium phosphate buffer in the presence of methyl [3-^{3}H]-β-alanate. After a 50 hr incubation period all reaction mixtures remained clear and had developed a brown colour, the intensity of which varied with the initial concentration of NADA. The reddish colour observed after 2 hr was less evident in the mixtures containing no amino ester. In translucent light the solutions containing initially 10^{-2} M NADA resembled in colour that of tanned brown cuticle of e.g. many lepidopterous pupae.

Rotary evaporation (bath temperature: 35°C, condenser temperature: −10°C) of the reaction mixtures showed that a maximum of 2.4% of the total [^{3}H]-radioactivity was present in the distillate after an incubation time of 100 hr. Aliquots were analyzed by HPLC on Biogel P-2 after an incubation time of 50 hr.

Figure 1 shows the elution profiles of radioactivity obtained from the two reaction mixtures containing initially 10^{-2} M [1-^{14}C]-NADA (0.32 μCi/mmole), either in the absence [Fig. 1(a)] or in the presence [Fig. 1(b)] of 10^{-2} M methyl [3-^{3}H]-β-alanate (2.64 μCi/mmole). The [^{14}C] distribution is very similar in both chromatograms: In Fig. 1(a), 47.2% of the total [^{14}C] activity is eluted between 15 and 35 ml; the corresponding value in Fig. 1(b) is 35.1%. Another 40.2% [Fig. 1(a)] and 44.2% [Fig. 1(b)], respectively, chromatographs between 35 and 55 ml. The peak with centre at 75 ml is unchanged NADA. In Fig. 1(b), only 7.6% of the [^{3}H]-radioactivity migrates with the first major [^{14}C]-peak, from which a MR of 4.6 is calculated. Similar elution profiles were observed from HPLC analysis of the other reactions with 10^{-1}, 10^{-3} and 10^{-4} M NADA (see Table 1). The amounts of [^{14}C]-activity present in the first HPLC peak were 5.5%, 33.2% and 27.7%, with MR values of 23.7, 1.8 and 0.3, respectively.

Table 1. Mushroom tyrosinase-catalyzed oxidation of [1-^{14}C]-NADA in the presence of methyl [3-^{3}H]-β-alanate: substrate concentrations and colour changes.

Concentration of NADA	10^{-1}	10^{-2}	10^{-2}	10^{-3}	10^{-4}
Specific activity of NADA (μCi/mmole)	0.032	0.32	0.32	3.2	32
Concentration of methyl β-alanate	10^{-2}	10^{-2}	0	10^{-2}	10^{-2}
Colour after 2 hr	pale yellow	red-brown	brown	red	pink
Colour after 50 hr	brown	brown	brown	light brown	pale brown
% of NADA oxidized after 50 hr	17	83	90	100	100

Reactions were performed in 100 ml 0.05 M potassium phosphate buffer pH 7.2. Enzyme concentration: 0.1 mg/ml ≡ 50 U/ml. Specific activity of methyl β-alanate: 2.64 μCi/mmole.

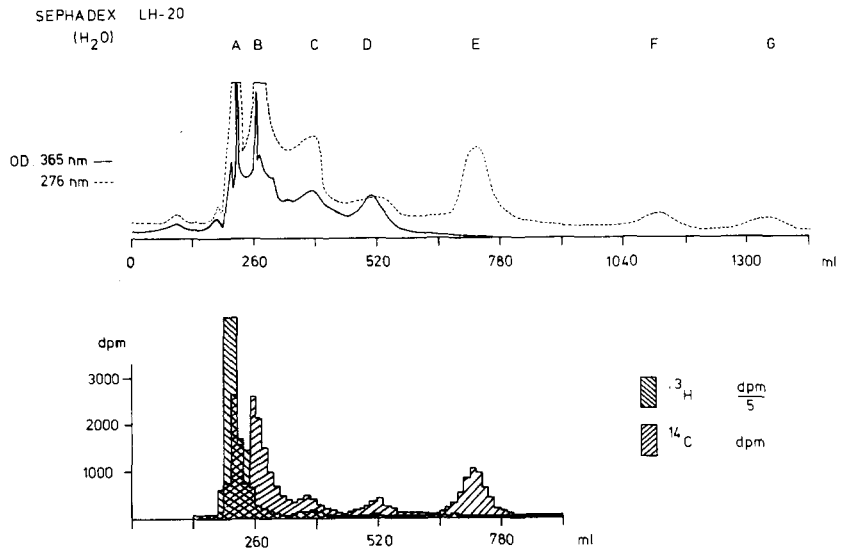


Fig. 2. Elution profiles of radioactivity (lower trace) and optical density from the reaction with 10^{-2} M [1-^{14}C]-NADA and methyl [3-^{3}H]-β-alanate on a 2.2 cm i.d. x 100 cm Sephadex LH-20 column (H_2O).

Ion exchange chromatography of the 10^{-2} M reaction mixture on a 1.5 cm i.d. × 60 cm Dowex 50-X2 (37–74 μm) column at 40±0.5°C with a stepwise gradient formed from (a): 0.2 M sodium acetate buffer pH 4.13, and (b): 1.0 M sodium acetate buffer pH 8.0 containing 2.0 M sodium chloride resulted in the elution of all the [^{14}C] activity, which was associated with reaction products of NADA in three distinguishable peaks between 80 and 200 ml at pH 4.2 (99% buffer A and 1% buffer B). These were followed by unchanged [1-^{14}C]-NADA at pH 4.4 and methyl [3-^{3}H]-β-alanate at pH 7.0. The amount of [^{3}H] radioactivity present in the reaction products indicated MR values between 2.1 and 3.7.

A more complex composition of the reaction mixtures is revealed by chromatography on Sephadex LH-20 as shown for the 10^{-2} M reaction in Fig. 2. There were four fractions denoted as A (160–240 ml), B (240–340 ml), C (340–460 ml), and D (460–650 ml) that eluted faster than NADA (peak E). Peaks F and G, each containing approx. 2% of the total [^{14}C] activity were yellow in colour and were not formed in the absence of methyl β-alanate. They incorporated only small amounts of the amino ester (MR = 2.6 and 3.2, respectively). When the material eluting in peak A (73.7% ^{3}H; 16.4% ^{14}C; MR = 0.2) was analyzed by HPLC on Biogel P-2 (Fig. 3), separation of the label into two regions was observed. With the first peak (15–30 ml) eluted 67.8% of the [^{14}C] activity of fraction A, which was 11.7% of the total [^{14}C] activity. It also contained 8.5% of the [^{3}H]-activity of fraction A, or 6.8% of the total. From this an MR of 1.7 is calculated for the material in the first HPLC peak. The second HPLC peak accounts for 87.2% of the eluted [^{3}H] activity (65.2% of total) and 28.3% of the eluted [^{14}C] activity (4.3% of total). Recording of an ^{1}H-NMR spectrum of the material eluting with the second HPLC peak showed the presence of unchanged methyl β-alanate. Fraction B from Sephadex LH-20 (Fig. 2) (16.8% ^{3}H; 34.9% ^{14}C; MR = 2.1) was further resolved in the same way into two mixtures of products by HPLC (Fig. 4). The first one (15–30 ml) contained 9.7% of total [^{3}H] activity and 29.0% of total [^{14}C] activity (MR = 3.0), and the second (30–50 ml) showed an MR of 0.9. Analogously, the material from fraction C (Fig. 2) (2.8% ^{3}H; 13.5% ^{14}C; MR = 4.8), when subjected to HPLC, showed separation into components with MR = 7.9 (1.3% ^{3}H; 10.2% ^{14}C) and MR = 2.3 (1.3% ^{3}H; 3.0% ^{14}C).

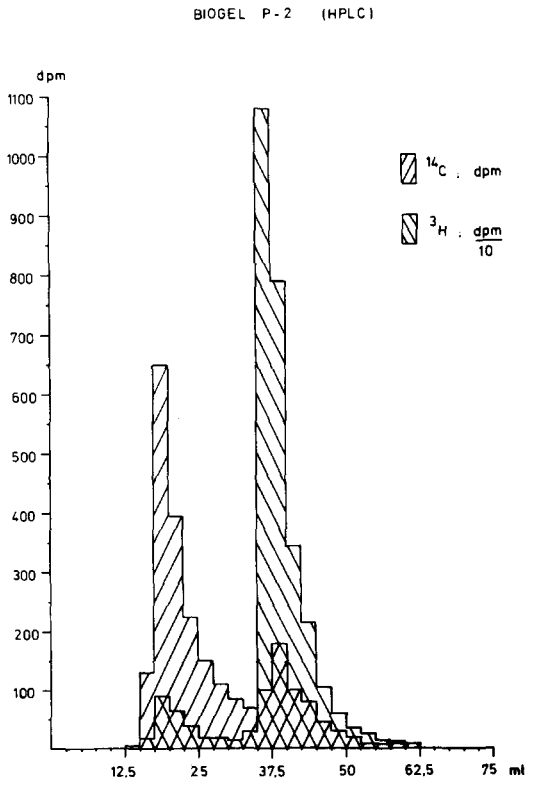


Fig. 3. Radioactivity distribution of fraction A from Fig. 2 on Biogel P-2.

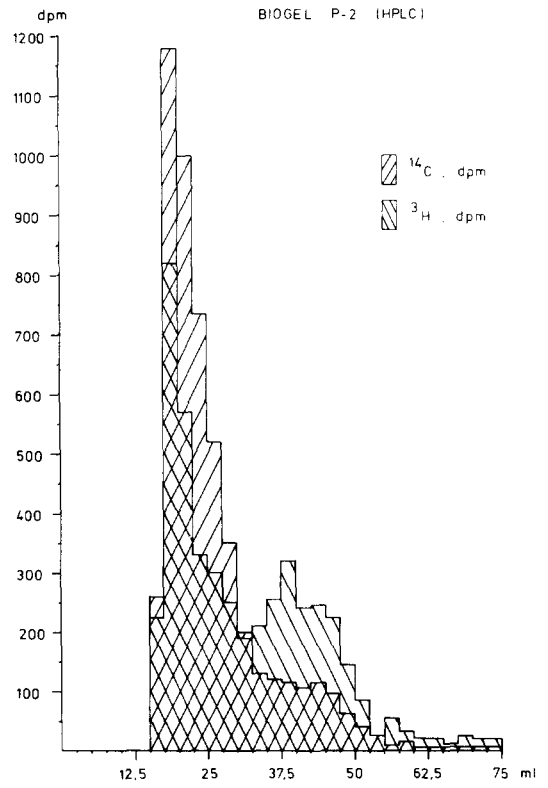


Fig. 4. Radioactivity distribution of fraction B from Fig. 2 on Biogel P-2.

Chromatographic analysis of other reaction mixtures obtained after oxidation of NADA in the presence of methyl β-alanate resulted always in principally the same u.v. absorption and radioactivity profiles. The majority of products eluted on Sephadex LH-20 as a brown coloured material in fractions A and B. On further analysis by HPLC, fraction A yielded a large fast eluting portion of oxidation products in the first peak and another one containing mostly methyl-β-alanate in addition to smaller amounts of oxidation products. Fraction B was always composed predominantly of fast eluting components. From a duplicate of the experiment described above the following MR values were found: Fraction A, HPLC first peak: 7.2% ^{3}H; 13.8% ^{14}C; MR = 1.9. Fraction B, HPLC first peak: 4.7% ^{3}H; 32.5% ^{14}C; MR = 7.0. Similarly, the reaction with 10^{-3} M [1-^{14}C]-NADA and 10^{-2} M methyl [3-^{3}H]-β-alanate yielded: Fraction A, HPLC first peak: 1.9% ^{3}H; 21.6% ^{14}C; MR = 1.2. Fraction B, HPLC first peak: 1.8% ^{3}H; 42.7% ^{14}C; MR = 2.4.

The substances eluting from Sephadex LH-20 in regions B and C after drying formed a brittle, darkly reddish-brown coloured resin. When an aliquot of the combined fractions B and C was subjected to dialysis (Sigma dialysis tubes, Cat. No. 250–9 U) for 96 hr against eight changes of distilled water, 19.6% of the [^{3}H] activity and 16.2% of the [^{14}C] activity present originally remained with the materials in the dialysis tube. This fraction upon evaporation yielded a blackish-brown water soluble resin.

Elementary analysis of the reaction product mixtures eluting in fractions B and C on Sephadex LH-20 gave a composition corresponding to $C_{7.9}H_{10.1}NO_{3.9}$ and $C_{8.1}H_{10.2}NO_{3.7}$, respectively. In the UV spectrum (H_2O) of fraction B general absorption with a low intensity maximum at 281 nm was observed, and in that of fraction C an additional maximum at 325 nm was observed. The ^{1}H-NMR spectrum (90 MHz; D_2O, internal reference: HDO at $\delta = 4.80$ ppm) of fraction B showed broad signals at $\delta = 7.3$–6.5 ppm (aromatic H), 3.9–3.7 ppm with a sharp singlet at 3.80 ppm ($-OCH_3$), 3.7–3.1 ppm ($>N-CH_2-$), 3.1–2.4 ppm ($-CH_2-$), and 2.1–1.8 ppm ($-NCOCH_3$) with relative intensities of 3:3:6:6:6. In the decoupled ^{13}C-NMR spectrum (22.6 MHz; D_2O/5% CD_3OD; internal reference: CD_3 at 49.0 ppm) intensive and sharp signals appear at 174.7 and 22.7 ppm ($-NCOCH_3$). A rather large number of different aromatic carbon atoms are indicated by broad groups of signals between 147 and 115 ppm. There are three groups of signals at 41, 35 and 32 ppm for the side chain carbon atoms, which are also detected in an analogous sample obtained by oxidation of NADA in the absence of methyl β-alanate. There appear also low intensity signals between 76 and 72 ppm, most probably resulting from –CHOH– groupings.

A sample of [1-^{14}C, 2-^{3}H)-NADA (0.76 μCi ^{14}C/mmole; $^{3}H/^{14}C = 7.95 \pm 0.40$) was used for investigation of the extent of side chain oxidation. Reaction mixtures containing 50 U/ml mushroom tyrosinase and initially 10^{-2} M [1-^{14}C, 2-^{3}H]-NADA either in the presence of 10^{-2} M methyl β-alanate (unlabelled) or in its absence were evaporated after an incubation period of 100 hr. The amount of [^{3}H] activity recovered as ^{3}HOH was between 1.0 and 4.0% of the starting radioactivity. The corresponding values obtained from non-enzymic reactions varied between 0.4 and 0.6%. Chromatography on Sephadex LH-20 showed that 24 and 35%, respectively, of the NADA had remained unchanged in the enzyme-catalyzed reaction. The $^{3}H/^{14}C$ ratio of materials eluting in fractions A, B and C (cf. Fig. 2) had remained unaltered within the limits of error ($^{3}H/^{14}C = 8.10 \pm 0.40$), but the occurrence of side chain oxidation was confirmed by isolation of a substance eluting with peak centred at 520 ml (cf. Fig. 2, fraction D) that showed a $^{3}H/^{14}C = 4.14 \pm 0.20$. Based on mass spectral data (m/e 193, M^+-H_2O; m/e 151, $M^+-H_2O-CH_2CO$; cf. ANDERSEN and ROEPSTORFF, 1978) and on its ^{1}H-NMR spectrum (triplet at 4.53 ppm integrating for one proton), the compound is N-acetylnoradrenalin, which was formed in 1.9–3.1% yield in the enzyme-catalyzed reaction.

In order to see how the mushroom tyrosinase-catalyzed oxidation of NADA would compare with analogous experiments utilizing an insect enzyme system, a crude cuticle preparation of a tanning *Manduca sexta* pupa was reacted with doubly-labelled NADA. After extensive grinding of the cuticle in an all glass Potter-Elvehjem homogenizer with 5 ml 0.05 M potassium phosphate buffer pH 6.8, the homogenate was centrifuged for 15 min at 15,000 *g* and 2°C; grinding and centrifugation was repeated once with the sediment. Each sample (cuticle supernatant and resuspended sediment) was divided into two portions, to one of which an equal volume of phosphate buffer, saturated with phenylthiourea (PTU) was added. All

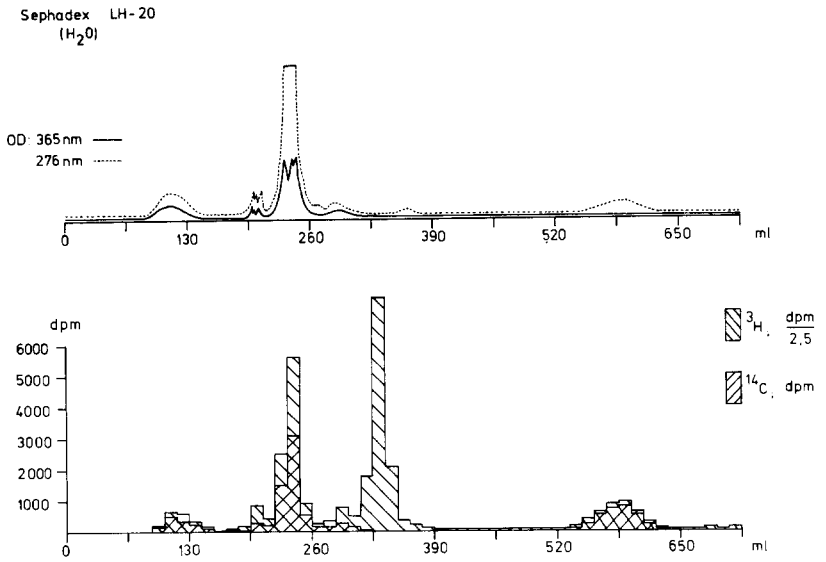


Fig. 5. Chromatography of supernatant from tanning *Manduca sexta* pupal cuticle after incubation with [1-^{14}C, 2-^{3}H]-NADA for 72 hr on a 2.2 cm i.d. × 100 cm Sephadex LH-20 column (H_2O).

four mixtures were made 0.7×10^{-2} M with respect to [1-^{14}C, 2-^{3}H]-NADA (3.73 μCi ^{14}C/mmole; ^{3}H/^{14}C = 5.34 ± 0.30) and adjusted to a final volume of 15 ml with 0.05 M phosphate buffer pH 6.8. Practically no change of colour was observed in the incubations containing PTU even after 72 hr at 27 ± 2°C, whereas the two without added PTU rapidly developed the typical reddish-brown colour.

Aliquots were withdrawn from the two incubations without added PTU after, 24, 48 and 72 hr and subjected to counting after centrifugation. In the incubation with cuticle supernatant, an unchanged concentration of radioactivity was observed in the 24 and 48 hr aliquots. A darkly coloured precipitate began to form after 60 hr causing a decrease in the concentration of radioactivity by 53% (^{3}H/^{14}C = 5.72 ± 0.30) in the 72 hr aliquot. Centrifugation of the reaction mixture was followed by chromatography on Sephadex LH-20 which resulted in the elution profile shown in Fig. 5 and Table 2. Compared with the ^{3}H/^{14}C ratio of the NADA added, there was a slight decrease in the ^{3}H/^{14}C ratio of materials eluting between 90 and 155 ml as well as between 220 and 280 ml. The peak of [^{3}H] activity at 330 ml was caused by ^{3}HOH and the substance eluting at 580 ml was identified as racemic N-acetyl-noradrenalin, showing no optical activity in the CD spectrum. There was no unreacted NADA detectable.

In the incubation with cuticle sediment, no precipitate was formed as far as could be judged by visual inspection of the reaction mixture containing insoluble cuticle particles. The concentration of radioactivity had remained unchanged during the 72 hr incubation period. Chromatography on Sephadex LH-20 revealed that 11.5% of the [^{3}H] activity was present as ^{3}HOH and 10.2% of the [^{14}C] activity as N-acetylnoradrenalin.

DISCUSSION

The chemical reactivity of N-acetyldopaquinone, generated enzymatically in presence of an aliphatic amine, was investigated. The properties of the products were compared with those expected from the proposed structure of sclerotin shown in Scheme 1. Methyl β-alanate was used as an amino group

Table 2. Relative distribution of radioactivity in incubations of [1-^{14}C-2-^{3}H]-NADA with *Manduca sexta* tanning pupal cuticle (see text) after Sephadex LH-20 chromatography (see Fig. 5).

Elution volume (ml)	Supernatant of cuticle homogenate*			Sediment of cuticle homogenate		
	% ^{3}H	% ^{14}C	^{3}H/^{14}C	% ^{3}H	% ^{14}C	^{3}H/^{14}C
66–169	6.9	10.5	3.84	4.7	6.0	4.43
170–221	5.0	5.4	5.37	6.9	8.2	4.75
222–273	31.2	42.0	4.33	65.0	65.0	5.67
274–312	5.2	9.1	3.33	3.3	3.8	4.92
313–390	37.7	3.1	71.48	11.5	2.1	30.50
391–533	2.2	3.0	4.20	2.7	4.1	3.78
534–650	10.9	25.6	2.49	5.5	10.2	3.10
651–780	0.9	1.3	4.11	0.4	0.5	4.02

*Since 53% of the starting radioactivity had precipitated with insolubles, only 47% was subjected to chromatography.

containing component; the corresponding amino acid, β-alanine, has been implicated in the sclerotization of cuticles in several insect species (BODNARYK, 1971; HACKMAN, 1974; ANDERSEN, 1979).

Analysis of the products of the reaction revealed a complex composition. ^{13}C-NMR spectroscopy of a selected, chemically inhomogenous fraction showed an undetermined number of aromatic and aliphatic carbon atoms. Incorporation of methyl β-alanate is much lower than expected from the classical quinone tanning hypothesis. The maximum amount of aminoquinone imine generated from 10^{-2} M NADA and 10^{-2} M methyl β-alanate may be calculated in the following way: peaks A, B and C (Fig. 2) together contain 93.3% of the [^{3}H] radioactivity and 64.8% of the [^{14}C] activity. After subsequent separations by HPLC, only 17.8% of the [^{3}H] activity remain with 50.9% of the [^{14}C] activity in the first peaks (15–30 ml; Figs. 3 and 4). Since Scheme 1 requires an MR of 0.5, only 8.9% of the [^{14}C] activity can be present as aminoquinone imine in those fractions, assuming all [^{3}H] activity has been incorporated into such a structure. However, this is unlikely, since components with an MR of 1 or higher may be present, too. Qualitatively the same result was obtained from the distribution of radioactivity and calculation of MR values when [1-^{14}C]-NADA was oxidized in the presence of a ten-fold molar excess of methyl [3-^{3}H]-β-alanate.

Since a significant fraction of materials eluting in fractions B plus C (see Fig. 2) proved to be non-dialyzable, it seems reasonable to assume that the reaction *inter alia* generates complex oligomeric hydroxyquinones, into which methyl β-alanate is incorporated statistically with an MR of 2.0. The formation of polyphenolic mixtures containing quinone groups during the oxidation of catechols in the absence of an amine is documented with a number of examples (NICOLAUS, 1968; LINDSEY, 1974). Furthermore, YASUNOBU *et al.* (1959) observed that the mushroom tyrosinase-catalyzed oxidation of C-terminal tyrosine-containing peptides leads to brown coloured, largely non-dialyzable products which were supposed to be polymers.

Side chain oxidation occurs on NADA in the mushroom tyrosinase-catalyzed reaction to a small extent. When either a soluble or an insoluble fraction of tanning *Manduca sexta* pupal cuticle was used as a sample of diphenoloxidase activity, the amount of [^{3}H] activity released from the β-carbon atom of NADA was much higher than in the reaction with mushroom tyrosinase. The identification of N-acetylnoradrenalin as one of the reaction products corresponds to the results of ANDERSEN and ROEPSTORFF (1978), who found this compound in hydrolysates of sclerotized—though not necessarily brown coloured—cuticle (see also ANDERSEN, 1972, 1976, 1979; KOEPPE and MILLS, 1975). The yield of N-acetylnoradrenalin isolated in these experiments was lower than the amount of ^{3}HOH formed. Besides the possibility of an exchange of ^{3}H in the side chain, e.g. via quinonemethides (cf. BECKER, 1974), slightly decreased ^{3}H/^{14}C ratios in some chromatographic fractions (Table 2) and the appearance of several types of aliphatic carbon atom signals in the ^{13}C-NMR spectrum of region B support the view that side chain oxidation products different from N-acetylnoradrenalin are generated additionally.

It has often been hypothesized that the appearance of darkly brown coloured cuticles is indicative of a sclerotization process which involves quinone tanning. This hypothesis rests mainly on model experiments investigating the reaction of nascent quinones with amino groups. On the other hand, compounds supporting the classical reaction scheme have so far not been identified as natural products occurring in insect cuticle. Besides the fact that sclerotized cuticle is highly resistant to structural analysis by chemical methods, the possibility should be considered that compounds of the type shown in Scheme 1 may, if they exist at all, be present in only very small amounts in tanned cuticles. The present study describes some experimental results that make a critical reappraisal of the classical quinone tanning theory necessary (cf. HACKMAN and GOLDBERG, 1977; VINCENT and HILLERTON, 1979). Since the colour of the reaction product mixtures resembles that of tanned cuticle, but the analytical procedures performed so far reveal a complex chemical composition and quite limited reaction of an aliphatic amine with nascent N-acetyldopaquinone, it is tempting to speculate that brown cuticle is tanned by polyphenolic mixtures rather than by the formation of crosslinkages between protein amino groups and a quinone in a molar ratio 2:1. Other types of chemical bond may be envisaged on the basis of these results: non-covalent interactions, comparable to the binding of vegetable tannins to proteins (GUSTAVSON, 1956), including those of insect cuticle (HACKMAN and GOLDBERG, 1977), as well as attachment of oxidized NADA units to tyrosine residues by phenolic coupling via free radicals. This latter possibility is supported by the fact that tyrosine residues in a number of proteins are subject to enzymatic oxidation (SIZER, 1953; CORY and FRIEDEN, 1967; see also NEVILLE, 1975). Candidates for the binding of NADA oxidation products by either mechanism are not only proteins and chitin, but also melanin, as is demonstrated by the precipitation of radioactivity with melanin-like material in the experiment with *Manduca sexta* pupal cuticle. Preliminary experiments showed that the oxidation of tyrosine in the presence of NADA indeed leads to binding of oxidation products of NADA with tyrosine–melanin. Further investigations will be necessary to clarify the question of whether the formation of compounds other than aminoquinone imine (Scheme 1) as observed in this study, does play an essential role in the tanning of insect cuticles.

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