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Biochemistry

Crosslink precursors for the dipteran puparium

(sclerotization/arylation/amino acid modification/protein bridging)

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ABSTRACT **During sclerotization of puparial proteins, tyrosine, lysine, and histidine were converted to highly basic aromatic metabolites. Peptides generated from the sclerotized cuticle with *N*-bromosuccinimide included the basic derivatives among the hydrolysis products. The absorbance maxima of the aromatic metabolites were 25 nm lower than those of the conventional tyrosyl peptides, with phenolic character poorly expressed or absent. Post-translational modification of the structural proteins preceded visual expression of tanning because aromatic conjugates also were present prior to pupariation. These results are consistent with a crosslinking mechanism favoring covalent bonding between protein chains.**

The decline in solubility and titer of hydrolase-susceptible linkages in the dipteran puparium coincides with enhanced uptake of phenolic precursors from the circulation (1, 2). Two models are favored for conversion of protomeric larval proteins to the dense, dehydrated, unreactive puparial matrix (Fig. 1). The schemes differ in (*i*) the role of aromatic substituents in crosslinking, (*ii*) the reactions driving the extrusion of water, (*iii*) the degree of polymerization of the bridge, (*iv*) the molecular weight of the modified proteins, and (*v*) the identity of the amino acid residues participating in matrix assembly. In Fig. 1*A*, specific residues along the primary chain are substituted by benzenoid groups (3, 4, 7–9). The crosslink is of a relatively low degree of polymerization, enabling the modified proteins to compress sufficiently to extrude the bulk of the aqueous domains. The model shows bridging by both ring substitution or side-chain addition, clearly distinguishing the structure from the alternate noncovalent proposal (Fig. 1*B*). In the noncovalent model, aromatic precursors are converted to a highly polymerized hydrophobic backbone (5). The proteins perturbed by the introduction of the new polymer dehydrate in the course of conformational changes that externalize nonpolar amino acid residues and increase hydrophobic interactions between proteins and the aromatic polymer. Changes in chemical stability are ascribed to shielding of protein and chitin by the poorly hydrated polyaryl component. The validity of the covalent model (Fig. 1*A*) can be established by examination of total acid hydrolysates for arylated amino acids cleaved from the linkage region (10). Because both the aromatic bridge and the proteins can be labeled separately, sidechains subject to modification can be identified. This approach has confirmed the prevalence of aromatic adducts of lysine and histidine in tanned puparia. The ring carbons are derived from tyrosine in puparial cases and from dopamine in larval cuticles. A portion of this work has appeared in abstract form (11).

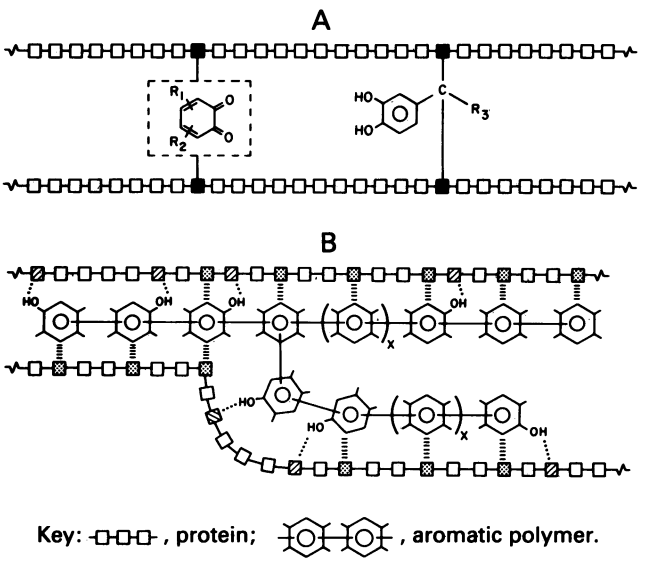

FIG. 1. Proposed structure for sclerotized puparial cuticle. (*A*) Covalently linked model with aromatic bridges between polypeptides. R_1, R_2, and R_3 can be aliphatic or aromatic. When aromatic, the sclerotizing agent is part of an oligomer with occasional links to protein (3, 4). (*B*) Noncovalently linked model with an aromatic polymer of high molecular weight with cuticle bound by hydrophobic interactions and hydrogen bonds (5, 6). ■, Amino acid in covalent crosslinking; ▩, amino acid in hydrophobic interaction; ▨, amino acid in hydrogen bonding. For details, see text.

MATERIALS AND METHODS

Preparation of Labeled Proteins. Larvae of *Sarcophaga bullata* were chilled and injected through the spiracular plates with 1 μl of buffered saline containing 1.0×10^5 dpm of labeled precursor (specific activity, 50 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels). Tyrosine and dopamine were incorporated to the maximum extent when injected into postfeeding, synchronized third-stage maggots, a stage when the rate of protein synthesis is low and arylation is high (12). All other precursor candidates were administered at the commencement of the last larval instar, coinciding with the period of greatest synthesis of cuticle protein. Values for radioactivity were normalized to 1.5×10^5 dpm per larva. Crosslinked peptides were generated from heavily sclerotized puparial cases by cleavage with *N*-bromosuccinimide at 30°C (13). For puparia labeled with [7-^{14}C]dopamine, cuticle powder was extracted with 20 M formic acid at 25°C, and the insoluble portion was heated to 100°C in 4 M acetic acid/0.03 M HCl under flowing nitrogen. The residue was suspended in 0.8 M NaOH/500 mM potassium borohydride and warmed to 70°C for 18 hr. Soluble products of reductive cleavage were acidified for precipitation of excess borate prior to chromatography (14). Larval protein CB-1 was prepared from maggots treated with [7-^{14}C]dopamine as described (2). The sample was hydrolyzed in 3 M 2-mercaptoethanesulfonic acid, diluted 100-fold with water, and passed through Dowex 50X8 (H^+). Material not retained was chromatographed on Dowex

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1X4 (formate) with a step gradient of 0.1 M formic acid, 0.1 M HCl/10 M formic acid, and 1 M HCl/10 M formic acid.

Analysis of Amino Acid Conjugates. Complete hydrolysis of proteins (1 mg/ml) for detection of basic conjugates was performed at 108°C in 5.8 M HCl or 3 M 2-mercaptoethanesulfonic acid at 60 mtorr after flushing of the sample with nitrogen. Radioactivity of heavily sclerotized samples was determined after oxidation with perchloric acid and hydrogen peroxide prior to scintillation counting (15). Arylated lysines and histidines were isolated as follows. Total hydrolysates (5.8 M HCl) were adjusted to pH 2.0 and applied to Dowex 50X4 (H^+) at 10 mg/3 ml of packed resin. The column was washed with 0.1 M ammonium acetate (pH 3.5), and all of the amino acids normally found in protein hydrolysates, with the exception of arginine, were eluted with 12 ml of 0.5 M ammonium acetate (pH 7.0). Lysine, histidine, tyrosine, glucosamine, and β-alanine emerged from the columns in the latter buffer. Aryl conjugates of lysine and histidine were eluted with 12 ml of 0.8 M ammonium hydroxide/20% methanol (vol/vol), pH 12.0, together with arginine. Authentic tyramine, dityrosine, and arterenol also were eluted in this fraction but were distinguishable from the aromatic adducts by thin-layer and affinity chromatography.

Chemicals. L-[*ring*-^{14}C]Tyrosine was prepared by the action of tyrosine phenol-lyase (EC 4.1.99.2) on [^{14}C(U)]phenol and S-methyl-L-cysteine (16). *N*-[*ring*-^{3}H]Acetyldopamine was synthesized according to Karlson *et al.* (17). The affinity support *N*-[*N'*-(*m*-dihydroxyborylphenyl)succinamyl]-aminoethylcellulose was synthesized from aminoethylcellulose and 3-aminophenylboronic acid (18).

RESULTS

Incorporation of Crosslink Precursors. When equivalent levels of labeled amino acids were administered to maggots at the commencement of the last instar and the puparial case was assayed for radioactivity, then tyrosine, histidine, arginine, phenylalanine, tryptophan, and dopamine were incorporated at a level in excess of 5% of the isotope load. Lysine, cysteine, alanine, and *N*-acetyldopamine were labeled to a slightly lesser extent (Table 1). Addition of labeled precursor to the integument was not related to amino acid composition because cysteine, tryptophan, and methionine were essentially absent from the hydrolysates. Each of the 10 remaining amino acids were incorporated at levels less than half that of the above precursors. The metabolic fate of each precursor was monitored by separation of products of acid hydrolysis on Dowex 50 with an elution program that released basic conjugates at pH 12.0 and the remaining amino acids (with the exception of arginine) in buffers at pH 3.5 and 7.0. Tyrosine, the amino acid with the highest level of incorporation, was converted in part to a metabolite of a more basic nature than the parent compound (Fig. 2). The relative distribution of isotope in the three eluting buffers was: pH 3.5, 5.7%; pH 7.0, 32.4%; pH 12.0, 61.9%. Not only tyrosine, but lysine, histidine, arginine, tryptophan, and phenylalanine contributed radioactivity in excess of 30% to the pH 12 buffer. The elution patterns for histidine and lysine (Fig. 2) showed that radioactivity and UV character were both associated with the pH 12 eluates, demonstrating that the two amino acids were converted to arylated products of a basic nature.

When the remaining precursors with isotope distribution patterns in excess of 30%—namely, phenylalanine, arginine, and tryptophan—were examined by thin-layer chromatography and amino acid analysis, it was evident that only lysine and histidine were subject to modification during sclerotization. Metabolites of phenylalanine were indistinguishable from those derived from tyrosine as assessed by thin-layer chromatography. Radioactivity in hydrolysates from arginine-treated populations was restricted to arginine *per se*. Tryptophan was recovered in the pH 12 fraction as a degradation product of ommochrome, kynurenine, and the parent amino acid (2, 9, 20). Alanine and cysteine were not subject to arylation, and their contribution to the isotopic complement was ascribed to catabolism followed by incorporation into nonessential amino acids. Conversion of tyrosine to a basic conjugate was not an artefact arising from the hydrolytic milieu. Nonradioactive cuticle hydrolyzed in the presence of 2×10^5 dpm of L-[^{14}C(U)]tyrosine or lysine and processed in the usual manner failed to contribute basic components to the pH 12 eluate from Dowex 50. The basic adducts derived from tyrosine or lysine were chromatographed on the amino acid analyzer in the region between lysine and arginine. After reduction with sodium borohydride, the samples were bleached to a straw-yellow color with a significant decline in UV absorbance (Fig. 2). In the reduced state, only a weak response to the *p*-nitroaniline test for phenols was observed.

Table 1. Incorporation of [^{14}C]amino acids and aminodiphenols into puparial cases

Precursor	^{14}C Total in cuticle, %	Fraction, % pH 12	Fraction, % pH 3.5 + 7.0	Residue, mol %
Tyrosine	10.7	61.9	38.1	3.6
Histidine	8.1	39.5	54.5	3.6
Arginine	8.1	38.0	41.9	2.4
Tryptophan	5.3	50.0	46.3	0.05
Phenylalanine	5.2	31.6	34.2	3.1
Lysine	3.4	33.0	64.0	3.6
α-Alanine	3.1	17.2	79.7	8.6
Cysteine	3.0	15.4	63.1	0.05
β-Alanine	32.7	16.4	83.6	11.4*
Dopamine	14.8	ND	ND	0.2
N-Acetyldopamine	4.4	ND	ND	ND

Each larva received 1.0×10^5 dpm of labeled precursor at the commencement of the last larval instar. The puparium was harvested 8 days after metamorphosis. Portions of the washed and powdered cases were hydrolyzed with 5.8 M HCl for amino acid analysis and ion-exchange chromatography for assessment of total incorporation per puparium after oxidation with $HClO_4/H_2O_2$. The hydrolysates were applied to the Dowex 50X4 (H^+) columns (1 × 3 cm), and the acidic, neutral, and moderately basic amino acids and phenols were eluted with 12 ml of 0.1 M ammonium acetate (pH 7.0). After elution of the highly basic aryl conjugates with 12 ml of 0.8 M ammonium hydroxide/20% methyl alcohol (vol/vol) radioactivity was determined by scintillation counting. ND, not determined.
* From Bodnaryk (19).

Conjugated Aromatics from Borate-Soluble Components. When labeled amber-colored puparial cases were extracted with sodium tetraborate/NaDodSO$_4$ at pH 9.1, partially sclerotized radioactive polypeptides were solubilized, accounting for 15% of the dry weight. The borate extracts were dialyzed and the retained material was hydrolyzed. The presence of basic adducts was confirmed by the Dowex-50 method. When an aliquot of the hydrolyzed sample from lysine-labeled animals was chromatographed on Sephadex LH-20, a radioactive peak was observed that absorbed in the UV region and did not coincide with lysine with respect to mobility (Fig. 3). Thin-layer chromatography confirmed that the isotope was present in a substance distinct from lysine. A substance with identical chromatographic properties was recovered from the tyrosine-labeled proteins. The radioactive areas were weakly fluorescent and could be distinguished from tyrosine, dopamine, 3,4-dihydroxyphenylalanine, di- or trityrosine, arterenol, or conventional basic tyrosyl peptides by thin-layer chromatography and

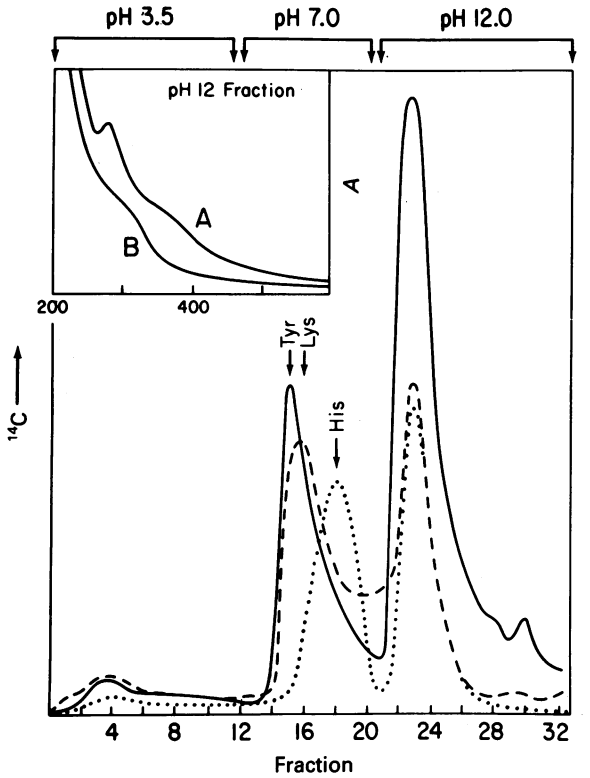

FIG. 2. Dowex 50 column (3 cm × 1 cm) chromatography of puparial hydrolysates. Sclerotized cases from populations administered ^{14}C-labeled amino acids were digested with 5.8 M HCl at 108°C, evaporated, and applied to the columns at pH 2.0. Amino acids and conjugates were eluted with a step pH gradient. (*Inset*) Representative absorption spectrum of the pH 12 fractions before (curve A) and after (curve B) reduction with sodium borohydride.

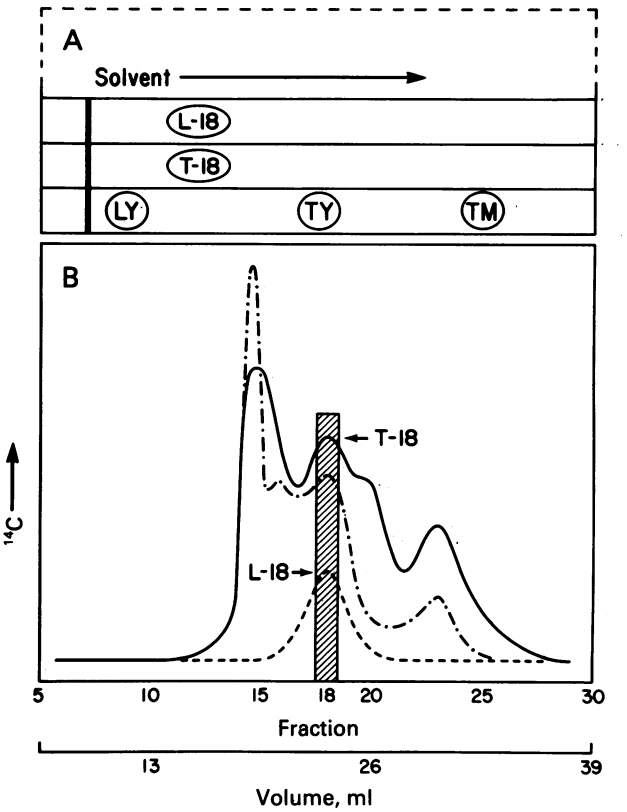

FIG. 3. Sephadex LH-20 column (30 cm × 1 cm) chromatography (*B*) and thin-layer chromatographic identification (*A*) of aryl conjugates. After administration of ^{14}C-labeled precursors, $B_4O_7^{-2}$/NaDodSO$_4$ extracts of puparia were hydrolyzed and chromatographed with 50% methyl alcohol as eluant. Fraction 18 was applied to cellulose thin-layer supports and irrigated with 1-butanol/acetic acid/water, 4:1:5 (vol/vol). The circled areas represent radioactive sectors. The guide compounds are lysine (Ly) (– – –), tyrosine (Ty) (——), and tyramine (Tm). ·–·–, A_{280}.

affinity matrix binding *o*-dihydric phenols at alkaline pH. When Dowex 50 eluates (pH 12) were reduced with sodium borohydride and applied to this support at pH 8, ≈95% emerged in the void volume and was not retained (Fig. 4). Virtually identical elution patterns were observed for hydrolysates with label derived from lysine, tyrosine, or histidine.

The major radioactive UV-absorbing moieties were not substituted dopamines or ketocatechols as judged by failure to bind to the affinity column. The minor component retained by the affinity matrix responded to ninhydrin and to the molybdate test for *o*-diphenols and resembled a catechol–lysine adduct from cockroach egg cases (10). The origin of the radioactivity derived from tyrosine was established by administration of L-[*ring*-^{14}C]tyrosine to distinguish the aromatic region of the molecule from the side chain. Recovery was identical with that of L-[^{14}C(U)]tyrosine. When the three labeled borate extracts were digested with papain instead of acid and the fragments were separated by thin-layer chromatography/electrophoresis, one-fourth of the peptides incorporated isotope from all three precursors (results not shown). Evidently a significant number of fragments contained both lysine and histidine linked to tyrosine or its metabolites.

Conjugate Distribution in Heavily Sclerotized Puparia. In the absence of tryptophan, *N*-bromosuccinimide cleaves proteins at the carboxyl terminus of tyrosyl and histidyl residues (21). This reagent is particularly valuable in the case of puparia because precursor proteins from the larval integument contain no tryptophan or cysteine to confound the identity of the loci subject to oxidation and hydrolysis (2, 13). In addition, a substantial portion of the aromatic crosslinks are unreactive to *N*-bromosuccinimide because of blocking of phenolic hydroxyls to afford aryl ethers (7). After removal of moderately sclerotized proteins by extraction with tetraborate, exposure of the residue to *N*-bromosuccinimide generated a mixture of fragments separable by chromatography on Dowex 50 (Fig. 5). The intact peptides were eluted at pH 3.5, 7.0, and 12.0 and were hydro-

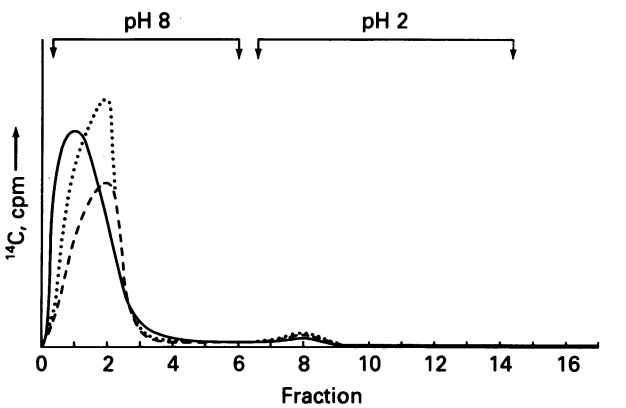

FIG. 4. Affinity chromatography of puparial hydrolysates. At pH 8, *o*-dihydric phenols are retained; at pH 3, the ligands are released. Hydrolysates from animals treated with L-[*ring*-^{14}C]tyrosine (——) or [^{14}C(U)]histidine (·····) and lysine (– – –) were chromatographed on Dowex 50 as described in Fig. 2 for elimination of unconjugated components. Material eluting at pH 12 was applied to the affinity support.

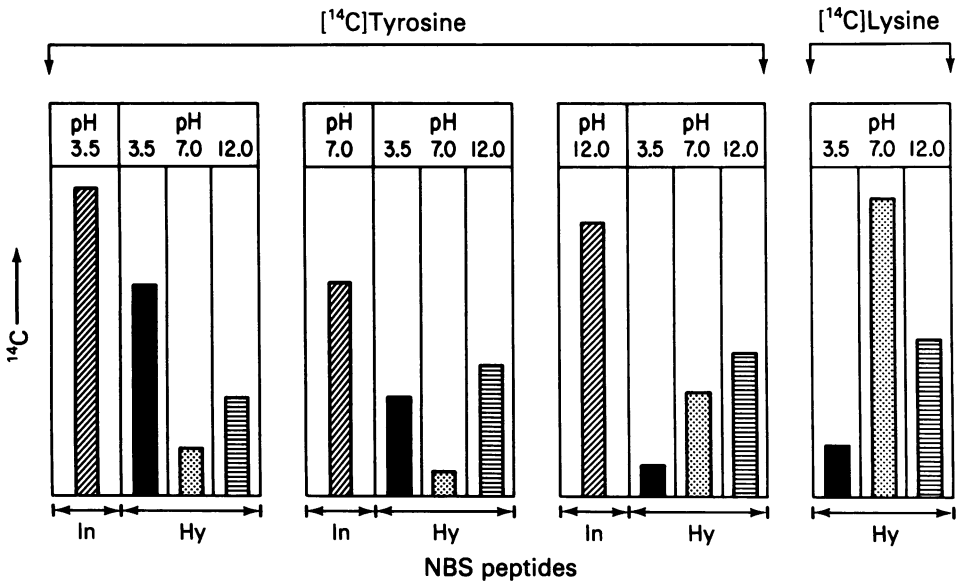

FIG. 5. Dowex 50 chromatography of sclerotized peptides before and after acid hydrolysis. Labeled puparia were cleaved with *N*-bromosuccinimide at 30°C and chromatographed to resolve acidic, neutral, and basic peptides (13). Isolates were hydrolyzed in 5.8 M HCl and analyzed for basic conjugates as set forth in Fig. 2. Peptides labeled with lysine were recombined after separation for conjugate analysis. In, intact peptides; Hy, hydrolyzed peptides.

lyzed for isolation of the basic conjugate on Dowex 50. Lysine-treated animals showed essentially the same distribution of intact peptides among the three pH classes. It is evident that the aryl-lysine conjugates were recovered in good yield.

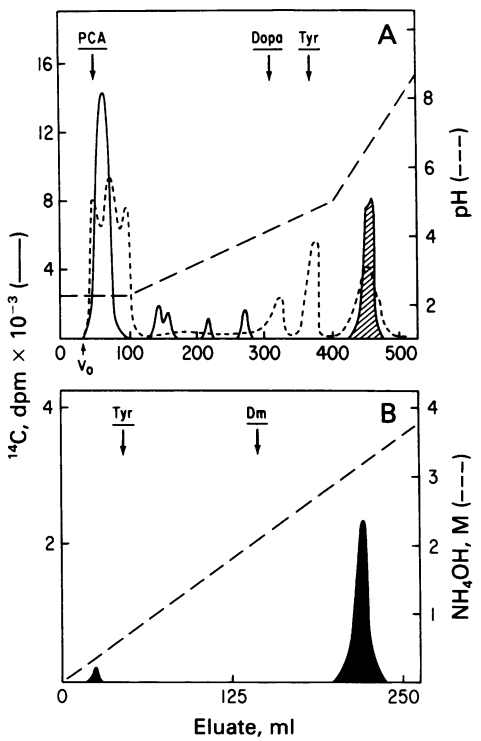

FIG. 6. Dowex 50 chromatography of metabolites of [7-^{14}C]dopamine from prepuparial (late third stage larval) cuticle. (*A*) Fraction CB-I was hydrolyzed in 3 M 2-mercaptoethanesulfonic acid and chromatographed on Dowex 50 (pyridinium; 50 cm × 1 cm) with a linear pH gradient – – –, A_{280} in arbitrary units. (*B*) The pale yellow fraction (▨) in *A* was rechromatographed on Dowex 50 (H^+; 15 cm × 1 cm) with a gradient of ammonium hydroxide in 20% methyl alcohol. The larger of the shaded peaks darkened in the course of column development. Markers are tyrosine, dopamine (Dm), protocatechuic acid (PCA), and 3,4-dihydroxyphenylalanine (Dopa).

Incorporation of [7-^{14}C]Dopamine. In the course of sclerotization, 14.8% of the radioactivity from dopamine was incorporated into the puparium, a level in excess of that observed from tyrosine (Table 1). These findings would be obtained if the catecholamine followed tyrosine in the metabolic scheme leading to biogenesis of the crosslink. When samples were hydrolyzed in 5.8 M HCl and chromatographed on Bio-Gel P-10 in 1 M acetic acid at 60°C, a single radioactive component was eluted from the column, with an UV absorption spectrum identical with that of the aryl–lysine adduct eluted from Dowex 50 at pH 12 (results not shown).

In addition to conversion of [^{14}C]dopamine to crosslinks in the sclerotized puparium, fraction CB-I from the late third instar larvae, consisting of unsclerotized protein bound to chitin, acquired radioactivity prior to the onset of pupariation (2, 12). After hydrolysis in 3 M 2-mercaptoethanesulfonic acid, 36% of the radioactivity was recovered as a basic metabolite retained by Dowex 50 (H^+). When chromatographed on Dowex 50 (pyridinium) at 55°C with a gradient from pH 2 to pH 9, 77% of the radioactive material emerged as an unpigmented peak at pH 6.4–7.1 (Fig. 6*A*). The peak fraction was taken up in an atmosphere of air at room temperature with a linear gradient of 0.5–4 M NH_4OH, pH 11.7/20% methanol (vol/vol). A single dark band developed on the column that eluted at the alkaline extreme of the gradient (Fig. 6*B*). Spot tests for phenols and amino acids were negative. Fraction CB-I also released an acidic metabolite of dopamine on hydrolysis with 2-mercaptoenthanesulfonic acid, with properties resembling an addition product of the thio acid with a fragment of the catecholamine. Ion pairing with trioctylamine in chloroform transferred 90% of the radioactivity into the alkaline organic phase along with the excess acid. This material was not retained by borylcellulose and was coeluted with 2-mercaptoethanesulfonic acid on Dowex 1 and Dupont Zorbax ODS, with a methanol gradient. Examination by NMR at 270 MHz in the region 7–9 δ failed to reveal aromatic protons.

DISCUSSION

After incorporation into the puparial case, tyrosine, lysine, and histidine administered to maggots at the threshold of puparium

formation were converted to basic substances distinguishable from the parent amino acids (Table 1). The metabolites could be resolved from lysine, tyrosine, and histidine by chromatography on Dowex 50 (Fig. 2), Sephadex LH-20, and cellulose thin-layer plates (Fig. 3), and affinity supports retaining *o*-dihydric phenols and derivatives (Fig. 4). Products of similar or identical chromatographic properties were recovered from peptides generated from the sclerotized proteins by cleavage of unmodified tyrosyl residues with *N*-bromosuccinimide (Fig. 5). This pattern of isotope distribution was in keeping with a mechanism wherein modification of lysyl and histidyl residues is accomplished through arylation by a tyrosine metabolite after translation (1, 7, 22). That a substantial number of the lysine and histidine residues were modified by conjugation with carbon derived from the tyrosine ring was shown by the good recovery of tyrosine in the pH 12 fraction, 61.9%, and the sum of radioactivities for lysine plus histidine, 67.9% (Table 1). Radioactive components eluted from Dowex 50 in the pH 12 buffer differed markedly from authentic basic peptides with tyrosine in the primary stucture, not only in UV character ($A_{280}/A_{254} = 0.24$) but also in chromatographic mobility and response to reagents specific for unsubstituted phenols.

Arguments favoring the model in Fig. 1*B* question the significance of the decline in lysine ε-amino groups during pupariation and the role of the polymeric aromatic pigment deposited as a crosslinker as the cuticle hardens (4–6). It is now evident, however, that a portion of the lysyl and histidyl residues are subject to modification by an aromatic component derived from the ring of tyrosine—a situation best explained by the synthesis of covalent crosslinks of the type outlined in Fig. 1*A*. In this respect, coupling of quinones with the ε-amino group of lysine through 1,4-reductive addition of the Michael type to afford stable conjugates has been reported in plants (23). The poor response to molybdate and a borylcellulose affinity support speaks against a side chain-substituted *o*-diphenol in sarcophagid puparia. The ability of *N*-bromosuccinimide to generate peptides that released arylated histidine and lysine on acid hydrolysis (Fig. 5) was also in accord with multiple ring substitutions (21, 24). The model in Fig. 1*B* stresses the importance of the resistant aromatic polymer remaining after acid or alkaline hydrolysis of puparia. Distinction between the polymer responsible for dehydration and melanic puparial pigment is not offered by proponents of this scheme. Concerning the abundant puparial pigment, chemical evidence favors a typical melanin pigment rather than a lignin-like plasticizer. Long-chain aromatic polymers require free-radical intermediates for assembly, adding protein either during polymerization or after synthesis of the benzenoid matrix. Free radical-mediated reactions are of low specificity, hence model 1B would afford aryl conjugates of many different amino acids. Table 1 shows that this was contrary to the findings because the participation of lysine and histidine in conjugate formation exceeded most amino acids by a factor of three or more.

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