

ANALYSIS OF COCKROACH OOTHECAE AND EXUVIAE BY SOLID-STATE ^{13}C -NMR SPECTROSCOPY

KARL J. KRAMER,^{1,*} ALLYSON M. CHRISTENSEN,² THOMAS D. MORGAN,¹ JACOB SCHAEFER,²
THOMAS H. CZAPLA³ and THEODORE L. HOPKINS³

¹U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, KS 66502, ²Department of Chemistry, Washington University, St Louis, MO 63130 and ³Department of Entomology, Kansas State University, Manhattan, KS 66506, U.S.A.

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Abstract—Sclerotized oothecae from four species of cockroaches, *Periplaneta americana*, *P. fuliginosa*, *Blatta orientalis* and *Blattella germanica*, were examined by solid-state ^{13}C -nuclear magnetic resonance and chemical analyses. The oothecae were composed of protein, water, calcium oxalate, diphenolic compounds, lipid, and uric acid. Calcium oxalate was the major soluble component in egg cases of *P. americana*, *P. fuliginosa*, and *B. orientalis*. Oothecae of *B. germanica* had approx. 10-fold less calcium oxalate and extractable diphenols than the other species. The major diphenolic compound extracted in cold dilute perchloric acid was 3,4-dihydroxybenzoic acid. Exuviae from *P. americana*, *B. germanica*, *Gromphadorhina portentosa*, *Blaberus craniifer*, and *Leucophaea maderae* also were examined by solid-state ^{13}C -NMR. They contained protein, diphenols, and lipid, as well as chitin, which accounted for 30–42% of the organic content, depending upon the species.

INTRODUCTION

Cockroach oothecae and cuticle are highly sclerotized proteinaceous structures, which are stabilized by cross-linking, dehydration, and impregnation with phenolic metabolites (Brunet, 1980; Hepburn, 1985; Andersen, 1985; Kramer and Hopkins, 1987; Sugumarin, 1988; Hopkins and Kramer, 1991). Whereas protein and diphenolic compounds are common to both, other components, such as carbohydrate, minerals, and organic acids, are more variable (Stay *et al.*, 1960; Hackman and Goldberg, 1960). For example, chitin is present in the exuviae, but is absent from the oothecae. Previously, we have studied the metabolism of tyrosine in several cockroach species during cuticle sclerotization and melanization, and quantified the extractable diphenols in cuticle (Czapla *et al.*, 1988, 1989a,b). A complex pattern of *o*-diphenol accumulation was observed. To obtain a better understanding of the structure of cockroach oothecae and exuviae in their native states, we undertook a noninvasive examination of these structures using solid-state nuclear magnetic resonance (NMR) spectroscopy. The cockroach ootheca remains an attractive system in which to study the chemistry of sclerotization because its organic composition consists mainly of protein, including a laccase (Pau *et al.*, 1971; Whitehead *et al.*, 1960). Oxalic acid and simple phenolic compounds such as 3,4-dihydroxybenzoic acid can also be extracted with aqueous acid (Atkinson *et al.*, 1973; Stay *et al.*, 1960). The solid-state ^{13}C -NMR data, together with data obtained from conventional chemical analysis, provided us with a

compositional analysis of the chemical structures of cockroach oothecae and exuviae.

MATERIALS AND METHODS

Insects and sample collection

All species of cockroaches (*Periplaneta americana*, *P. fuliginosa*, *Blattella germanica*, *Blatta orientalis*, *Gromphadorhina portentosa*, *Blaberus craniifer*, and *Leucophaea maderae*) were reared in containers bedded with wood shavings at $28 \pm 2^\circ\text{C}$ with a photoperiod of 16L:8D. Water and Purina® Lab Chow were provided *ad libitum*. *P. fuliginosa* and strains of *B. germanica* were obtained from Drs Donald Mullins and Mary Ross, respectively, Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, Va (Ross and Cochran, 1966). Oothecae were collected after the eggs hatched, and exuviae were collected after adult ecdysis. Samples were washed with water, air dried, milled to a 40 micron particle size, and stored at -20°C .

Nuclear magnetic resonance spectroscopy

Cross-polarization magic-angle spinning (CPMAS) ^{13}C -NMR spectra of oothecal and exuvial powders were obtained at 50.3 MHz by using 2 ms cross-polarization transfers from protons at a 38 kHz radiofrequency field strength, followed by proton decoupling performed at 95 kHz. Natural-abundance powdered samples ($< 850 \mu\text{m}$ particle size) were spun at 3.205 kHz in a zirconium dioxide double-bearing hollow rotor that holds 1 ml vol (Schaefer *et al.*, 1981). In these experiments, 150 mg composite samples were centered in the rotor using Kel-F sample holders. Spectra were collected under spinning sideband suppression conditions (Dixon, 1982). Chemical shifts are reported in parts per million downfield from external tetramethylsilane. Chemical composition was estimated by integration of resonances at 144 ppm for diphenol content, 104 ppm for chitin, and 33 ppm for lipid (after correcting for contributions from diphenol and protein carbons) (Schaefer *et al.*, 1987; Kramer *et al.*, 1989a,b). Protein content for cockroach exuviae was determined by integrating peaks between 55 and 60 ppm after subtracting contributions from chitin

*To whom all correspondence should be addressed at: USGMRL, ARS, USDA, 1515 College Avenue, Manhattan, KS 66502, U.S.A. or BITNET address: KJRAME@KSUVM.

carbons. Concentrations of protein and oxalate in cockroach oothecae were determined by deconvolution and integration of peaks at 175 and 172 ppm, respectively, using NMRI software (New Methods Research Inc., East Syracuse, N.Y.).

Mineral and moisture analyses

Elemental analyses of the oothecae were performed using the graphite furnace atomic absorption method at the Kansas State University Emission Spectroscopy Laboratory. Moisture content of the oothecal powder was measured by gravimetric analysis after heating for 1 h at 130°C. Ash content was determined after heating the sample at 800°C for 10 min, 925°C for 16 h, and 1000°C for 4 h.

Amino acid, diphenol, and uric acid analyses

Samples for amino acid analysis were hydrolyzed in 6 M HCl containing 5% phenol at 110°C for 24 h *in vacuo* and quantitated by cation exchange chromatography with post-column ninhydrin derivatization. Milled oothecae (5 mg) were homogenized in glass tissue grinders containing 0.3 ml of 0.3 M perchloric acid with α -methyldopa ($6 \mu\text{g ml}^{-1}$) as an internal standard for quantitation of electroactive compounds (Morgan *et al.*, 1987). Uric acid was also extracted from *B. germanica* oothecae in 0.5% lithium carbonate, but it was recovered at only about 50% of the levels found in the perchlorate extract. Some samples of embryonic membranes or individual oothecal walls were homogenized in glass tissue grinders without prior milling. The extracts were analyzed by reversed-phase liquid chromatography with amperometric, dual electrode detection. Uric acid was quantitated by the current flow at +0.8 V on the upstream electrode, whereas the diphenols were quantitated at -0.1 V on the downstream electrode. A 5% dilution of the extract was used for quantitation of 3,4-dihydroxybenzoic acid. The mobile phase contained 13% (v/v) methanol, 0.12 mM sodium octyl sulfate, and 0.1 M phosphoric acid or acetic acid adjusted with NaOH to pH 3.0 or 4.5, respectively. The lower pH was appropriate for analysis of most of the compounds of interest, but the higher pH was required to resolve 3,4-dihydroxybenzylalcohol from an unknown compound. Standard compounds were obtained from Sigma or Aldrich Chemical Company except for 3,4-dihydroxyphenylethanol, which was a gift from Dr Peter F. Sorter of Hoffman La Roche, Inc. (Nutley, N.J.), and 3,4-dihydroxybenzylalcohol, which was prepared by reduction of 3,4-dihydroxybenzylaldehyde with sodium borohydride in ethanol.

RESULTS

Moisture, ash and cation composition of cockroach oothecae

While milling and extracting samples of cockroach egg cases, we observed that the empty oothecae of *B. germanica* appeared more flexible and less brittle than those of *P. americana*, *P. fuliginosa*, and *B. orientalis*. This difference may have been due in part to the relative amounts of inorganic and organic constituents. Moisture values for the oothecal powders were similar, ranging from approx. 7–11%. Ash weights and the amounts of inorganic cations are shown in Table 1. The less brittle egg cases of *B. germanica* had only 1.47% ash, compared to 9.83–14.09% for oothecae of other species. This result indicated that *B. germanica* oothecae were more organic in nature than the others. Calcium accounted for about 8% of the dry wt of *P. americana*, *P. fuliginosa*, and *B. orientalis* oothecae, but only 0.5% in the *B. germanica* oothecae, a 16-fold difference. Other inorganic cations (Na, Mg, K) were detected at <0.3% levels in all of the egg cases.

Table 1. Ash and major inorganic cations of oothecae from four species of cockroaches*

Species	Ash	Ca	Na	Mg	K
<i>P. americana</i>	11.28	7.85	0.04	0.09	<0.10
<i>P. fuliginosa</i>	9.83	7.50	0.07	0.10	<0.10
<i>B. orientalis</i>	14.09	8.05	0.11	<0.10	0.15
<i>B. germanica</i>	1.47	0.52	0.28	0.25	0.14

*Percent of dry weight. Mean value of two determinations from a composite sample. P and Zn were <0.10% of dry wt.

Solid-state ^{13}C -NMR of cockroach oothecae

Solid-state ^{13}C -NMR spectroscopy was used to determine the relative amounts of organic constituents present in oothecae from four species of cockroaches. Figure 1 shows the spectra for three of the species examined. The assignments of the chemical shifts are listed in Table 2 and the relative organic compositions in Table 3. Protein was the major component in all of the egg cases. The oothecae of *B. germanica* were 95% protein, whereas the other oothecae were only 86–88% protein. Oxalate, which exhibited a sharp signal at 172 ppm in the ^{13}C -NMR spectra, accounted for 7–8% of the organic components in all of the oothecae, except that of *B. germanica*, which was <1% oxalate. Diphenols

COCKROACH EGG CASES

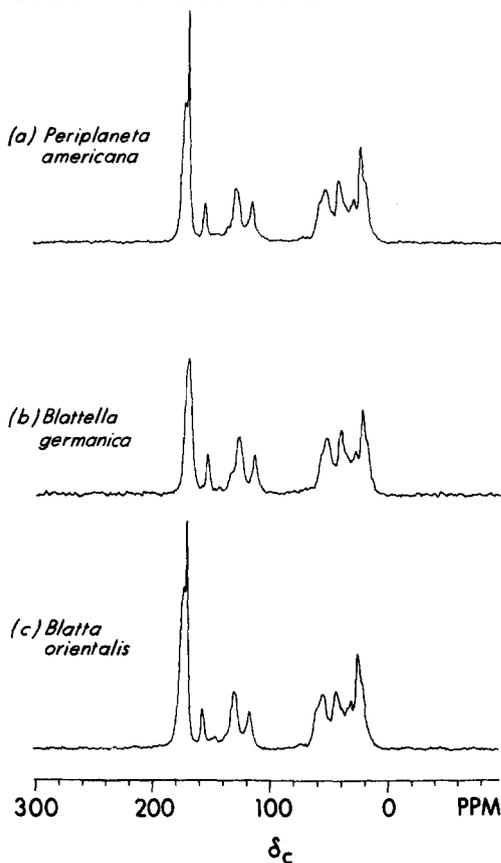


Fig. 1. Natural-abundance cross-polarization magic-angle spinning ^{13}C -NMR spectra of oothecae from (a) *P. americana*, (b) *B. germanica*, and (c) *B. orientalis*. The scale is in parts per million downfield from tetramethylsilane (TMS) used as an external reference.

Table 2. Assignments for chemical shifts in CPMAS ^{13}C -NMR spectra of cockroach oothecae and exuviae*

δ value (ppm)	Assignment
175	Carbonyl carbons in protein, phenolic and lipid acyl groups
172	Carbonyl carbons of oxalate
157	Phenoxy carbon of tyrosine
145	Phenoxy carbons of diphenolic compounds
138, 130	Aromatic carbons
117	Tyrosine carbons 3 and 5
104	Carbon 1 in GlcNAc
82	Carbon 4 in GlcNAc
75	Carbon 5 in GlcNAc
72	Carbon 3 in GlcNAc
60	α -Carbon in amino acids Carbon 6 in GlcNAc
55	α -Carbon in amino acids Carbon 2 in GlcNAc
30–50	Protein, phenolic, and lipid aliphatic carbons
26, 22	Methyl carbons in protein, phenols, and lipids

*See spectra in Fig. 1. Assignments made by comparison to solution and solid state ^{13}C -NMR spectra of model compounds and sclerotized structures (Schaefer *et al.*, 1987; Kramer *et al.*, 1987, 1988).

ranged from 3 to 6% in all of the egg cases, and lipid was only 1% of the total carbons. No carbohydrate carbons were evident. The oothecal proteins or oothecins were rich in amino acids with aromatic side chains, as demonstrated by the substantial resonances between 115–140 ppm.

The oothecae from *P. americana* were extracted with cold 1.2 N HCl, and both the residue and dried extract were subjected to solid-state ^{13}C -NMR analysis. As shown in Fig. 2, oxalate was the major acid-soluble organic component. Only minor amounts of protein and diphenolic compounds were extracted relative to the amount of oxalate. The ^{13}C -NMR spectrum of the acid insoluble residue from *P. americana* oothecae resembled the spectrum of *B. germanica* oothecae [compare Fig. 1(b) with Fig. 2(b)]. These results demonstrated that a major organic difference between the oothecae was the amount of oxalate present.

Diphenol and uric acid analyses of cockroach oothecae

The amounts of electroactive organic compounds in the oothecae used for NMR analysis were determined by liquid chromatography with electrochemical detection after extraction with 0.3 M perchloric acid. The major diphenol in the extracts of all species was 3,4-dihydroxybenzoic acid (protocatechuic acid), which eluted at 16.1 min in the HPLC profile (Fig. 3). Other diphenols, such as 3,4-dihydroxybenzylalcohol, 3,4-dihydroxyphenylethanol, catechol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxybenzaldehyde occurred at much lower levels. When the pH of the mobile phase used for the

Table 3. Organic compositions of oothecae from four species of cockroaches*

Component	Relative amount (%) in			
	<i>P. americana</i>	<i>P. fuliginosa</i>	<i>B. orientalis</i>	<i>B. germanica</i>
Protein	87	86	88	95
Oxalate	8	7	7	<1
Diphenol	4	6	4	3
Lipid	1	1	1	1

*Relative % of total carbons estimated by ^{13}C -NMR.

COCKROACH EGG CASES (*P. americana*)

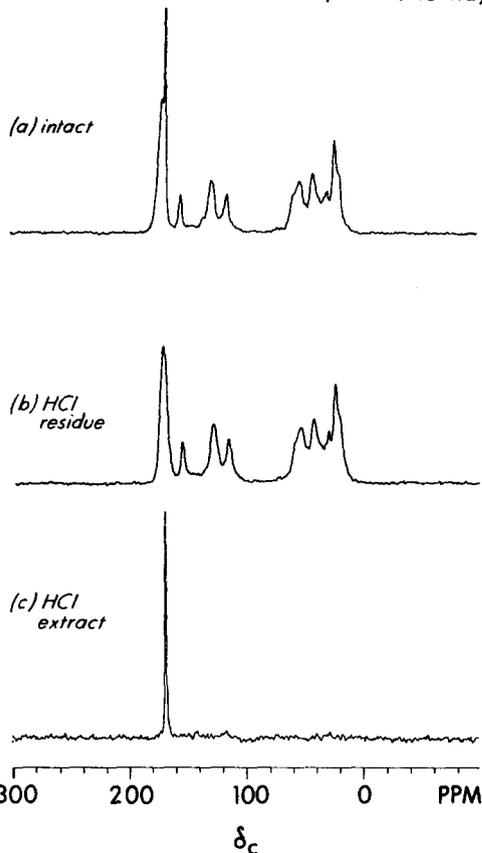


Fig. 2. Natural-abundance, cross-polarization magic angle spinning ^{13}C -NMR spectra of (a) *P. americana* oothecae, (b) residue remaining after acid extraction, and (c) dried extract soluble in 1.2 M HCl.

chromatography was increased from 3 to 4.5, an unknown compound, which had coeluted with 3,4-dihydroxybenzylalcohol at 8.5 min, moved to an earlier retention time, allowing quantitation of the latter. 3,4-Dihydroxybenzoic acid also eluted earlier at the higher pH. Although 3,4-dihydroxybenzylalcohol and 3,4-dihydroxybenzaldehyde were present in appreciable amounts in *P. fuliginosa*, 3,4-dihydroxybenzoic acid was the only diphenol present in large amounts in all four species (Table 4). The concentration of 3,4-dihydroxybenzoic acid ranged from 31 to 53 $\mu\text{mol g}^{-1}$ dry wt in the oothecae of *P. americana*, *P. fuliginosa*, and *B. orientalis*, but was only 3 $\mu\text{mol g}^{-1}$ in *B. germanica*. This 18-fold difference was somewhat surprising, because the total amount of diphenol (bound plus extractable diphenols) was similar in all of these species (see Table 3).

Oothecae from *B. germanica* contained uric acid at 9 $\mu\text{mol g}^{-1}$ dry wt, which eluted at 5.3 min (Table 4). The levels of uric acid were about 10-fold lower in the other species. The internal membranes that partition the eggs had not been removed from these oothecal samples. Therefore, we determined the amount of electroactive compounds in extracts of membranes and walls. The membranes contained $2.18 \pm 0.71 \mu\text{mol}$ ($n = 3$) of uric acid per g of wet wt, and the oothecal

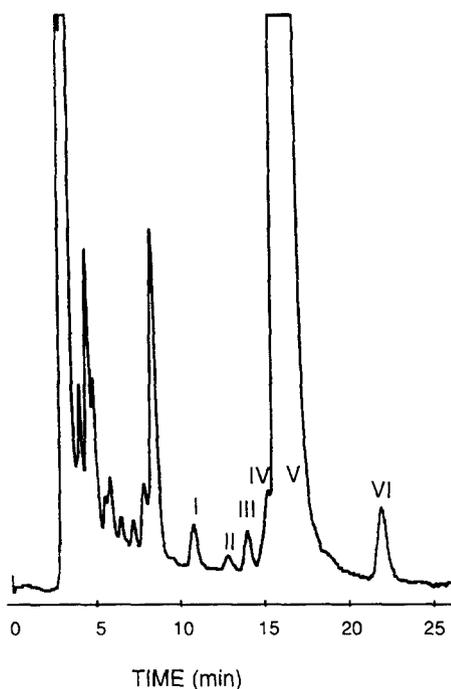


Fig. 3. Chromatogram obtained by reversed phase LC of an acidic extract of egg cases (0.4 mg) from *B. orientalis*. Amperometric detection was performed on the downstream electrode at -0.1 V after oxidation at $+0.8$ on the upstream electrode. Full scale was 20 nA. Peak V produced about 10 μ A of current flow at its maximum. Peak identification: I, α -methyl dopa (internal standard); II, 3,4-dihydroxyphenylethanol; III, catechol; IV, 3,4-dihydroxyphenylacetic acid; V, 3,4-dihydroxybenzoic acid; VI, 3,4-dihydroxybenzaldehyde.

walls contained 1.10 ± 0.10 μ mol g^{-1} ($n = 3$). Thus, appreciable amounts of urate were present in both structures. The value of 9 μ mol g^{-1} for uric acid in the original composite sample used for NMR analysis suggested that some of the *B. germanica* oothecae may contain substantially higher levels of urate. The amount of 3,4-dihydroxybenzoic acid in the oothecal walls of *B. germanica* was 2.06 ± 0.34 μ mol g^{-1} wet wt ($n = 3$), whereas the corresponding value for the internal membranes was 0.57 ± 0.06 ($n = 3$). These results indicated that 3,4-dihydroxybenzoic acid and uric acid were the major electroactive compounds extracted from the oothecal wall of *B. germanica*, whereas the oothecae of the other three species contained much higher amounts of the former compound. The oothecal wall of *P. americana* contained

29.59 μ mol g^{-1} wet wt of 3,4-dihydroxybenzoic acid and 0.46 μ mol g^{-1} of uric acid.

Analysis of amino acid residues of cockroach oothecae

The amounts of several amino acids were determined after acid hydrolysis of the oothecae. Glycine was the major amino acid in the oothecae of the four cockroach species, followed by tyrosine or leucine (Table 5). Proline and valine were also relatively abundant, but no other amino acid exceeded 0.25 mmol g^{-1} . Phenylalanine was much less abundant than tyrosine. Lysine, which has an ϵ -amino group, and histidine, which has a weakly basic imidazolium function, were two of the amino acids with nucleophilic side chains that were present in the oothecae. Although similar amounts of recoverable lysine were present in the four species, the amount of recoverable histidine was higher in the oothecae of *B. germanica* than in the other three species. β -Alanine, an amino acid found in many sclerotized cuticular structures (Kramer and Hopkins, 1987), and glucosamine, a hydrolysis product of chitin, were absent in the hydrolysates.

Solid-state ^{13}C -NMR of cockroach exuviae

Figure 4 and Table 6 show the ^{13}C -NMR spectra and organic composition, respectively, of exuviae from several cockroaches, including mutants of *B. germanica* that differ in cuticular pigmentation. A comparison of these spectra with those of the oothecae revealed that chitin carbons were present and oxalate carbons were absent in the exuviae. Protein (44–61%) and chitin (30–42%) were the major organic constituents of the exuviae, followed by diphenols (4–13%) and lipid (1–2%). The exuviae of the yellow, orange, and black mutant strains of *B. germanica* had less protein and more chitin than that of the wild-type strain.

DISCUSSION

Chemical composition

The use of solid-state NMR for the examination of sclerotized insect structures, such as cockroach egg cases and exuviae, revealed that they were composed mainly of protein, with lesser amounts of other materials such as diphenolic compounds and lipid. Highly variable levels of carbohydrate and minerals may also be present, ranging from 0 to 40% chitin and from a few percent to >50% mineral salts (Kramer *et al.*, 1988). Sclerotization of oothecae and cocoons involves protein and oxidized diphenolic compounds. However, in cuticular structures where

Table 4. Electroactive compounds extracted with 0.3 M $HClO_4$ from sclerotized oothecae*

Component	Amount extracted from			
	<i>P. americana</i>	<i>P. fuliginosa</i>	<i>B. orientalis</i>	<i>B. germanica</i>
3,4-Dihydroxybenzoic acid	30.88	53.30	49.24	2.93
3,4-Dihydroxybenzaldehyde	0.12	0.61	0.13	0.17
3,4-Dihydroxybenzylalcohol	—	1.08	—	—
Catechol	tr	tr	0.05	0.06
Uric acid	0.94	0.35	0.90	8.94

*Unit = μ mol g^{-1} dry wt. Mean value of two determinations from a composite sample. Tr = trace level detected. Trace levels were <0.02 μ mol g^{-1} for catechol. *P. americana*, *B. orientalis*, and *B. germanica* had <0.10 μ mol g^{-1} 3,4-dihydroxybenzylalcohol. Trace levels of 3,4-dihydroxyphenylethanol (<0.02) and 3,4-dihydroxyphenylacetic acid (<0.06) were also present in the extracts.

Table 5. Partial amino acid compositions of hydrolysates of cockroach oothecae*

Amino acid	Species			
	<i>P. americana</i>	<i>P. fuliginosa</i>	<i>B. orientalis</i>	<i>B. germanica</i>
Glycine	1.76	1.95	1.69	2.78
Histidine	0.15	0.09	0.11	0.37
Leucine	0.75	0.83	0.76	0.99
Lysine	0.14	0.11	0.14	0.16
Phenylalanine	0.17	0.18	0.14	0.23
Proline	0.43	0.41	0.35	0.42
Tyrosine	0.74	0.68	0.68	1.07
Valine	0.29	0.28	0.25	0.31

*Unit = mmol g⁻¹ dry wt. Mean value of two determinations from a composite sample. Amino acids not shown were <0.25 mmol g⁻¹.

chitin is a second major component, both polysaccharide and protein may covalently interact with free radical or quinonoid compounds derived from diphenols. The covalent bonds that form during sclerotization are poorly characterized, although cross-links between protein side chains of histidine and both the aromatic and/or aliphatic carbons of diphenolic compounds such as N-β-alanyldopamine have recently been observed using solid-state NMR spectroscopy (Schaefer *et al.*, 1987; Christensen *et al.*, unpublished data).

Results of chemical and NMR analyses of cockroach egg cases revealed substantial differences in the levels of calcium, oxalic acid and 3,4-dihydroxybenzoic acid. The semi-quantitative solid-state ¹³C-NMR data showed that the diphenol levels of sclerotized oothecae varied by a factor of two between species, even though the acid-extractable diphenols varied by a factor of 18. Less than 25% of the diphenolic compounds were extracted by cold 10% HCl, indicating that most of the diphenols were tightly bound to the insoluble matrix of the oothecae.

COCKROACH EXUVIAE

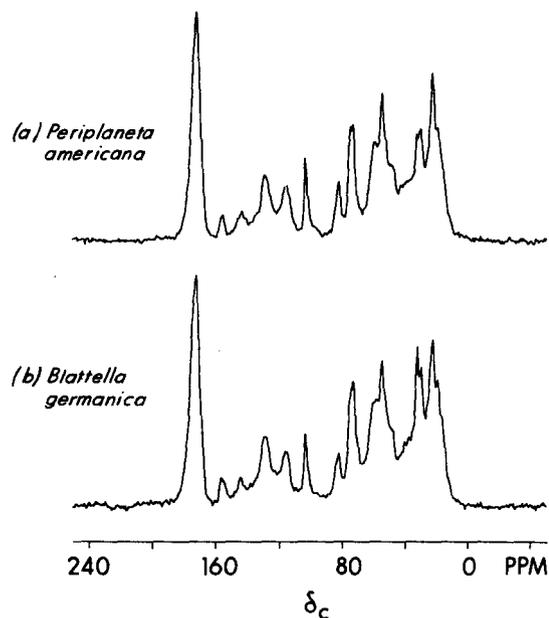


Fig. 4. Natural-abundance cross-polarization magic-angle spinning ¹³C-NMR spectra of exuviae from (a) *P. americana* and (b) *B. germanica*.

Table 6. Organic composition of exuviae from five species of cockroaches*

Species	Relative amount (%) of			
	Protein	Chitin	Diphenol	Lipid
<i>P. americana</i>	49	38	11	2
<i>B. germanica</i>				
Wild type	59	30	9	2
Yellow	44	41	13	2
Orange	46	40	12	2
Black	51	38	9	2
<i>G. portentosa</i>	53	38	8	1
<i>B. craniifer</i>	52	42	5	1
<i>L. maderae</i>	61	35	4	1

*Relative % of total carbons estimated by ¹³C-NMR.

Oxalic acid

Little or no oxalate was evident in the NMR spectra of *B. germanica* oothecae or exuviae from the four cockroach species. However, oxalate was very conspicuous in spectra of oothecae of *P. americana*, *P. fuliginosa*, and *B. orientalis*. Stay *et al.* (1960) detected calcium oxalate crystals in these species by X-ray diffraction; crystals were absent in oothecae of cockroach species that carry their oothecae internally, but were found in 18 of the 19 oviparous species that were examined. We found that oxalate represented about 8% and <1% of the total carbons of the empty oothecal cases of *P. americana* and *B. germanica*, respectively. The data from Stay *et al.* (1960) for these two species were about 7.5 and 0.2% dry wt, respectively, and the data from Hackman and Goldberg (1960) were 5.2 and 0.3%. Our calcium determinations for *P. americana* and *B. germanica* were in agreement with those of Hackman and Goldberg (1960). Stay *et al.* (1960) reported that *B. germanica* had a greater concentration of calcium oxalate crystals in the notched edge of the oothecae and in the walls close to this structure, which is split open during hatching. Rajulu and Renganathan (1966) reported that calcium oxalate may be largely responsible for the extreme resistance of *P. americana* oothecae to hot alkali. Apparently, many species of cockroaches use calcium oxalate in a mineralization process, which makes the oothecae both hard and brittle. The oothecae of the praying mantid, *Orthodera ministralis*, also contains calcium oxalate (Hackman and Goldberg, 1960). In a few insect species, such as the face fly, *Musca autumnalis*, mineralization involves calcium phosphate salts instead of oxalate to produce a hard, brittle, puparial cuticle (Roseland *et al.*, 1985; Grodowitz *et al.*, 1987).

Uric acid

Much of the dry weight of cockroach oothecae could be metabolites of uric acid. Urates play a central role in the physiology of cockroaches and probably serve as a reservoir of nitrogen that can be used for further metabolism (reviewed by Cochran, 1985). Although the products of uric acid degradation in bacteriocytes and other cells of the fat body have not been identified, glycine or glyoxylate are produced from the central portion of the uric acid molecule in other organisms. Residues of glycine in the proteins of the oothecal wall accounted for 13–17% of the dry wt of the wall in *P. americana*,

P. fuliginosa, and *B. orientalis*. Oxalate accounted for 7–8% of the organic content in these species, and glyoxylate is a likely precursor of oxalate. In addition to a speculative role as a precursor of components of the oothecal wall, uric acid may be a source of nutritional nitrogen for the embryos (Mullins and Keil, 1980). Mullins and Keil (1980) measured 3 μmol of uric acid in newly formed oothecae of *B. germanica*, but only 1 μmol in the oothecal case plus newly hatched cockroaches. In this species, we detected about 0.3 nmol of uric acid per ootheca remaining in the internal membranes and 1 nmol in the oothecal wall itself. Although these amounts represented only a small fraction of the original amount present in newly formed oothecae, uric acid may play a role in the structure of the oothecal wall. It has also been found in some cuticular structures, such as the light-reflecting layers of certain scarab beetles and in the wings of certain butterflies (Caveney, 1971; Lafont and Pennetier, 1975).

Diphenols and amino acids

Solid-state ^{13}C -NMR data showed that the diphenol levels in sclerotized oothecae varied between 3 and 6% of the organic content (or about 3–5% of the dry wt). The chemical analyses demonstrated that extractable 3,4-dihydroxybenzoic acid was about 0.48–0.82% of the dry wt for *P. americana*, *P. fuliginosa*, and *B. orientalis*, but only 0.04% of the weight for *B. germanica*. Therefore, extractable 3,4-dihydroxybenzoic acid accounted for 14–22% of the total diphenols that were detected by NMR in the first three species, but only 2% of the total in *B. germanica*. The higher percentage of covalently bound diphenol in *B. germanica* was surprising, because the oothecal wall of this species was more flexible than those of the other three species and was translucent instead of dark brown in color. Solid-state ^{13}C -NMR data for total diphenol content of exuviae agreed with data on extractable diphenols reported by Czaplá *et al.* (1989a,b), in that the yellow and orange strains of *B. germanica* had the highest content of diphenols and *L. maderae* the least. The chemical compositions of exuviae from yellow and orange mutant *B. germanica* strains were nearly identical. Wild-type and black mutant exuviae had higher protein contents but less chitin and diphenols.

3,4-Dihydroxybenzoic acid was originally identified as a component of cockroach oothecae by Pryor (1940). More recently, Atkinson *et al.* (1973) detected 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde in extracts of oothecae from *P. americana*. We identified these same diphenols in each of the species examined, and we also found 3,4-dihydroxybenzylalcohol in *P. fuliginosa*. In both the present study and that of Atkinson *et al.* (1973), 3,4-dihydroxybenzylalcohol was not detected in extracts from tanned oothecae of *B. germanica*, even though its glucoside appears to be the sole storage form of diphenols in the left collateral gland of this species (Pau and Acheson, 1968). It is possible that all of the alcohol is oxidized to its quinone in the oothecal wall, but it appears more likely that the main role of the alcohol is that of a precursor of the acid.

In contrast to cockroach oothecae, the major diphenols in cockroach exuviae were the N-acylated

derivatives of dopamine and norepinephrine (Czaplá *et al.*, 1988, 1989a,b). N- β -Alanyl and/or N-acetyl derivatives were abundant in the exuviae of *P. americana*, *B. germanica* and *L. maderae*. N-Malonyl derivatives of dopamine and norepinephrine were the most abundant diphenols in the oothecae of certain mantid species (Yago and Kawasaki, 1984; Kramer *et al.*, 1989a). All of the above diphenols were probably derived from tyrosine (Brunet, 1980; Kramer and Hopkins, 1987). The reason for such variability in the diphenols used for sclerotization is unknown.

Oxidation reactions of phenols lead to cross-links and harden the exoskeleton and egg case (Kramer and Hopkins, 1987; Hopkins and Kramer, 1990). The covalent bonds that form during sclerotization are poorly characterized, except for cross-links between histidyl residues of protein and the β -carbon and aromatic carbons of catecholamines in the pupal cuticle of *Manduca sexta* (Schaefer *et al.*, 1987; Christensen *et al.*, unpublished data). The major difference in the amino acid compositions of the oothecae of the four species that we examined was the high concentration of histidyl residues in *B. germanica*. The oothecal walls of *B. germanica* differed from the other species in that they were translucent instead of an opaque dark brown, but similar amounts of bound phenols were present in the oothecae of these four species. These observations suggest that the nucleophilic imidazole ring of the abundant histidyl residues in *B. germanica* may react with *o*-quinones more rapidly, thereby decreasing the extent of subsequent tanning reactions of quinones that result in color formation. A previous study found that the amount of recoverable lysine decreased substantially during tanning of the oothecal wall of *P. americana* (Hackman and Goldberg, 1963). We recovered similar amounts of lysine from the hardened oothecae of the four cockroach species. Further work is needed to determine how and to what extent lysine and histidine react with quinones during sclerotization of oothecae from these species.

The ^{13}C -NMR spectra provided information about relative abundance of aromatic and aliphatic amino acids in cockroach exuviae and oothecae. The aromatic carbon region (110–150 ppm) of the ^{13}C -NMR spectrum of *Blaberus giganteus* exuviae that was obtained by Peter *et al.* (1984) was similar to the spectra that were obtained in the present study, suggesting that similar amounts of aromatic amino acids and phenolic compounds were present in cockroach exuviae. The spectra of cockroach oothecae had major resonances at 115–140 ppm, which was consistent with the high concentration of tyrosyl residues present. The concentrations of tyrosine, leucine, and glycine in the oothecae were consistent with the occurrence of a pentapeptide repeat sequence, Gly-Tyr-Gly-Gly-Leu, as part of the structure of oothecins in *P. americana* oothecae (Pau, 1987a,b). Our amino acid analyses were similar to those of Hackman and Goldberg (1971) for *P. americana*, and our data showed that tyrosine, leucine, and glycine were the most abundant amino acids in *P. fuliginosa*, *B. orientalis* and *B. germanica* as well. Another strong resonance in the ^{13}C -NMR spectra of the oothecae that occurred at 40–50 ppm may be assigned to aliphatic carbons of amino acids and

other compounds. Glycine, the most abundant amino acid, has one aliphatic carbon. Aliphatic carbons of leucine and lysine contributed substantially to resonance in the 40–50 ppm region as well (Hackman and Goldberg, 1963; the present study).

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