

## Aromatic Cross-Links in Insect Cuticle: Detection by Solid-State <sup>13</sup>C and <sup>15</sup>N NMR

JACOB SCHAEFER,\* KARL J. KRAMER, JOEL R. GARBOW, GARY S. JACOB,† EDWARD O. STEJSKAL,‡ THEODORE L. HOPKINS, ROY D. SPEIRS

Cross-polarization magic-angle-spinning nuclear magnetic resonance spectroscopy has been used to determine insect cuticle composition and cross-link structure during sclerotization or tanning. Unsclerotized cuticle from newly ecdysed pupae of the tobacco hornworm, *Manduca sexta* L., had a high protein content with lesser amounts of lipid and chitin. Concentrations of chitin, protein, and catechol increased substantially as dehydration and sclerotization progressed. Analysis of intact cuticle specifically labeled with carbon-13 and nitrogen-15 revealed direct covalent linkages between ring nitrogens of protein histidyl residues and ring carbons derived from the catecholamine dopamine. This carbon-nitrogen adduct was present in chitin isolated from cuticle by alkaline extraction and is probably bound covalently to chitin. These data support the hypothesis that the stiffening of insect cuticle during sclerotization results primarily from the deposition of protein and chitin polymers and their cross-linking by quinonoid derivatives of catecholamines.

THE INSECT EXOSKELETON IS A COMPLEX extracellular cuticular structure whose chemical nature and physical properties vary with functional demands. It is composed primarily of protein, chitin, and water, the interactions of which, although poorly understood, largely determine structural and mechanical properties (1). During sclerotization (strengthening), the cuticle becomes stiffer, drier, and resistant to chemical and physical degradation. It has been proposed that catecholamines act as dehydrating agents, protein denaturants, and precursors of cross-linking agents for the presclerotized protein-chitin matrix (2). Proposed cross-linking intermediates include *o*-quinones or *p*-quinone methides, whose formation from catechols is catalyzed by oxidative enzymes in the newly secreted cuticle.

Because tanned cuticle is an intractable material and the putative cross-linking agent or agents are highly reactive and transient, the analytical techniques used in the past to study the chemistry of sclerotization have not yielded convincing proof for the cross-linked structures or proposed intermediates. However, recent studies of bacterial cell walls (3) have shown that the relatively new solid-state spectroscopic method, cross-polarization magic-angle-spinning (CPMAS) nuclear magnetic resonance (NMR) (4), makes it possible to evaluate cross-linked structures in intact biological tissue.

We report here the results of experiments in which CPMAS <sup>13</sup>C and <sup>15</sup>N NMR were used to determine in situ the relative concentrations of and covalent interactions among catecholamine, protein, and chitin in pupal cuticle of the tobacco hornworm,

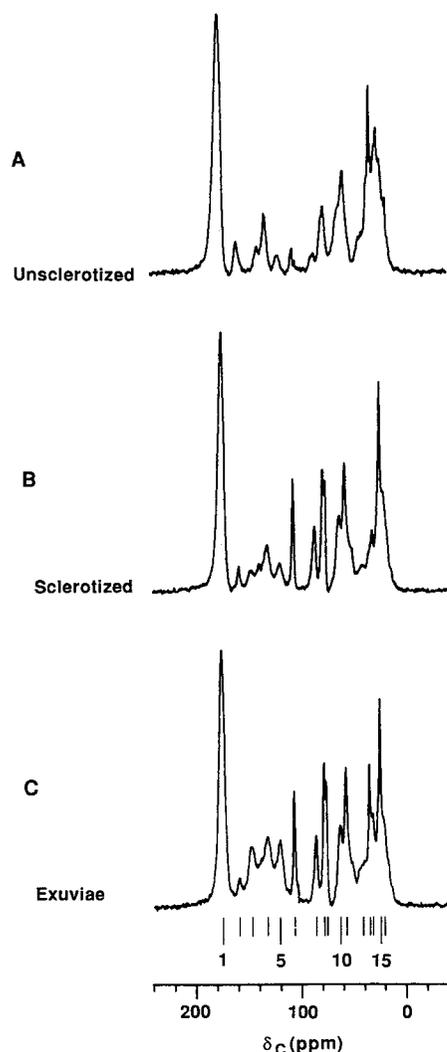
*Manduca sexta* L. The analytical method depends on the ability to identify and quantify in intact cuticle the natural-abundance <sup>13</sup>C levels, as well as <sup>13</sup>C-<sup>15</sup>N covalent bonds, between catechols or chitin labeled with <sup>13</sup>C and protein enriched in <sup>15</sup>N-histidine.

At ecdysis, *M. sexta* pupal cuticle is soft and colorless except for small tanned areas on the abdomen (5). In a few hours the cuticle stiffens and becomes dark brown. The natural-abundance <sup>13</sup>C CPMAS NMR spectra of cuticle can be used to estimate concentration changes of the major organic components during cuticular tanning or sclerotization (6). Protein carbons contribute to <sup>13</sup>C resonances (lines) 1, 2, 4, 5, and 10 to 16 (7) (Fig. 1C and Table 1). The broad peptide backbone  $\alpha$ -carbon peaks between 55 and 62 parts per million (ppm) (lines 10 and 11) generally diagnose protein levels (8). The well-resolved sharp lines between 74 and 104 ppm (lines 6 to 9) are due to the 2-acetamido-2-deoxy-D-glucopyranoside (GlcNAc) carbons and so reflect chitin content. The oxygenated aromatic carbon

J. Schaefer, J. R. Garbow, G. S. Jacob, E. O. Stejskal, Physical Sciences Center, Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167.  
K. J. Kramer, U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1515 College Avenue, Manhattan, KS 66502, and Department of Biochemistry, Kansas State University, Manhattan, KS 66506.  
T. L. Hopkins, Department of Entomology, Kansas State University, Manhattan, KS 66506.  
R. D. Speirs, U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502.

\*Present address: Department of Chemistry, Washington University, St. Louis, MO 63130.  
†Present address: Department of Biochemistry, University of Oxford, Oxford OX1 3QU, England.  
‡Present address: Department of Chemistry, North Carolina State University, Raleigh, NC 27695.

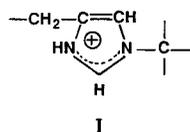
peak at 144 ppm (line 3) monitors catechols, and the sharp methylene carbon peak at 30 ppm (line 14) can be used to estimate amounts of lipid. Fast cross-polarization rates for protonated carbons, long proton rotating-frame lifetimes, and high concentrations of protons ensure representative relative NMR intensities under the conditions used in these experiments (8). When required, absolute concentrations of carbons can be determined by comparison with an external standard.



**Fig. 1.** Natural-abundance CPMAS  $^{13}\text{C}$  NMR spectra of (A) newly ecdysed and relatively unsclerotized *M. sexta* pupal cuticle, (B) 3-day-old sclerotized pupal cuticle, and (C) pupal exuviae consisting primarily of highly sclerotized exocuticle. Assignments for lines 1 to 16 are given in Table 1. The scale is in parts per million downfield from tetramethylsilane (TMS) as an external reference. Spectra were obtained at 50.3 MHz through the use of 2-msec cross-polarization transfers from protons and 50-kHz radio-frequency fields. The dried samples were contained in a cylindrical double-bearing rotor spinning at 3.2 kHz. Residual spinning sidebands were suppressed by pulse techniques. Additional details and references may be found in (8).

As the cuticle sclerotizes, proteins become progressively less soluble (9); the relative levels of protein, chitin, and catechol increase; and those of lipid and water decrease (10) (Fig. 1, A and B). The outer heavily sclerotized portion of the cuticle or exuvium contains approximately twice as much protein, 18 times as much chitin and catechol (Fig. 1C), and one-ninth as much water as unsclerotized cuticle does (10).

Since the proteins in sclerotized cuticle are difficult to extract, it has long been believed that they are stabilized by cross-links involving covalent bonds to other cuticular components (1, 2). Nitrogen-containing amino acid side chains in cuticular proteins are potential nucleophiles for the formation of carbon-nitrogen cross-links with components such as quinonoid intermediates. Our CPMAS  $^{15}\text{N}$  NMR spectral data (7) of *M. sexta* pupal cuticle labeled by injection of L-[1,3- $^{15}\text{N}_2$ ]histidine into wandering-stage larvae (11) support the formation of carbon-nitrogen cross-links. The  $^{15}\text{N}$  NMR spectrum of unsclerotized cuticle shows protonated and nonprotonated histidyl ring nitrogen peaks at 140 and 225 ppm, respectively (Fig. 2A), as well as a natural-abundance amide nitrogen peak at 100 ppm. In 3-day-old sclerotized cuticle, a new histidyl signal is observed at 155 ppm that builds up during the time course of sclerotization (Fig. 2B) and becomes the major nitrogen resonance in pupal exuviae (Fig. 2C). The dipolar dephasing produced by delayed  $^1\text{H}$  decoupling (12) has little effect on the new signal (Fig. 2D), which shows the nitrogen to be nonprotonated. Its chemical shift indicates a histidyl nitrogen attached to either an aliphatic or aromatic carbon (13). From the relative intensity of the new peak, it is estimated that one-third to one-half of all histidyl residues in exuviae have undergone a posttranslational substitution to a structure (I) of the form

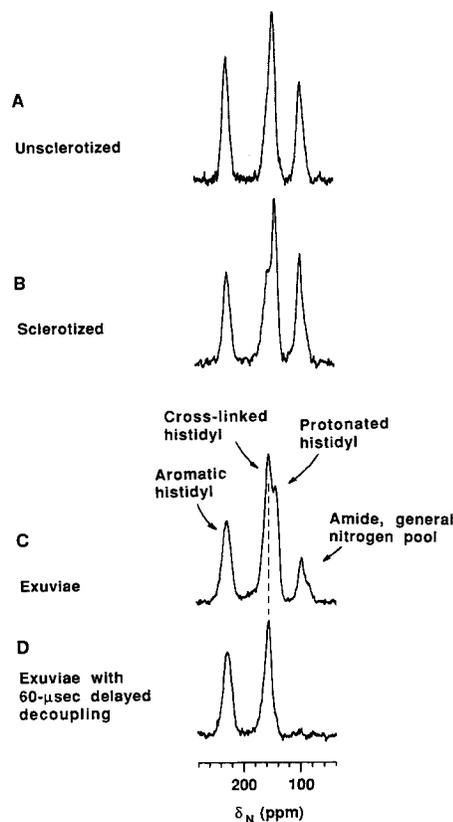


Preliminary  $^{15}\text{N}$  NMR results involving labeling by [ $\epsilon$ - $^{15}\text{N}$ ]lysine suggest that the  $\epsilon$ -amino nitrogen of lysine also forms carbon-nitrogen cross-links in *M. sexta* pupal cuticle, although these are less numerous than the histidyl nitrogen cross-links.

Proteins with substituted histidyl nitrogens seem to be tightly coupled to chitin in sclerotized cuticle. The  $^{15}\text{N}$  NMR spectrum of pupal chitin prepared by alkali extraction (14) from exuviae labeled with [1,3- $^{15}\text{N}_2$ ]histidine shows only two peaks: a natural-abundance amide peak at 100 ppm and the

substituted histidyl ring nitrogen peak at about 150 ppm (Fig. 3A). Delayed decoupling experiments (12) on this material demonstrate that the 150-ppm peak consists of two equal components, one protonated and shifted 1 to 2 ppm upfield, and the other nonprotonated and shifted slightly downfield. This result is consistent with the shifts expected (15) for the substituted ring structure shown above. About one-third of the total  $^{15}\text{N}$  label in the intact cuticle (determined by integration of the spectrum) was recovered in the chitin preparation, which means that virtually all of the substituted histidyl rings are coupled to chitin. These rings remain bound to chitin but are no longer part of the cuticular proteins that have been hydrolyzed away (14).

The natural-abundance  $^{13}\text{C}$  NMR spectrum of the pupal chitin preparation confirms the presence of aromatic or olefinic carbons (Fig. 3C). However, the total inten-



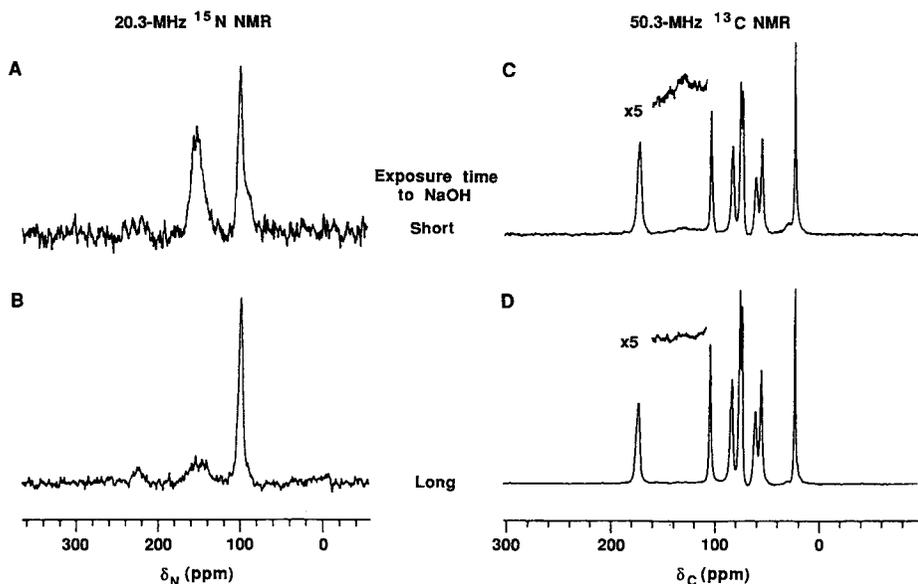
**Fig. 2.** CPMAS  $^{15}\text{N}$  NMR spectra of L-[1,3- $^{15}\text{N}_2$ ]histidine-labeled (A) newly ecdysed unsclerotized pupal cuticle, (B) 3-day-old sclerotized pupal cuticle, (C) pupal exuviae, and (D) pupal exuviae under delayed decoupling. Spectra were obtained at 20.3 MHz through the use of matched spin-lock cross-polarization transfers with 1-msec contacts and 35-kHz radio-frequency fields. Only nonprotonated nitrogen resonances survive delayed decoupling. The vertical display for the top two spectra is approximately twice that of the bottom two. The horizontal scale is in parts per million downfield from solid ammonium sulfate as an external reference.

sity of the aromatic carbon peaks between 120 and 160 ppm is 3% of the integrated intensity of the  $^{13}\text{C}$  NMR spectrum and so cannot be accounted for exclusively by the ring carbons of histidyl residues (16). Therefore, aromatic compounds like catechols must also be present in the chitin preparation. Prolonged exposure of this preparation to hot basic solution eventually leaves only chitin (Fig. 3, B and D).

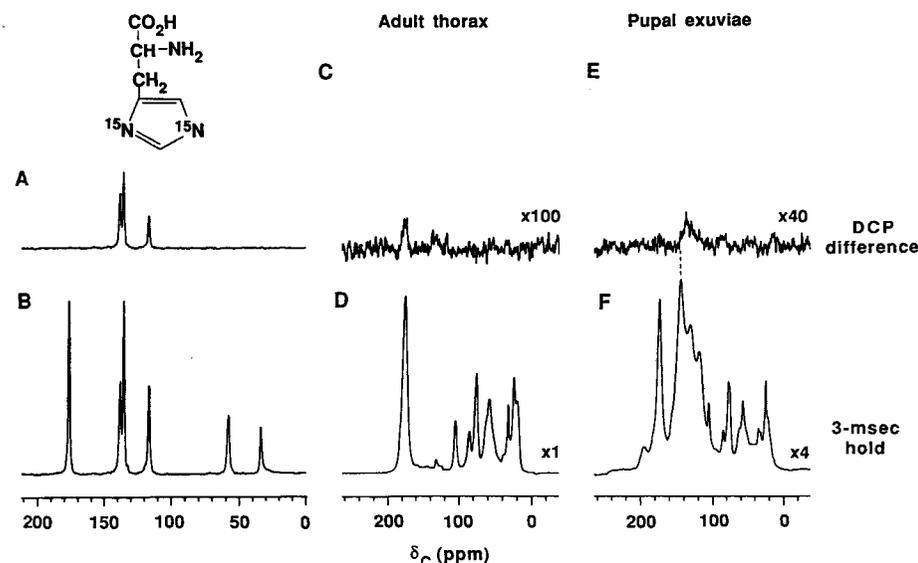
The proteins coupled to pupal chitin do not seem to be bonded directly. This conclusion is based on the results of double cross-polarization (DCP) MAS  $^{13}\text{C}$  NMR experiments, which were used to detect the presence of  $^{13}\text{C}$ - $^{15}\text{N}$  bonds (17). A DCP experiment separates by difference (18) those  $^{13}\text{C}$  atoms adjacent to  $^{15}\text{N}$  atoms from all other  $^{13}\text{C}$  atoms. For example, with L-[1,3- $^{15}\text{N}_2$ ]histidine, only the three carbons in the

imidazole ring bonded to  $^{15}\text{N}$  atoms are detected by DCP signals (Fig. 4A), whereas all carbons in histidine are detected by single cross-polarization signals (Fig. 4B). The intensities of these three carbon DCP peaks can be used to identify the individual  $^{13}\text{C}$ - $^{15}\text{N}$  bonds in the labeled histidine (17). The DCP  $^{13}\text{C}$  NMR spectrum of sclerotized cuticle from adults reared from larvae fed uniformly labeled [ $^{13}\text{C}$ ]glucose (19) and injected with L-[1,3- $^{15}\text{N}_2$ ]histidine has difference signals at 180 and 140 ppm (Fig. 4C). The intensity of the 180-ppm DCP difference signal relative to that of the normal signal (Fig. 4D) (a ratio of 0.001) arises from the natural-abundance  $^{15}\text{N}$  (0.3%) of the amide nitrogens (20) in chitin (labeled to 8%  $^{13}\text{C}$  by the glucose) and proteins. We attribute the weak DCP difference signals at 140 ppm to natural-abundance  $^{13}\text{C}$  adjacent to labeled [ $^{15}\text{N}$ ]histidyl nitrogens (21). The absence of any other DCP difference signal rules out direct histidyl nitrogen linkages to chitin carbon atoms.

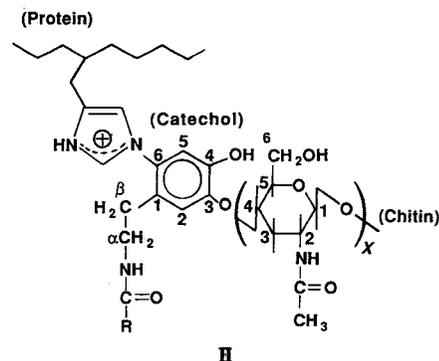
The C-N cross-links in *M. sexta* cuticle are formed instead between histidyl ring nitrogens and catecholic ring carbons. The DCP difference  $^{13}\text{C}$  NMR spectrum of pupal exuviae from larvae injected with both L-[1,3- $^{15}\text{N}_2$ ]histidine and [*ring*- $^{13}\text{C}_6$ ]dopamine (22) shows a peak at 135 ppm, slightly upfield from the major oxygenated ring carbon peak at 144 ppm (Fig. 4, E and F). This peak does not appear in DCP spectra of exuviae labeled only with L-[1,3- $^{15}\text{N}_2$ ]histidine. The 135-ppm chemical shift is consistent with an aromatic carbon next to nitrogen (23). Although the exact structure of the histidyl-catecholamine ring adduct is unknown, the data of Figs. 2, 3, and 4 are consistent with a product (see structure II, where R =  $\text{CH}_2\text{-CH}_2\text{-NH}_3^+$ ) from an



**Fig. 3.** CPMAS  $^{15}\text{N}$  NMR (A and B) and  $^{13}\text{C}$  NMR (C and D) spectra of chitin extracted by alkali treatment from L-[1,3- $^{15}\text{N}_2$ ]histidine-labeled sclerotized *M. sexta* pupal cuticle. The natural-abundance  $^{15}\text{N}$  100-ppm amide peak (A and B) and the eight major natural-abundance  $^{13}\text{C}$  peaks (C and D) all arise from chitin.



**Fig. 4.** Single (bottom) and double (top) CPMAS  $^{13}\text{C}$  NMR spectra of L-[1,3- $^{15}\text{N}_2$ ]histidine (A and B), thoracic cuticle of *M. sexta* adults labeled with L-[1,3- $^{15}\text{N}_2$ ]histidine and [ $^{13}\text{C}$ (U)] glucose (C and D), and *M. sexta* pupal exuviae labeled with [1,3- $^{15}\text{N}_2$ ]histidine and [*ring*- $^{13}\text{C}_6$ ]dopamine (E and F). Only  $^{13}\text{C}$  atoms directly coupled to  $^{15}\text{N}$  atoms have resonances in the double-CPMAS (DCP) difference spectra. The  $^{13}\text{C}$  label from glucose appears predominantly in chitin (D), while that from dopamine enhances the intensity of the aromatic region of the spectrum between 120 and 160 ppm (F). Data accumulation involved 35,000 scans (A and B), 400,000 scans (C and D), and 900,000 scans (E and F), respectively. The last of the three accumulations was a 2-week experiment. The histidine sample weighed 470 mg; the thorax sample, 346 mg; and the exuviae sample, 161 mg.



*o*-quinone sclerotization mechanism (1, 2). By this mechanism a cross-link results from nucleophilic attack of the 1- or 3-nitrogen of the imidazole ring on the 2-, 5-, or 6-ring carbon of an *o*-quinone derivative of *N*- $\beta$ -alanyldopamine. *N*- $\beta$ -Alanyldopamine is the major catecholamine derived from dopamine found in tanning *M. sexta* pupal cuticle (24). A catecholamine-chitin carbon-oxygen

**Table 1.** Chemical assignments of resonances (7) in the CPMAS <sup>13</sup>C NMR spectra of *M. sexta* cuticle.

Resonance	δ-Value (ppm)*	Assignment
1	172	Carbonyl carbon in chitin, protein, lipid, and catechol
2	155	Phenoxy carbon in tyrosine; guanidino carbon in arginine
3	144	Phenoxy carbons in catechols
4	131	Aromatic carbons
5	121	Tyrosine carbons 3 and 5; imidazole carbon 4; catechol carbons 2 and 5
6	104	GlcNAc carbon 1
7	85	GlcNAc carbon 4
8	75	GlcNAc carbon 5
9	74	GlcNAc carbon 3
10	62	GlcNAc carbon 6; amino acid α-carbon
11	55	GlcNAc carbon 2; amino acid α-carbon
12	44	Amino acid and catechol aliphatic carbons
13	33	Amino acid, catechol, and lipid aliphatic carbons
14	30	Lipid aliphatic carbons
15	23	Methyl carbons in chitin, protein, lipid, and catechol amino acid methylene carbons
16	19	Amino acid and lipid methyl carbons

\*δ-Values relative to external TMS reference.

linkage is drawn between the phenoxy carbon 3 and the GlcNAc carbon 4, but phenoxy carbon 4 as well as other GlcNAc carbons may participate in the conjugate linkage. If we assume that cuticular proteins have a random distribution of residues, of which about 10% are histidines, then the most heavily cross-linked exuviae examined have a histidine-catechol-chitin coupling once every 20 units along the protein chain—a high cross-link density (25). Except for the covalent bonding of catechols with sulfhydryl groups in proteins, the formation of catechol adducts with either protein nucleophilic side chains or chitin residues has been little studied (26).

Additional direct cross-links between histidyl ring nitrogens and the β-carbon of dopamine may form as a result of a β-sclerotization mechanism where an acyl dopamine with an α, β-unsaturated side chain or a p-quinone methide structure is the reactive intermediate (2). However, the DCP <sup>13</sup>C NMR spectrum of pupal exuviae from fifth-instar larvae injected with both L-[1,3-<sup>15</sup>N<sub>2</sub>]histidine and β-[<sup>13</sup>C]dopamine was too weak to permit an unambiguous assignment of any DCP difference peak, even after a 10-day data accumulation. Most of the <sup>13</sup>C label from dopamine appeared in a carbon with a chemical shift of 78 ppm, which indicates the formation of an oxygenated β-<sup>13</sup>C adduct (23) such as N-β-alanyl-norepinephrine, which is found at levels of micromoles per gram in pupal exuviae (24), or perhaps a β-carbon-O-alkylated adduct between N-β-alanyldopamine and a seryl, threonyl, tyrosyl, aspartyl, glutamyl, or GlcNAc residue. A minor carbonyl group signal from label observed at 195 ppm (27) is attributed to catabolism of the β-methylene carbon of dopamine to an aldehyde

group, perhaps 3,4-dihydroxybenzaldehyde (1).

Solid-state NMR analysis of *M. sexta* pupal cuticle provides direct evidence that aromatic cross-links derived from catecholamines stabilize the chitin-protein matrix during the process of sclerotization. Covalent bonds form between carbons of the catechol ring and nitrogens of the imidazole ring of cuticular proteins. That chitin may also be involved in the cross-link structure is indicated by tight binding of the histidyl-catecholamine adduct to chitin isolated from cuticle by hot alkali extraction. Solid-state NMR data on natural-abundance <sup>13</sup>C in protein, chitin, catechol, and lipid during cuticulogenesis support the general scheme (1, 2) for assembly of the pupal procuticle with increasing amounts of protein and chitin secreted after ecdysis as well as the gradual accumulation of catechols during sclerotization.

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- Assignment of resonances was made by comparison to solution and solid-state spectra of model com-

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  - On the basis of relative concentrations of cuticular components from Fig. 1 and from gravimetric analysis, newly ecdysed *M. sexta* pupal cuticle is approximately 14% protein, 2% chitin, 1% catechol, 3% lipid, and 80% water. Sclerotized cuticle (3 days after ecdysis) is 20% protein, 25% chitin, 3.5% catechol, 1.5% lipid, and 50% water. Pupal exuvium is 32% protein, 36% chitin, 18% catechol, 5% lipid, and 9% water. NMR spectra were normalized for direct intensity comparison of lyophilized samples.
  - Labeled histidine (10 to 14 mg) (99% atomically labeled <sup>15</sup>N, MSD Isotopes, Montreal, Canada) was dissolved in 0.3 ml of water, the pH was adjusted to 6.8, and the solution was filtered through a 0.5-μm Millex-PF filter (Millipore, Bedford, Massachusetts), and injected into a wandering fifth-instar larva through an abdominal proleg.
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  - DCPMAS NMR spectra were obtained by using matched spin-lock transfers first from <sup>1</sup>H to <sup>13</sup>C, and then from <sup>13</sup>C to <sup>15</sup>N. If the <sup>15</sup>N radio-frequency field is on resonance and its amplitude satisfies a magic-angle-spinning carbon-nitrogen Hartmann-Hahn condition (17), a spin-lock transfer from <sup>13</sup>C to <sup>15</sup>N drains polarization from <sup>13</sup>C. A direct difference experiment between single and double cross-polarization procedures therefore results in the accumulation of DCPMAS difference <sup>13</sup>C signals arising exclusively from <sup>13</sup>C directly bonded to <sup>15</sup>N. The fraction of <sup>13</sup>C with <sup>15</sup>N neighbors can be determined quantitatively from such difference spectra.
  - To label cuticular chitin with <sup>13</sup>C, 400 mg of [<sup>13</sup>C(U)] D-glucose (90% atomically labeled, MSD Isotopes, Montreal, Canada) were admixed with 2 g of artificial diet and fed to each larva.
  - The carbon-nitrogen contact was maintained for 3 msec, during which time the polarization transfer from a carbonyl carbon to an amide nitrogen is only half completed.
  - The <sup>15</sup>N distribution for this sample was intermediate to those shown in Fig. 2, B and C; the total <sup>15</sup>N incorporation was greater than for the pupal exuviae sample, although less per milligram of tissue.
  - To label catechols, 5 mg of [<sup>13</sup>C<sub>6</sub>]dopamine (99% atomically labeled, MSD Isotopes, Montreal, Canada) dissolved in 0.05 ml of water were injected into a wandering-stage larva.
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27. This is an isotropic chemical shift. A minor peak near 190 ppm also appears in the spectrum of the *ring-<sup>13</sup>C* labeled cuticle (Fig. 4F), but this peak is a spinning sideband not removed by pulse techniques because of extensive <sup>13</sup>C-<sup>13</sup>C dipolar coupling among aromatic carbons.
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