Sterols in Mollusks and Crustacea of the Pacific Northwest[1]

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ABSTRACT

There are at least eight major sterols in mollusks and cholesterol predominates in shrimp and crab of the Pacific Northwest. Depending on the analytical method used, significantly different values for total sterols in mollusks are obtained. Using combined gas liquid chromatography for Δ^5 sterols and a modified Liebermann-Burchard reaction for $\Delta^{5,7}$ sterols, an average total sterol and cholesterol content in oysters (*Crassostrea gigas*) of 170 ± 21 mg and 51 ± 10 mg/100 g, respectively were found. Approximately one-half these amounts were found in 7 other mollusks. Dungeness Crab (*Cancer magiter*) and Pacific shrimp (*Pandalus joradani*) contain 50 and 144 mg cholesterol/100 g, respectively. For these crustacea, this represents 99% of the total sterols present. In mollusks, the percent nonesterified sterols ranges from 49.6 to 100%, with a mean of 73%. Only free cholesterol is present in Dungeness crab. Proximate composition of all samples, including oysters analyzed over a 9-month period is reported.

The cholesterol content of mollusks and crustacea for human dietary information is periodically reported (1-3). More detailed reviews indicate that different sterols are found in mollusks (4-5) and crustacea (6-7). The continuing debate over the role that cholesterol may play in the development (8) and pathology (9) of coronary heart disease supports the evaluation of cholesterol levels in different foods.

Mollusks are unique among all foods in the diversity of sterols found other than cholesterol. However, the metabolic relationship between these other sterols and cholesterol in man and animal models has only been examined to a limited degree (10-12). Crustacea and marine finfish appear similar to other vertebrates where cholesterol predominates as the primary sterol. Determining the quantitative and qualitative sterol composition of mollusks and crustacea indigenous to the Pacific Northwest was the primary objective of this study. Greatest effort was given to the analysis of oysters (*Crassostrea gigas*).

Because of the mixture of sterols in mollusks, accurate analytical measurement of these compounds continues to present some difficulty (3). To overcome this problem, gas liquid chromatography (GLC) was used to quantitate the Δ^5 sterols and a modified Liebermann-Burchard (LB) reaction permitted measurement of the $\Delta^{5,7}$ sterols. (Numbers after the symbol "Δ" indicate the position of the double bond in the basic cyclopentanoperhydrophenanthrene ring.) This colorimetric procedure allows for the determination of the $\Delta^{5,7}$ sterols which are otherwise lost in a thin layer chromatograph (TLC) purification step prior to GLC analysis.

Another common deficiency in reporting sterol levels of seafoods, is that most surveys are of limited: (a) sample number; (b) sample description; (c) information on geographical location; and (d) seasonal variation. This investigation attempted to minimize these shortcomings.

MATERIAL AND METHODS

Samples

Mollusks and crustacea were obtained fresh from various locations along the Oregon and Washington coasts during the period March 1978-May 1979. Specific information on each sample is detailed in Table I. Oysters obtained in 3 sizes over a 9-month period from the same location were examined (all oyster samples were obtained during the last week of each month). Twelve oysters in each size group were homogenized to provde a composite from which subsamples were drawn for analysis. Values are reported on a wet-wt basis per 100 g. Canned oysters were obtained from a local parent company distributor (Astoria, OR) but were a product of Korea. The canned shrimp sample was from a lot previously analyzed for minerals (13). All samples, whether assayed individually or as a composite, were homogenized drained samples from which subsamples were removed.

Proximate Analyses

Protein (total N × 6.25), ash and moisture were measured using AOAC procedures (14-16). Fat was determined gravimetrically from an aliquot of the total lipid extract according to Folch et al. (17). All sample homogenizations and lipid extractions were made within 24 hr of sample harvest.

Sterol Determinations

From the total lipid chloroform/methanol (2/1, v/v) extract (17) an aliquot was removed that contained an estimated 1-3 mg total sterols. After removing the extracting solvent under a stream of N_2, the residual lipid was saponified with 5 mL 1 N KOH in 80% ethanol for 30 min at 80 C in a Teflon-lined capped tube. The tube was allowed to cool, and 5 mL distilled water and 10 mL heptane added. Four fractions, (3 were 1-mL each and 1 was 5-ml) of the heptane upper layer were removed for sterol analyses as indicated in Figure 1. The heptane was removed under a stream of N_2. One mL of glacial acetic acid was added to two of the 1-mL fractions and 2-mL acetone-absolute ethanol (1:1, v/v) to the third. Total sterol content was determined from one tube containing 1 mL glacial acetic acid by the colorimetric LB reaction (18) as outlined by Abell et al. (19) except that all absorbance readings were made after 14 min. To the nonsaponifiable fraction dissolved in acetone-absolute ethanol, 1 drop glacial acetic acid and 1 mL 1% digitonin in 50% ethanol were added (20). After allowing to stand overnight at RT, the precipitate was isolated by centrifugation and washed with: (a) acetone/ether (1/2, v/v); (b) acetone/water (1/1, v/v); (c) acetone, and then dried under N_2. The digitonides were dissolved in 1 mL glacial acid and total sterols measured as previously described. Total sterols determined colorimetrically by the LB reaction are reported against a pure cholesterol standard (Sigma Chemical Co., St. Louis, MO) as such with or without prior digitonin precipitation.

Advantage of the fast acting property of $\Delta^{5,7}$ sterols with LB reagent was taken to measure this class of compound as previously described for skin sterols (21,22). Standard LB absorbance curves at 620 nm were prepared for cholesterol and 7-dehydrocholesterol at concentrations of 0.4 mg and 0.1 mg per reaction tube, respectively. In all determinations of standards and unknowns, 5 mL LB reagent (ice cold 50 mL acetic anhydride and 2.6 mL sulfuric acid) was added to sterol(s) dissolved in 1 mL glacial acetic

[1]Portions of this paper were presented at the AOCS Meeting, San Francisco, CA, April, 1979.

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TABLE I

Summary of Samples Analyzed

Sample (date harvested)	Specie	Origin of samples	(Number of samples or composites (number per composite)	Average weight (g)
Mollusks				
Butter clam no. 1 (9/12/78)	*Saxidomus giganteus*	Paulsbo, WA	3	84.5 ± 16.5
Butter clam no. 2 (3/11/78)	*Saxidomus giganteus*	Tillamook Bay, OR	1 (4)	9.3 ± 1.7
Cockles no. 1 (3/11/78)	*Clinocardium nuttallii*	Tillamook Bay, OR	1 (12)	27.5 ± 3.7
Cockles no. 2 (3/11/78)	*Clinocardium nuttalli*	Tillamook Bay, OR	1 (12)	53.4 ± 6.4
Geoducks (9/12/78)	*Panope generosa*	Paulsbo, WA	3	687.6 ± 125.4
Horse clams (9/12/78)	*Tresus capax*	Paulsbo, WA	3	202.9 ± 42.8
Little necks (9/12/78)	*Venerapis staminea*	Paulsbo, WA	3 (12)	27.5 ± 1.5
Mussels (12/26/78)	*Mytilus edulis Linneaus*	South Jetty, Warrenton, OR	1 (36); 1 (18); 1 (12)	3.1 ± 1.1; 7.5 ± 1.2
Oysters, fresh (9/78-5/79)	*Crassostrea gigas*	Willipa Bay, WA	See footnote d	
Oysters, canned[a] (1978)	Unknown	Product of Korea	3 cans	227g/can
Razor clams 3/28/79)	*Siliqua patula*	Seaside, OR	1 (12)	20.3 ± 4.8
Crustacea				
Dungeness crab (5/1/79)	*Cancer magister*	Seaside, OR	See footnote e	1.2kg
Shrimp:				
fresh cooked[b] (5/1/79)	*Pandalus jordani*	Off Newport, OR coast	1	raw count, 220-280/kg
IQF[b] (5/1/79)	*Pandalus jordani*	Off Newport, OR coast	1	raw count, 220-280/kg
canned[c] (9/27/75)	*Pandalus jordani*	Southern WA coast	1 can	128g/canned[f]

[a]Commercially canned oysters; entire drained content of can analyzed.
[b]Commercially processed and subsequently individually quick frozen (IQF) and reanalyzed; salt added to IQF product.
[c]See Gordon and Roberts (1977) for complete sample description.
[d]Complete description of fresh oysters given in Tables II and VIII.
[e]Picked body and leg meat of one crab, commercially processed with salt; intestine also analyzed.
[f]Drained weight.

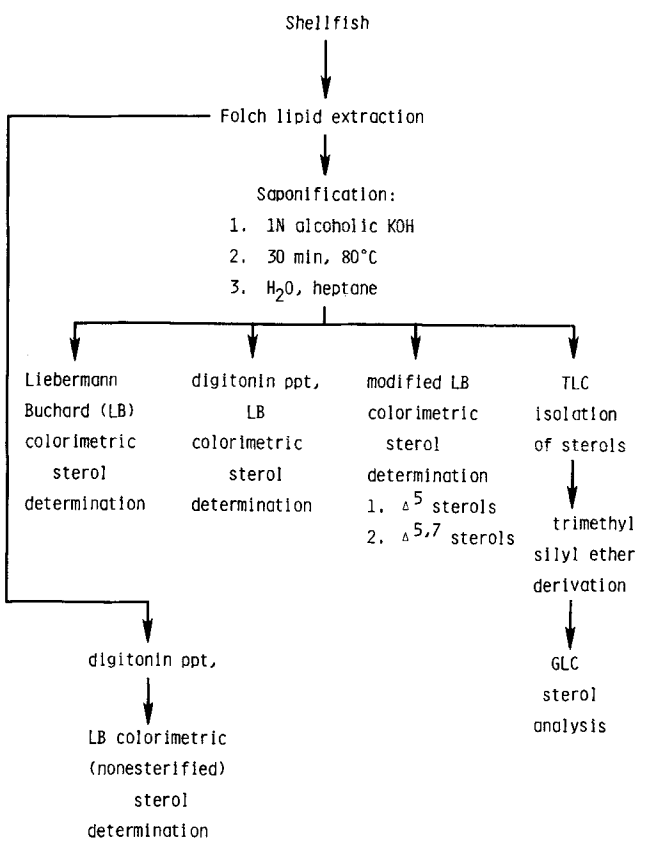

FIG. 1. Extraction and analysis scheme for sterols in mollusks and crustacea.

acid. Upon addition of the LB reagent, the reaction was timed and absorbance read at 30-sec intervals while maintaining the reaction temperature at 25 C. Maximum color development was observed after 2 min with 7-dehydrocholesterol and 14 min for cholesterol. Absorbance of unknowns handled in a similar manner were recorded at 2 and 14 min after the start of the reaction. For calculation, the two absorbance readings for standard cholesterol (2 and 14 min) are multiplied by 2.5 and those for 7-dehydrocholesterol are multiplied by 10. Standard color intensity units (L) were obtained. Calculation for total Δ^5 sterols and $\Delta^{5,7}$ sterols in mg per reaction tube are from equations 1 and 2.

$$1.\ \text{mg } \Delta^5 \text{ sterols} = \frac{R_2 \times L_1\Delta^{5,7} - R_1 \times L_2\Delta^{5,7}}{L_2^C \times L_1^{\Delta^{5,7}} - L_1^C \times L_2^{\Delta^{5,7}}}$$

$$2.\ \text{mg } \Delta^{5,7} \text{ sterols} = \frac{R_1 \times L_2^C - R_2 \times L_1^C}{L_2^C \times L_1^{\Delta^{5,7}} - L_1^C \times L_2^{\Delta^{5,7}}}$$

R_1 = absorbance of unknown during LB reaction after 2 min.
R_2 = absorbance of unknown during LB reaction after 14 min.
L_1^C = color intensity of 1 mg cholesterol after 2 min.
L_2^C = color intensity of 1 mg cholesterol after 14 min.
$L_1^{\Delta^{5,7}}$ = color intensity of 1 mg 7-dehydrocholesterol after 2 min.
$L_2^{\Delta^{5,7}}$ = color intensity of 1 mg 7-dehydrocholesterol after 14 min.

The basic techniques for isolation and GLC analysis of sterols were modeled after those of Miettinen et al. (23). The fourth aliquot (5-mL) of the heptane layer (Fig. 1) was reduced in volume under N_2 and the lipid applied to a 20 x 20 x 0.5 mm Silica Gel G TLC plate. Development

TABLE II

Retention Times of Standard Trimethylsilyl Ether Sterols Relative to Trimethylsilyl Ether 5α-Cholestane

Trimethylsilyl ether sterol	Relative retention time[a]	Free sterol empirical formula	Free sterol systematic nomenclature
5α-Cholestane	1.00		
C_{26}-Sterol	1.45	$C_{26}H_{42}O$	22-*trans*-24-norcholesta-5,22-dien-3β-ol
22-Dehydrocholesterol	2.06	$C_{27}H_{44}O$	22-*cis/trans*-cholesta-5,22,dien-3β-ol
Cholesterol	2.30	$C_{27}H_{46}O$	cholesta-5-en-3β-ol
Brassicasterol	2.50	$C_{28}H_{46}O$	24-methylcholesta-5,22-dien-3β-ol
Desmosterol	2.50	$C_{27}H_{44}O$	cholesta-5,24-dien-3β-ol
Lathosterol (Δ^7 Cholesterol)	2.55	$C_{27}H_{46}O$	5α-cholest-7-en-3β-ol
7-Dehydrocholesterol	2.55	$C_{27}H_{44}O$	cholesta-5,7-dien-3β-ol
24-Methylenecholesterol	3.05	$C_{28}H_{46}O$	24-methylenecholesta-5-en-3β-ol
Stigmasterol	3.28	$C_{29}H_{48}O$	24-ethyl-cholesta-5,22-dien-3β-ol
β-Sitosterol	3.80	$C_{29}H_{50}O$	24-ethyl-cholesta-5-en-3β-ol
Stigmastanol (fucosterol)	3.82	$C_{29}H_{52}O$	(24E)-24-ethylidene-cholesta-5-3n-3β-ol

[a]Column: 2 m × 2 mm id glass containing 3% SE-30 on 100/120 Gas Chrom Q operated at 230 C with 75 mL/mL carrier (He) gas. See Fig. 4.

TABLE III

Proximate Composition of Mollusks and Crustacea[a]

Sample:	Moisture	Protein	Fat (%)	Ash	CHO[b]
Mollusks					
Butter clams no. 1	78.4 ± 0.9	14.1 ± 0.6	1.5 ± 0.1	1.6 ± 0.1	4.4
Butter clams no. 2	–	–	1.7[c]	–	–
Cockles no. 2	85.7[d]	10.3[d]	0.9[c]	2.2[d]	0.9
Cockles no. 2	85.4	10.8	1.7	2.1	–
Geoducks	77.7 ± 3.4	13.7 ± 0.2	1.9 ± 0.4	1.3 ± 0.0	5.4
Horse clams	76.0 ± 0.9	16.4 ± 0.3	2.0 ± 0.1	1.4 ± 0.1	4.2
Little necks	78.5 ± 1.3	12.9 ± 0.6	1.8 ± 0.1	1.7 ± 0.1	5.1
Mussels	81.5 ± 0.7	12.1 ± 0.3	1.8 ± 0.5	1.8 ± 0.2	2.8
Oysters, fresh[e]					
9/19/79	80.1 ± 1.2	11.01±0.6	3.8 ± 0.5	1.3 ± 0.1	3.8
10/25/79	81.1 ± 0.7	9.6 ± 0.4	3.6 ± 0.6	1.4 ± 0.1	4.3
11/30/79	82.9 ± 0.6	8.6 ± 0.5	3.0 ± 0.1	1.3 ± 0.1	4.2
12/28/79	80.6 ± 0.8	9.7 ± 0.6	3.7 ± 0.5	1.3 ± 0.1	5.2
1/29/79	82.7 ± 0.8	8.3 ± 0.5	3.1 ± 0.2	1.1 ± 0.1	4.8
3/1/79	83.5 ± 0.4	7.4 ± 0.2	2.5 ± 0.1	1.3 ± 0.4	5.3
3/29/79	81.4 ± 0.6	8.6 ± 0.2	3.1 ± 0.3	1.1 ± 0.0	5.8
5/3/79	79.0 ± 0.7	9.0 ± 0.1	3.6 ± 0.8	1.3 ± 0.0	7.1
5/30/79	78.4 ± 0.4	10.8 ± 0.1	3.9[c]	1.3 ± 0.17	5.6
Oysters, canned	78.5 ± 1.0	9.1 ± 0.8	4.7 ± 0.6	1.9 ± 0.1	5.8
Razor clams	81.1[d]	14.3[d]	1.1[c]	1.4[d]	2.1
Crustacea					
Dungeness crab, leg	77.6[d]	19.3[d]	1.0[c]	1.6[d]	0.5
Dungeness crab, body	80.0[d]	17.7[d]	1.0[c]	1.4[d]	–
Dungeness crab, intestine	87.4[d]	8.6[d]	1.1[c]	2.5[d]	0.4
Shrimp:					
fresh cooked	78.8[d]	19.7[d]	1.6[c]	1.0[d]	–
IQF	79.7[d]	17.7[d]	1.5[c]	1.0[d]	–
canned	–	–	1.7[c]	–	–

[a]Mean ± SD of three samples analyzed in duplicate, except fat not measured in duplicate.

[b]Carbohydrate (CHO) by difference.

[c]One observation.

[d]Mean duplicate measurement.

[e]Three composites of 12 oysters analyzed each period; each composite consisted of three weight groups: 20.4 ± 2.7g; 31.8 ± 1.9g; 51.4 ± 4.9g for 9 month period. See Table IV.

was in hexane/ethyl ether/glacial acetic acid (90/30/1, v/v/v). Visualization of the sterol band (Rf = 0.3) was with a brief exposure to iodine vapor. Cholesterol was used as a standard. The sterol band was removed from the plate and the lipid washed from the adsorbent with chloroform/methanol (2/1, v/v). Recovery of Δ^5 sterols from TLC plates was 96 ± 3% as determined colorimetrically; no correction was made for this estimated loss. To the sterols in chloroform/methanol was added 250 μg 5-α-cholestane in 1 mL heptane. All solvents were removed under N_2 and 0.3 mL dry silylating reagent was added and the reaction allowed to stand at RT for 30 min. The silylating reagent consisted of dimethylformamide/hexamethyldisilazane/trimethylchlorosilane (40/40/1), v/v/v) dried over silica gel. Sterol silyl ether derivatives were separated on a 2 m × 2 mm (id) glass column containing 3% SE-30 on 100/120 Gas Chrom Q. A Hewlett-

TABLE IV

Protein, Lipid, Total Sterol and Cholesterol Cholesterol Versus Size in Pacific Coast Oyster[a]

Size (g)	Protein (%)	Lipid (%)	Total sterols (mg/100g)	Cholesterol (mg/100g)
20.4 ± 2.7[b]	8.9 ± 1.2	3.1 ± 0.5	163.8 ± 16.2	49.8 ± 8.7
31.8 ± 1.9[c]	9.3 ± 1.3	3.2 ± 0.5	174.1 ± 25.8	58.2 ± 12.2
51.4 ± 4.9[d]	9.0 ± 1.9	3.5 ± 0.8	190.4 ± 12.3	57.1 ± 4.4
$p<0.01$	NS	NS	NS	NS

[a]Mean ± SD of 18 composite samples, 12 oysters per composite, for the 6 months Sept. 1978-Feb. 1979.
[b,c,d]Values followed by a different superscript are significantly different.

Packard Model 7610A gas liquid chromatograph was used with the following operating parameters: column temp 230 C (isothermal); injection temp 300 C; detection temp 260 C. The relative retention times of pure sterol silyl ether standards compared to 5-α cholestane are presented in Table II. Pure sterol standards were obtained from various commercial sources and from Dr. William Connor, University of Oregon Health Sciences Center, Portland, OR.

Nonesterified sterols were measured from an aliquot of the original Folch extract (Fig. 1). The solvent was removed and the residue redissolved in 2 mL acetone-aboslute ethanol (1/1, v/v). Colorimetric determination of the nonesterified sterols precipitated with digitonin was as previously described.

RESULTS AND DISCUSSION

The proximate composition of mollusks and crustacea are reported in Table III. All mollusks were high in moisture content compared to other marine products (13,24). Oysters appear to be slightly lower in protein and higher in fat than other mollusks examined. Protein and lipid content of oysters are not influenced by size (Table IV). Over 9 consecutive months (Sept. 1978-May 1979), a trend of lower lipid levels during the first three months of the year was observed (Table III).

It was observed that the unsaponifiable extract of mollusks produced a very fast-acting chromogen upon addition of LB reagent. In addition, the color of this quick reaction was a blue-red vs the characteristic green obtained with cholesterol. Other investigations have indicated that mollusks contain $\Delta^{5,7}$ sterols (4), but this class of component(s) has not been quantitated in previous studies. A series of three publications by Bauman and Associates (21,22,25) indicated that Δ^{7} and $\Delta^{5,7}$ sterols were the cause of the early color reaction with LB reagents.

The contribution of the $\Delta^{5,7}$ sterols to the LB absorbance curve appears a problem only with mollusks and not with other seafoods such as crustacea and finfish (Fig. 2). Finfish, rockfish (*Sebastodes pinniger*) and ling cod (*Ophiodon elongatus*) unsaponifiable lipids were extracted and assayed in the same manner as for oysters and shrimp. Absorbance curves in Figure 2 were not accomplished at identical sterol concentrations. The difference in absorbance characteristics of three pure sterols found in mollusks is shown in Figure 3. While the molar extinction coefficient (E) for cholesterol is very low after 2 min, the E for 7-dehydrocholesterol is maximum. Absorbance of these two compounds after 2 and 14 min was used as the basis for calculating $\Delta^{5,7}$ sterols and will be discussed in relationship to all methods used to measure mollusk sterols. Absorption curves in Figures 2 and 3 are not intended to represent equivalent sterol concentrations.

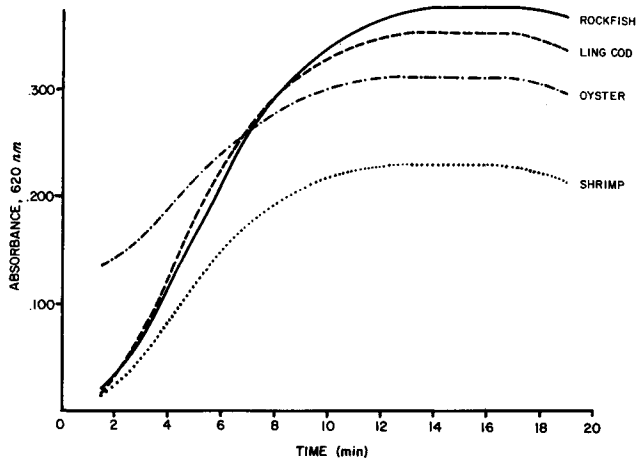

FIG. 2. Change in absorbance (620 mm) vs time during the Liebermann-Burchard reaction on sterol fractions isolated from finfish, oysters and shrimp.

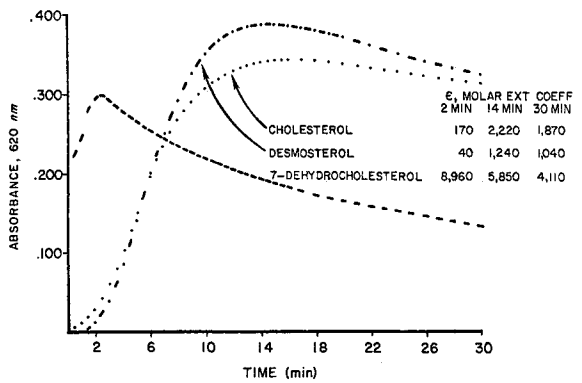

FIG. 3. Change in absorbance (620 mm) vs time during the Liebermann-Burchard reaction on three standard sterols found in mollusks.

Levels of total sterols determined in mollusks and crustacea by four procedures are presented in Table V. Each method results in significantly different results. Oysters have the highest average lipid content and they also contain the highest total sterols from among all mollusks examined. Lower sterol levels are found in other mollusks with correspondingly lower lipid content. The difference in sterol levels in two Butter clam samples could be attributed to seasonal variations or origin of samples. Size is not a major factor in varying sterol levels per unit mass as evidenced by the two Cockle samples analyzed and examination of oysters (Table IV).

Problems associated with the colorimetric LB reaction for the quantitation of mollusk sterols have been discussed by Kritschevsky and DeHoff (3). Statistical evaluation of results obtained on oyster samples assayed by all four methods (17 samples) are shown in Table VI. Using the direct LB reaction on the total unsaponifiable fraction, the highest mean sterol content of 198.0 mg/100 g was found. Precipitating an identical unsaponified lipid extract with digitonin and then employing the LB reaction gave a mean value of 146.8 mg/100 g. The difference in these two values is primarily attributed to the presence of the $\Delta^{5,7}$ sterols and the high E of the samples assayed without digitonin precipitation. Since cholesterol was used as the standard, the value of 191.8 mg/100g is excessively high. Precipitation of $\Delta^{5,7}$ sterols with digitonin was not accomplished as

TABLE V

Total Sterols in Mollusks and Crustacea as Determined by Different Analytical Methods

	Liebermann Burchard reaction		Modified Liebermann Burchard reaction			GLC analysis	
	total sterols						
	w/o digitonin (mg/100g)	w/digitonin (mg/100g)	Δ^5 sterols (mg/100g)	$\Delta^{5,7}$ sterols (mg/100g)	Sum $\Delta^5+\Delta^{5,7}$ (mg/100g)	GLC sterols (mg/100g)	Sum GLC + $\Delta^{5,7}$ (mg/100g)
Mollusks							
Butter clams no. 1	99.4 ± 15.5	102.1 ± 9.2	74.3 ± 13.0	7.8 ± 1.2	82.2 ± 14.2	87.1	93.6
Butter clams no. 2	146.0	111.3	101.8	16.5	118.3	115.4	131.9
Cockles no. 1	66.2	71.7	56.1	5.6	61.7	59.8	65.4
Cockles no. 2	63.2	67.9	46.7	5.7	52.4	64.4	70.1
Geoducks	101.4 ± 18.0	91.9 ± 24.5	60.7 ± 8.1	13.9 ± 4.4	74.5 ± 11.9	70.8	84.1
Horse clams	121.3 ± 1.9	114.2 ± 5.7	90.7 ± 3.3	7.6 ± 0.8	98.4 ± 2.5	92.8	99.6
Little necks	108.5 ± 7.1	104.8 ± 4.6	78.6 ± 5.9	8.7 ± 1.0	87.3 ± 6.0	80.2	87.8
Mussels	127.9 ± 8.3	83.8 ± 9.9	76.4 ± 8.9	25.9 ± 2.2	101.4 ± 6.9	57.6	84.6
Oysters, fresh	188.9 ± 23.9 (25)[a]	148.7 ± 19.8 (22)	132.7 ± 22.4 (25)	21.4 ± 7.4 (25)	154.5 ± 25.5 (25)	148.0 ± 16.2 (20)[b]	169.8 ± 20.5 (20)[b]
Oysters, canned	170.8 ± 20.1	149.5 ± 19.1	141.8 ± 15.5	14.6 ± 3.8	156.4 ± 17.5	153.8 ± 24.3	168.3 ± 25.8
Razor clams	70.3	71.0	68.0	2.5	70.5	65.9	68.4
Crustacea							
Dungeness crab, leg	55.6	58.8	47.8	0	47.8	46.5	46.5
Dungeness crab, body	61.1	65.5	67.9	0	67.9	55.1	55.1
Dungeness crab, intestine	58.2	60.8	59.8	0	59.8	61.7	61.7
Shrimp:							
fresh cooked	177.8	–	121	0	121	161.4	161.4
IQF	158.1	–	113	0	113	129.2	129.2
canned	156.8	–	120	0	120	142.6	142.6

[a]Number of observations in parenthesis; not all uniformly paired observations.

[b]$\Delta^{5,7}$ equals 21.8 ± 8.2 for 20 samples analyzed by GLC.

TABLE VI

Statistical Comparison of Methods used to Determine Total Sterols in Oysters

Method	Mean[a,b] (mg sterol/100g)
Liebermann Burchard (LB) reaction without digitonin ppt	198.0[c]
GLC analysis + $\Delta^{5,7}$ sterols by modificified LB reaction	173.5[d]
Modified LB Δ^5 reaction; sum of Δ^5 and $\Delta^{5,7}$ sterols	158.3[e]
Liebermann Burchard reaction after digitonin ppt	146.8[f]

[a]Mean value of 17 observations with pooled SD ± 16.4.

[b]Common determinations made on samples from September, 1978 (3); October (3); November (3); December (1); January, 1979 (1); February (2); March (3); May (1).

[c,d,e,f]Values not followed by the same superscript are significantly different ($P<0.01$).

evidenced by the complete lack of the fast-acting chromogen(s) upon addition of the LB reagent. It has previously been reported that $\Delta^{5,7}$ sterols ([26] - Matsumoto, 1955, cited in ref. 4) will not complex with this saponin. An alternative explanation is that the $\Delta^{5,7}$ sterols are precipitated by digitonin, but they are somewhat soluble and lost in the washing steps. Although the amount of sterol determined after digitonin precipitation is without interference of the $\Delta^{5,7}$ sterols, this value is low. The error (low value) now results from the presence of desmosterol (and brassicasterol) which has a lower E (Fig. 3), compared to cholesterol. Both brassicasterol and desmosterol appear similar in this property (3). Oysters are believed to contain more brassicasterol than desmosterol, but this may not be true for other mollusks.

Using the modified LB reaction, a total sterol level in oysters (17 samples) of 158.3 mg/100 g was found (Table VI). More important than the total value obtained by this modification, was the ability to quantitate the $\Delta^{5,7}$ sterols. However, $\Delta^{5,7}$ values are only offered as a best approximation. Because of the various color reactions occurring in the LB reaction with mollusk sterols, any of the three methods with modifications employed in this study give significantly different results. The mean value of 158.3 mg/100g in 17 oyster samples is the sum of 135.2 mg Δ^5 sterols and 23.1 mg $\Delta^{5,7}$ sterols as determined by the modified LB reaction.

While the colorimetric LB reaction with or without modification may offer a quick method of estimating total sterol in mollusks, the method of choice for both qualitative and quantitative measurements is GLC. However, sample preparation prior to GLC analysis also results in disappearance of $\Delta^{5,7}$ sterols. This loss is attributed to the TLC isolation step which appears to cause complete destruction. These compounds are prone to air oxidation (4). Quantitation of Δ^5 sterols by GLC in 17 oyster samples average 150.6 mg/100g (Table VI). This amount, plus the $\Delta^{5,7}$ sterol(s) determined by the modified LB reaction gave the best estimate of total sterols in oysters, 173.5 mg/100g (Table VI). Concurrently, this combination of methods gives the best estimate of total sterols in all mollusk samples analyzed (Table V). In comparing total sterol levels obtained by the different methods, the differences are not as great for mollusks other than oysters, and in some cases agree very well (i.e., razor clams).

The variation in results produced by different analytical methods with oysters cannot be evaluated as extensively with crustacea because of the smaller number of samples. With crustacea containing mainly cholesterol, less problem was envisioned than with mollusks. The means for total sterols in 3 samples of Dungeness crab, leg, body and intestine (Table V), by 4 methods, were not significantly different ($P<0.05$). The reason for this variation is not apparent.

The GLC analysis of mollusk sterols examined in this study routinely produced a chromatogram typical of the one presented for oyster silyl ether sterols (Fig. 4). Based on silyl ether sterol standards (Table II) and previous reports in the literature, mollusks of the Pacific Northwest contain at least 8 primary sterols. Some problems still re-

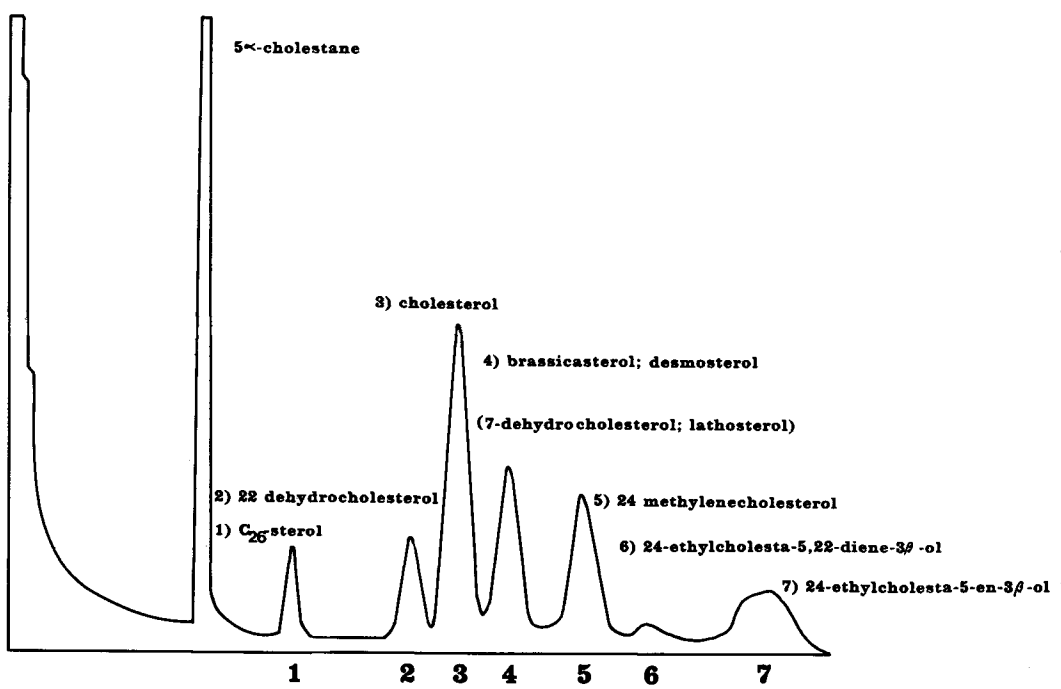

FIG. 4. Gas liquid chromatographic separation of oyster (*Crassostrea gigas*) sterols – trimethyl silyl ether derivatives.

TABLE VII

Mean Sterol Levels in Oysters(*Crassostrea gigas*) over a Nine-Month Period (Sept. 1978-May 1979)

Sterol	Mean ± SD[a,b] (mg/100g)	Range (mg/100g)
C_{26}-Sterol	15.9 ± 4.2	9.4 - 28.6
22-Dehydrocholesterol	14.5 ± 1.2	11.2 - 17.1
Cholesterol	50.7 ± 9.5	36.4 - 69.2
Brassicasterol; Desmosterol	26.7 ± 4.0	20.4 - 32.6
$\Delta^{5,7}$ sterol(s) – (7-Dehydrocholesterol)	21.8 ± 8.2[c]	11.1 - 41.8[c]
24-Methylenecholesterol	26.3 ± 4.3	18.9 - 33.8
24-ethylcholestra-5,22-diene-3β-ol	0.7 ± 1.2	0.1 - 3.5
24-ethylcholestra-5-en-3β-ol	15.5 ± 4.2	5.5 - 21.3
Totals	169.8 ± 20.5	133.5 - 202.4

[a]20 composite samples, 12 oysters per composite.
[b]All values determined by GLC; for exception see footnote c.
[c]Quantitation by modified Liebermann-Burchard reaction.

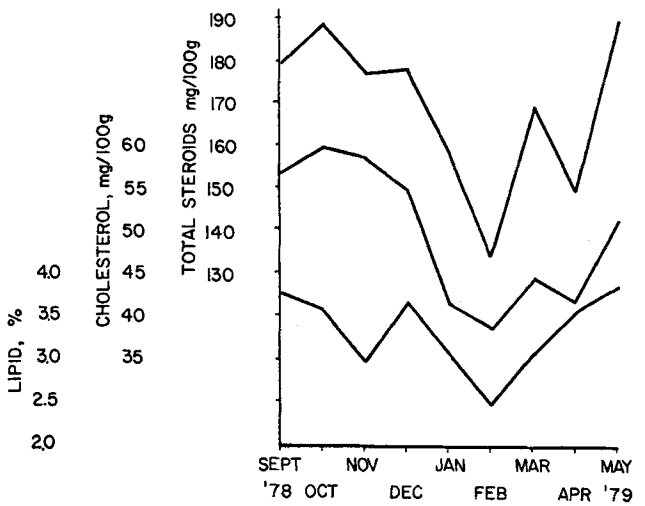

FIG. 5. Seasonal variation of total lipid, cholesterol and total sterols in oysters (*Crassostrea gigas*).

main in accurately quantitating and separating those sterols which elute after cholesterol (Fig. 4) and have an RRT greater than 2.3 (Table II). The previous mention to the loss of $\Delta^{5,7}$ sterols prior to GLC analysis may be advantageous. Pure brassicasterol and demosterol, as their silyl derivatives, have RRT of 2.5 under the conditioning employed in this study (Table II). Derivatized 7-dehydrocholesterol has an RRT of 2.55. A mixture of all three compounds produces a single peak with an RRT of 2.50. Having no $\Delta^{5,7}$ sterols in samples prepared for GLC analysis reduces the complexity of peak 4 (Fig. 4). The resulting peak 4 is a mixture of only two compounds, brassicasterol and desmosterol. Both these compounds have been reported in mussels (*Mytilus edulis*) by Idler and Wiseman (5) with a ratio of desmasterol to brassicosterol of 1:1.2. In the same report, these authors reported that 2.81% of the total sterol content of Eastern oysters (*Crassostrea virginica*) was brassicasterol and none was desmasterol. The presence of desmasterol in any mollusk or anthropod is not unrealistic. This compound is an intermediate in the biosynthesis of cholesterol as is lathosterol, another compound of questioned quantitative importance in mollusks. However, as intermediates these levels are not high. While oysters may not contain much desmasterol, the level of this compound in other mollusks examined in this study is left unresolved.

Only two major peaks are prevalent after 24-methylenecholesterol (Fig. 4). These peaks have been assigned the identity of 24-ethylcholesta-5, 22-diene-3β-ol and 24-ethylcholesta-5-en-3β-ol and should have RRT times nearly identical to the standards stigmasterol and β-sitosterol, respectively. Teshima et al. (27) have shown the two compounds 24-ethylcholesta-5, 22-diene-3β-ol (porifersterol) and 24-ethylcholesta-5-en-3β-ol (clinosterol) having a 24β-configuration, are present in *Crassostrea virginica*. It can only be assumed, these two sterols exist in Pacific Coast mollusks. Actual sterochemistry of sterol was not accomplished in this study. The possible presence of (24E)-24-ethylidene-5-en-3β-ol peak, is considered very likely, but was not confirmed. Another sterol possibly present in oysters is (24Z)-24-ethylidenecholesta-5-en-3β-ol (isofucosterol) which has recently been shown to be synthesized in this mollusk (28). This compound has a similar RRT with 24-ethylcholesta-5-3β-ol. Peak 7, as shown in Figure 4, was not uniformly symmetrical in all chromatograms. The mean level and range of individual sterols found in oysters over a nine month period are reported in Table VII. Cholesterol is the predominate sterol at 51 mg/100 g. Brassicasterol-desmosterol, 7-dehydrocholesterol and 24-methylenecholesterol are each present at about one-half this level. The colorimetric examination of $\Delta^{5,7}$ is not without problems. Early consideration was given to the presence of lathosterol in mollusks examined since it is an intermediate in cholesterol biosynthesis (29). However, Kind and Meigs (30) reported that lathosterol is unique and only found in the class *Amphineura* (chitans) of the phylun *Mollusca*. This reduced the complexity to considering only $\Delta^{5,7}$ sterols exist in oysters and other mollusks. Bivalves and particularly oysters are a source of provitamin D, and for that reason 7-dehydrocholesterol was selected as a standard and considered the sole $\Delta^{5,7}$ sterol present. This may be an incorrect assumption based on a recent report (27) which found seven $\Delta^{5,7}$ sterols in Eastern oysters (*Crassostrea virginica*) but whose identities could not be established. Ergosterol is one $\Delta^{5,7}$ sterol that potentially could be present. In this same report (27), a total of 39 sterols were found in the Eastern oysters. Thirteen were at levels of 1% (total sterol) or higher and three could not be identified, but were $\Delta^{5,7}$ sterols. If fucosterol and isofucosterol could have been separated and quantitated in this study, the major sterol composition between Eastern Coast and Pacific Coast oysters would likely be very similar. Eastern Coast oysters contained 1.5 and 4.6 percent of their total sterols as fucosterol and isofucosterol, respectively (27). The sterol composition of Eastern oysters obtained

TABLE VIII

Quantitative Distribution of Individual Sterols in Mollusks and Crustacea by GLC (Δ^5) and the Modified Liebermann-Burchard Reaction ($\Delta^{5,7}$)

Sample (mg/100g)	C_{26} Sterol (mg/100g)	22-dehydro-cholesterol (mg/100g)	cholesterol (mg/100g)	brassi-casterol[a] (mg/100g)	$\Delta^{5,7}$ sterols (mg/100g)	24-methylene-cholesterol (mg/100g)	24-ethylcholesta-5,22-diene-3β-ol (mg/100g)	24-ethylcholesta 5-en-3β-ol (mg/100g)	Sum (mg/100g)
Mollusks									
Butter clams no. 1	2.2	7.1	31.9	10.6	6.5	31.3	0.1	4.0	93.7
Butter clams no. 2	5.4	13.3	47.9	11.6	16.5	29.2	tr[b]	8.0	131.9
Cockles no. 1	3.7	8.8	19.9	7.6	5.6	18.8	tr	0.9	65.3
Cockles no. 2	3.6	8.2	18.1	8.8	5.7	20.0	–	5.7	70.1
Geoducks	2.3	4.9	32.1	6.4	13.3	15.8	2.4	6.9	84.1
Horse clams	2.8	7.0	42.4	9.5	6.8	27.4	0.1	3.7	99.7
Little necks	2.9	7.0	35.7	7.2	7.6	21.8	0.8	4.7	87.7
Mussels	3.1	7.2	25.8	8.9	27.0	10.4	tr	2.4	84.8
Oysters, canned	10.2 ± 1.4	17.01 ± 1.7	49.1 ± 7.8	29.7 ± 5.6	14.6 ± 3.8	30.9 ± 5.3	tr	16.9 ± 6.4	168.4
Razor clams	3.6	8.0	19.8	7.9	2.5	18.0	tr	8.7	68.5
Crustacea									
Dungeness crab, leg	0.1	0.4	45.9	–	–	0.1	–	–	46.5
Dungeness crab, body	0.2	0.5	54.2	–	–	0.2	–	tr	55.1
Dungeness crab, intestine	0.9	2.1	55.9	–	–	2.5	–	0.3	61.7
Shrimp									
fresh cooked	0.1	0.2	128.9	–	–	0.1	tr	0.1	129.4
IQF	–	0.1	142.4	–	–	0.1	tr	0.1	142.7
canned	0.3	0.2	160.5	–	–	0.5	tr	tr	161.6

[a]May include desmosterol.

[b]Trace amount.

TABLE IX

Percent Nonesterified Δ^5 Sterols in Mollusks and Dungeness Crab

	Percent nonesterified steroids[a]	
Mollusks		
Butter clams no. 1	89.0 ± 12.1	(3)[b]
Butter clams no. 2	74.0	(1)
Cockles no. 1	75.6	(1)
Cockles no. 2	81.2	(1)
Geoducks	52.6 ± 6.2	(3)
Horse clams	67.8 ± 3.7	(3)
Little necks	79.3 ± 7.7	(3)
Mussels	77.3 ± 6.2	(3)
Oysters, canned	49.6 ± 5.2	(17)
Razor clams	100+	(1)
Crustacea		
Dungeness crab, leg	95.3	(1)
Dungeness crab, body	100+	(1)
Dungeness crab, intestine	99.2	(1)

[a]Sterols precipitated with digitonin without saponification/sterols precipitated with digitonin after saponification X 100.
[b]Mean ± SD, number of samples in parenthesis.

from various geographical sources are very uniform (31). The extent to which de novo synthesis and dietary sterols meet the requirements for oysters or other mollusks is known.

A seasonal variation in total lipids, total sterols and cholesterol was evident for oysters examined over the 9-month period. The lowest level for all three categories was found in samples taken in February (Fig. 5). Idler, et al., have reported lowest lipid levels in August for scallops (32) and July for oysters (33). Even though oysters were not examined over a complete 12-month cycle in this study, a geographical difference may account for differences in reports.

While season and geographical source of samples may influence total sterol in mollusks, preliminary indications are that the ratio of individual sterols in oysters (34) may not change dramatically, and this may also be true for other mollusks. Teshima and Patterson (35) found that the proportion of cholesterol increased, whereas that of brassicasterol (24-methylcholesta-diene-3β-ol) and 24methylenecholesterol decreased during starvation.

The major difference between oysters and all other mollusks examined is the comparatively smaller amount of total sterols in the latter (Table VIII). However, qualitatively and, again, proportionally, they appear similar. The sterol components of a canned oyster from Korea, species unknown, is very similar to that of fresh oysters (*Crassostrea gigas*) obtained from Willipa Bay, Washington, A brief report on sterols in British shellfish (36) gives values that appear significantly higher than results reported here.

The striking difference between mollusks and crustacea is that the latter contain predominantly cholesterol, > 97% (Table VIII). Dungeness crab and shrimp contained no fast-acting sterols (Fig. 2) upon addition of LB reagent, indicating a lack of $\Delta^{5,7}$ sterols. The metabolism and ultimate distribution of sterols in 8 crustacea common to the waters of Japan has been examined (37). Cholesterol was the main sterol in all 8 species ranging from 78 to 100%. Other sterols detected, but not in every specie, included 22-dehydrocholesterol, brassicasterol, desmosterol and 24-methylenecholesterol. Quantitative vlaues were not reported. Gagosian (38) found shrimp (*Pandalus boreolis*) to have 130 ± 5 mg sterol/100 g wet wt. Cholesterol accounted for 94.3% and desmosterol for 4.2% of the total. Idler and Wiseman (6) also found that cholesterol is the principal sterol in shrimp (*Pandalus borealis*) and desmosterol was second when present. Only cholesterol has previously been reported in Dungeness crab (39).

Questions still remain as to the source and importance of all sterols in mollusks and crustacea. Since sterol esters may be in metabolic transit (40) while free sterols are permanently incorporated in tissue, the distribution of free and esterified sterols was examined. In man and most animals, approximately 70% of serum cholesterol is esterified. Other tissues in man have various levels of esterified cholesterol ranging from 7 to 83% (40). In beef and pork muscle only 6% of its cholesterol is esterified (40). The percent nonesterified sterols in mollusks, the percent nonesterified sterols range from 49.6 to 100% (see Table IX). Only free cholesterol is present in Dungeness crab. In fresh oysters, 60% of the sterols were nonesterified. In the Eastern oyster, 85% of the sterols were without an ester linkage (27). Another major difference between Eastern Coast vs Pacific Coast oysters was the higher lipid content of the latter, 0.98% vs 3.2%, respectively.

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Seed Viability and Aflatoxin Production in Individual Cottonseed Naturally Contaminated with *Aspergillus flavus*

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ABSTRACT

Individual cottonseed naturally contaminated with *Aspergillus flavus* Link were tested for viability and assayed for aflatoxin B_1. Of the 55 infected seeds tested, less than half (21) germinated normally, a rate much lower than expected for uninfected cottonseed. Aflatoxin levels in the seeds varied from below 300 ng/g to over 100,000 ng/g of seed. Statistical analyses indicate that the presence of aflatoxin is correlated with poor germination in cottonseed naturally contaminated with *A. flavus*.

Aflatoxin contamination of cottonseed is associated with field invasion by *Aspergillus flavus* Link (1). Lee and Russell found that seeds containing aflatoxin were randomly distributed throughout infected locks with generally no more than 2 or 3 of the 7-10 seeds per lock affected (2). It has recently been shown that maize seeds naturally contaminated with aflatoxin germinate poorly (3). The present study was undertaken to determine whether seed viability could be correlated with *A. flavus* infection and to determine whether toxin production could influence seed viability.

Seeds from locations in seven states including Alabama, Arizona, Arkansas, New Mexico, South Carolina, Tennessee and Texas were thoroughly delinted in sulfuric acid and surface sterilized in a solution of 2% sodium hypochlorite and 0.001% Triton X 100 for 2 min, rinsed three times with sterile deionized water and placed on potato dextrose agar plates. The seeds were incubated for seven days at room temperature, and only those found to be naturally contaminated with *A. flavus* were selected for use in this study. Seed viability was determined by the presence of a normal hypocotyl. Visible external fungal growth was removed from the seed and the individual seeds were analyzed for aflatoxin (4). A small plug of agar (approximately 1 cm^2) adjacent to the seed was also analyzed for aflatoxin by the same method. The Chi Square test was used to determine the significance of the differences observed.

Of the 55 seeds examined, 21 germinated normally. This is a germination rate of only 38%, much lower than the 90% rate expected for uninfected cottonseed under the same conditions (5). Eight of the 21 (38%) viable seeds contained aflatoxin, whereas 24 of the 34 (71%) nonviable seeds contained aflatoxin. Significant differences (P=0.05) in viability exist between seeds infected with toxigenic strains and those infected with nontoxigenic strains. Aflatoxigenic isolates of *A. flavus* were more likely to be found in nonviable than viable seed. The cause-effect relationship of this correlation remains to be established.

All isolates that produced aflatoxin in the agar also produced it in the seed. If the infecting fungus was toxigenic, viability of the infected seed did not prohibit aflatoxin production. However, only five of the seeds contained aflatoxin levels above 100,000 ng/g of seed. All of these were from nonviable seeds. All other aflatoxin positive seeds contained levels below 300 ng/g. The low levels of aflatoxin found in these seed as compared with levels detected in individual cottonseed in other studies (2,4), might be explained by differences in the extent of the fungal invasion into the seed. Studies examining this possibility are currently underway.

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