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Part 9 is Kennard et al. (1983).

Methoxychlor Metabolism in Chickens

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[^{14}C]Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] was given orally to intact, colostomized, and bile-fistulated chickens. Dosages were 0.2, 10, and 100 mg of methoxychlor/day for periods of 1 and 14 days. Recovery of ^{14}C was greater in carcasses of hens compared to roosters for comparable dosages and time. ^{14}C in eggs varied with dose and time and was predominantly in yolk. Methoxychlor and 26 metabolites were either identified or characterized: 22 metabolites in feces, 14 in urine, and 6 in bile. Metabolism included demethylation, ring hydroxylation, conjugation with glucuronic acid, dechlorination, dehydrochlorination, and formation of substituted benzophenones.

Methoxychlor [1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane] (1, Table I) is an insecticide used to control flies on livestock and in farm buildings. It has other insecticidal uses, but, for this paper, we are concerned with its direct contact by farm animals.

The metabolism or distribution of methoxychlor or [^{14}C]methoxychlor has been studied in mice, rats, and goats. Kapoor et al. (1970) reported five metabolites of [^{14}C]methoxychlor in urine and feces of mice. Woodward et al. (1948) observed no methoxychlor or bis(4-methoxyphenyl) acetic acid in urine of rats fed methoxychlor, and Weikel (1957) observed that ^{14}C was eliminated predominantly in feces of rats given [^{14}C]methoxychlor intravenously. We (Davison et al., 1982) identified 17 metabolites of [^{14}C]methoxychlor in urine and feces of goats and observed that an increase in size of the dose shifted the predominant route for elimination of ^{14}C-metabolites from urine to feces.

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We now report the metabolism of [^{14}C]methoxychlor by chickens.

MATERIALS AND METHODS

Chickens. One-year-old white leghorn hens and 4-5-month-old white leghorn roosters were used. Methoxychlor was given once daily in gelatin capsules at 0.2, 10, or 100 mg/chicken for 1 or 14 consecutive days (see Table II). Eight tenths to 6.9 μCi of [^{14}C]methoxychlor was given in each capsule, and three to six chickens were used in each treatment group. Feed and water were available ad libitum.

Urine and feces were collected from colostomized roosters, and bile was collected from bile-fistulated roosters. All chickens were killed 24 h after the last dose of [^{14}C]methoxychlor. The chickens were plucked, the feathers were discarded, various tissues were sampled, and then the remainder of the carcasses were ground and sampled. All samples except bile were lyophilized and stored in glass jars. Bile was stored either frozen or at 3 °C.

Methoxychlor and [^{14}C]Methoxychlor. Purity of

Table I. Metabolites Identified in Bile, Feces, and Urine from Chickens Given [^{14}C]Methoxychlor[a]

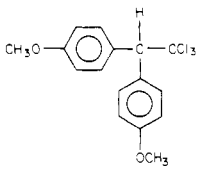
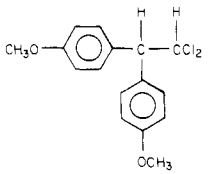
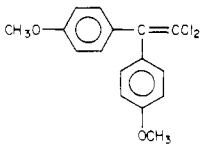
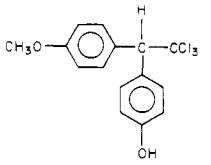
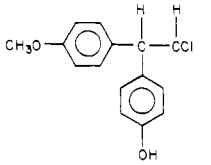
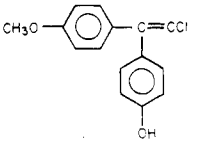
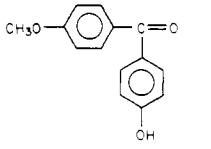
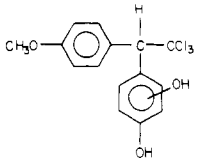
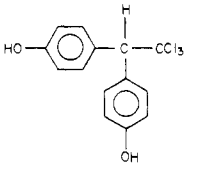
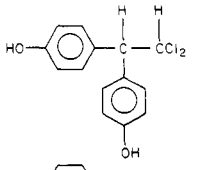
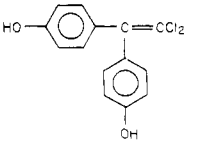
metabolite no.	metabolite structure	goat[b]	chicken	comments[c]
1		F, B	F	methoxychlor; hexane, ethyl acetate, methanol extracts; MS
2		F, B	F	hexane, ethyl acetate, methanol extracts; synthesized, MS
3		F, B	F	hexane, ethyl acetate, methanol extracts; synthesized, MS
4		F, B	F, U, B	hexane, ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative
5		F, B	F, U	hexane, ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative
6		F, U, B	F, U	hexane, ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative
7			F	ethyl acetate, methanol, water extracts; MS interpretation
8			F, U	methanol, water extracts; MS interpretation
9		U	F, U	ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative
10		F, U, B	F	ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative
11		F, U, B	F, U	ethyl acetate, methanol, water extracts; synthesized, MS on Me_3Si derivative

Table I (Continued)

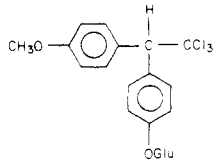
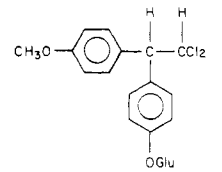
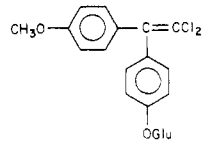
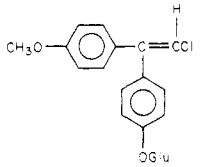
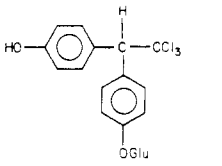
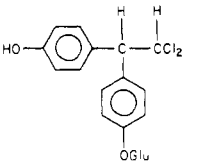
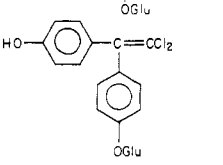
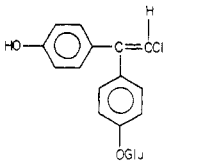
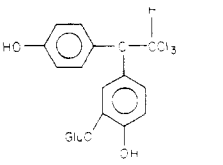
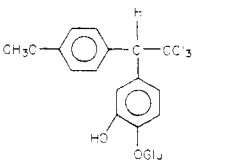
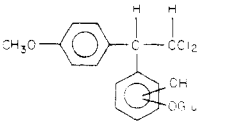
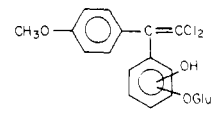
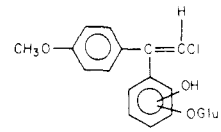
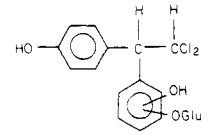
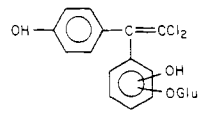
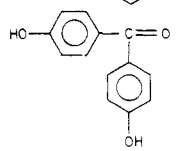
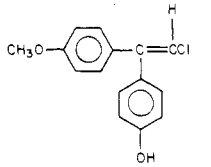
metabolite				
no.	structure	goat[b]	chicken	comments[c]
12			F, U	methanol, water extracts; MS interpretation
13		U	F, U, B	methanol, water extracts; MS interpretation
14			F, U, B	methanol, water extracts; comprised at least 37.6% of ^{14}C in bile at 100-mg dose; MS interpretation
15		U	F	methanol, water extracts; MS interpretation
16		U	B	MS interpretation
17		U	U, B	MS interpretation
18		U	U, B	MS interpretation
19		U	U	MS interpretation
20	 		F, U	water extract; MS interpretation, two isomers present, separated by 1.3 min on GC–MS; see text for NMR discussion
21		U	F	water extract; MS interpretation; two isomers present, separated by 1.5 min on GC–MS; isomers probably the same as for metabolite 20

Table I (Continued)

metabolite no.	structure	goat[b]	chicken	comments[c]
22			F, U	water extract; MS interpretation; isolated as both the per-Me_3Si and methyl ester-Me_3Si derivatives
23			F	water extract; MS interpretation; published spectrum is a mixture of the per-Me_3Si and methyl ester-Me_3Si derivatives
24			F	water extract; MS interpretation; published spectrum is a mixture of the per-Me_3Si and methyl ester-Me_3Si derivatives; two isomers were present, separated by 2.5 min on GC-MS
25			F	water extract; MS interpretation; published spectrum is a mixture of the per-Me_3Si and methyl ester-Me_3Si derivatives; two isomers present, separated by 2.6 min on GC-MS
26			F	methanol extract; synthesized, MS on Me_3Si derivative
27		F	F	ethyl acetate extract; synthesized, MS on Me_3Si derivative

[a] Abbreviations used are as follows: F, feces; U, Urine; B, bile; MS, mass spectrum; Me_3Si, trimethylsilyl. [b] Metabolites from goats were reported by Davison et al. (1982, 1983). [c] Comments pertain only to results obtained with chickens. Spectral comparisons were made with authentic samples; syntheses reported in this paper or in Davison et al. (1982).

methoxychlor was >99%, and the radiochemical purity of [*ring*-UL-^{14}C]methoxychlor, sp act. 9.03 mCi/mM, was >98% (Davison et al., 1982). 1,1-Dichloro-2,2-bis(4-methoxyphenyl)ethane (**2**, Table I) was identified as the impurity in the [^{14}C]methoxychlor.

Methoxychlor was weighed into gelatin capsules. [^{14}C]Methoxychlor dissolved in 0.2 mL of ethanol was added to the capsules, then 1 mL of corn oil was added, and the capsules were sealed for dosing.

Carbon-14 Analysis. Lyophilized materials and bile were combusted in a Packard Model 306 oxidizer and assayed as described by Davison et al. (1982). Triplicate analyses were done on samples collected from individual chickens.

Apparatus. Radioactive monitors, gas–liquid and high-pressure liquid chromatographic equipment, and the mass spectrometer used in these studies have been described (Davison et al., 1982). Capillary GC was done on a Hewlett-Packard 5790 gas chromatograph with a 12 m, 0.2 mm i.d., 0.33 μm film thickness cross-linked methyl silicone column.

Extraction of Fecal Metabolites. Lyophilized feces (20 g) were pooled according to the dose given to the chickens, then slurried with 250 mL of solvent, and allowed to stand overnight before the solvent was filtered. The feces were extracted at least 3 times each with hexane, ethyl acetate, methanol, and water in successive order. Extracts other than the water extract were concentrated in round-bottom flasks under vacuum.

Isolation of Metabolites. Metabolites were isolated by column, thin-layer (TLC), high-pressure liquid (HPLC), and gas–liquid (GC) chromatographic procedures [see supplementary material (see paragraph at end of paper regarding supplementary material) and Davison et al. (1982)]. TLC plates were silica gel G, 0.25 mm thick. HPLC was performed on a reverse-phase Radial-Pak C_{18} cartridge (Waters Associates, Inc., Milford, MA) eluted with a water to methanol gradient, except when the cartridge was eluted normal phase, methanol to water gradient, to separate urinary metabolites **11**, **17**, **18**, and **19** (glucuronides without a methoxy group; see Table I for metabolite structures) from **12**, **13**, **14**, **20**, and **22** (glucuronides with a methoxy group).

Liquid urine and bile, pooled according to the dose given to the chickens, were applied directly to Porapak Q columns (Davison et al., 1982). Isolation of metabolites from roosters given 0.2 mg of methoxychlor was not attempted.

Identification of Metabolites. After cleanup by liquid chromatography, most samples were derivatized with bis(trimethylsilyl)trifluoroacetamide containing 1% chlorotrimethylsilane (BSTFA). The samples were then analyzed by a GC equipped with an effluent splitter and a radioactive monitor (GC-RAM) to locate areas containing ^{14}C as an aid in interpreting subsequent GC–mass spectra (GC–MS). The metabolites were identified by interpretation of the mass spectra of the derivatized compounds (Figure 1) and by comparison of the spectra to those of authentic compounds. In some cases, nuclear magnetic resonance (NMR) spectra were used to further assist identification.

To locate the position of ring hydroxylation and conjugation, a sample containing four metabolites (**20**, **21**, **24**, and **25**), but predominantly metabolite **20** (1.2-mg total mass), was refluxed with 0.2 mL of benzyl chloride and 0.1

Table II. Recovery of ^{14}C from Laying Hens, Roosters, Colostomized Roosters, and Bile-Fistulated Roosters Given [^{14}C]Methoxychlor

sex, duration, and methoxychlor dose	N	% of dose										
		feces	urine	droppings	bile	gallbladder[a]	GI tract[b]	liver[a]	kidneys	carcass[c]	eggs	total recovered
hens, 1-day balance study[d]												
0.2 mg (5.8 μCi)	3			86.4			1.63	0.42	0.02	1.94	0.010	90.5
10 mg (5.8 μCi)	3			81.0			2.07	0.35	0.06	3.42	0.009	87.1
100 mg (5.8 μCi)	3			77.1			2.53	0.40	0.08	8.42	0.016	88.9
hens, 14-day balance study												
0.2 mg (0.8 μCi)/day	3			85.4		0.012	0.30	0.026	0.006	0.41	0.25	86.5
10 mg (0.8 μCi)/day	3			85.3		0.023	0.28	0.035	0.006	0.70	0.34	86.8
100 mg (0.8 μCi)/day	3			84.2		0.010	0.29	0.030	0.007	0.97	0.46	86.2
roosters, 1-day balance study[d]												
0.2 mg (6.9 μCi)	3			91.2		0.06	0.96	0.16	0.03	0.32		93.1
10 mg (6.9 μCi)	3			92.3		0.16	1.28	0.16	0.02	0.59		94.6
100 mg (6.9 μCi)	3			91.3		0.07	1.24	0.12	0.02	1.17		94.1
roosters, 14-day balance study												
0.2 mg (0.8 μCi)/day	3			85.3		0.028	0.11	0.025	0.005	0.006		85.5
10 mg (0.8 μCi)/day	3			85.3		0.010	0.11	0.030	0.002	0.011		85.5
100 mg (0.8 μCi)/day	3			86.2		0.014	0.19	0.031	0.003	0.021		86.5
colostomized roosters, 1-day metabolism study												
0.2 mg (4.8 μCi)	6	69.9	10.2				13.4	1.22	0.02	1.06		95.9
10 mg (4.8 μCi)	5	69.6	12.0				7.5	0.70	0.03	0.98		90.8
100 mg (4.8 μCi)	6	61.9	11.7				10.0	1.80	0.03	2.24		87.7
bile-fistulated roosters, 1-day metabolism study												
0.2 mg (3.5 μCi)	4			55.8	22.0		10.3	2.94	0.06	1.26		92.4
10 mg (4.8 μCi)	3			69.7	15.1		6.6	0.30	0.03	1.10		92.9
100 mg (4.8 μCi)	4			65.1	18.4		3.3	0.13	0.03	1.20		88.2

[a] Gallbladders from hens on the 1-day balance study were discarded without analysis. Gallbladders from colostomized and bile-cannulated roosters remained on the livers. [b] Includes contents. [c] Feathers, small samples of breast muscle, skin and adipose tissue, and the organs indicated were removed. [d] All chickens were killed 24 h after the last dose. All data were analyzed within the study statistically by analysis of variance. Significant treatment effects were detected in the percent ^{14}C eliminated in droppings ($P < 0.10$) or retained in kidneys and carcasses ($P < 0.05$) for hens on the 1-day balance study and were detected in the percent ^{14}C retained in carcasses ($P < 0.05$) for roosters on the 1-day balance study.

g of sodium methylate in 20 mL of methanol for 7 h. Then the methanol was evaporated, and water, ether, and 2.5 N NaOH were added and allowed to separate. The ether layer was discarded, and the aqueous layer was acidified with HCl and extracted with ether. The ether was evaporated, and the residue was dissolved in 1 mL of 0.05 M Na acetate buffer, pH 4.75. Twenty microliters of Helex p. enzyme was added and incubated at 38 °C overnight. The reaction mixture was extracted with toluene and the solvent evaporated. The residue was reacted with diazomethane, and the mass spectra were obtained by GC–MS using both packed and capillary columns. The two major compounds obtained were diphenyldichloroethene isomers, each containing two methoxy groups and one benzyloxy group, $M^{+\cdot}$ 414. The mass spectrum of one compound had peaks at m/z 414 (100%) and m/z 323 (18%); the other compound had peaks at m/z 414 (70%) and m/z 323 (100%).

2-[4-(Benzyloxy)-3-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene was synthesized by reacting 4-(benzyloxy)-3-methoxybenzaldehyde with trichloromethyllithium to form 1-[4-(benzyloxy)-3-methoxyphenyl]-2,2,2-trichloroethanol, which was reacted with anisole in methylene chloride with a mixture of acetic and sulfuric acids to yield 2-[4-(benzyloxy)-3-methoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane (Feil et al., 1973, 1975). The trichloroethane eluted at 29.4 mL on HPLC with acetonitrile–water (7:3) (Waters Associates C_{18} NOVAPAK RCM): MS m/z (rel intensity) 450 (3 Cl, 4.1 $M^{+\cdot}$), 333 (100, M – CCl_3), 242 (23), 241 (19), 213 (33); NMR (acetone-d_6) δ 3.75 (s, OCH_3), 3.81 (s, OCH_3), 5.08 (s, CH_2), 5.18 (s, CH), 6.90 (d, 3,5-HArOMe, J = 8.78 Hz), 6.98 (d, 5-HArOMeOBz, J = 8.95 Hz), 7.21 (d, 2-HArOMeOBz, J = 1.79 Hz), 7.64 (d, 2,6-HArOMe, J = 8.78 Hz), other assignments could not be made because of inadequate resolution. The trichloroethane was reacted with sodium hydride in tetrahydrofuran for 24 h at room temperature. The desired product eluted at 33 mL on HPLC (conditions as above): MS Figure 2; NMR (acetone-d_6) δ 3.79 (s, OCH_3), 5.11 (s, CH_2), 6.77 (dd, 6-HArOMeOBz, J = 8.3 and 1.75 Hz), 6.91 (d, 3,5-HArOMe, J = 8.75 Hz), 6.95 (d, 2-HArOMeOBz, J = 1.75 Hz), 7.02 (d, 5-HArOMeOBz, J = 8.3 Hz), 7.25 (d, 2,6-HArOMe, J = 8.75 Hz), 7.42 (m, phenyl).

2-[3-(Benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene was synthesized by reacting 3-(benzyloxy)-4-methoxybenzaldehyde with trichloromethyllithium to form 1-[3-(benzyloxy)-4-methoxyphenyl]-2,2,2-trichloroethanol, which was reacted with anisole in methylene chloride with methanesulfonic acid to yield 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane (Feil et al., 1973, 1975). The trichloroethane eluted at 24.6 mL on HPLC with acetonitrile–water (7:3) (Waters Associates C_{18} NOVAPAK RCM): MS m/z (rel intensity) 450 (3 Cl, 2.5, M^{+}), 415 (0.25, M – Cl), 380 (1.2, M – 2Cl), 333 (100, M – CCl_3), 242 (4.0), 241 (3.0), 213 (5.0); NMR (acetone-d_6) δ 3.78 (s, OCH_3), 3.82 (s, OCH_3), 5.14 (s, CH_2 and CH), 6.87 (d, 3,5-HArOMe, J = 8.75 Hz), 6.95 (d, 5-HArOMeOBz, J = 8.31 Hz), 7.18 (d, 2-HArOMeOBz, J = 2.19 Hz), 7.54 (d, 2,6-HArOMe, J = 8.75 Hz), other assignments could not be made because of inadequate resolution. The trichloroethane was reacted with sodium hydride in tetrahydrofuran for 24 h at room temperature. The desired product eluted at 32.4 mL on HPLC (conditions as above); MS Figure 2; NMR (acetone-d_6) δ 3.81 (s, OCH_3), 3.84 (s,

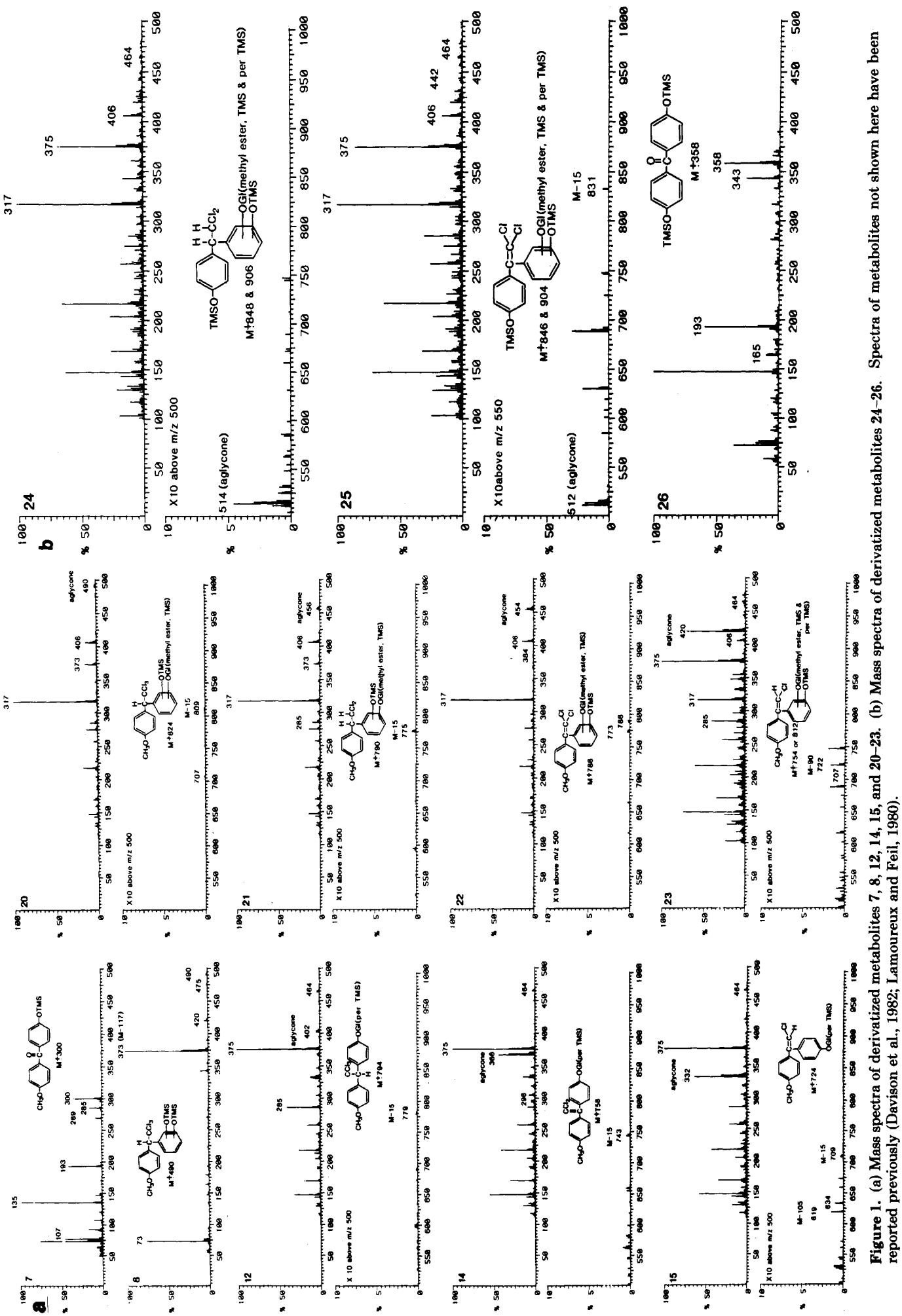


Figure 1. (a) Mass spectra of derivatized metabolites 7, 8, 12, 14, 15, and 20–23. (b) Mass spectra of derivatized metabolites 24–26. Spectra of metabolites not shown here have been reported previously (Davison et al., 1982; Lamoureux and Feil, 1980).

Table III. Methoxychlor Equivalents in Selected Tissues and Eggs of Chickens Given [^{14}C]Methoxychlor

sex, duration, and methoxychlor dose	equiv, μg/g of lyophilized tissue (material)									
	breast muscle	skin[a]	abdominal fat	heart	liver	kidneys	carcass[b]	eggshell[c]	egg white[c]	egg yolk[c]
hens, 1-day balance study[d]										
0.2 mg	0.005	0.052	0.097		0.49	0.090	0.045	<0.001	0.010	0.070
10 mg	0.40	0.60	1.07		3.34	1.51	0.57	0.005	0.091	0.063
100 mg	0.53	12.5	22.4		30.8	16.9	11.1	0.105	1.30	0.750
hens 14-day balance study										
0.2 mg/day	0.004	0.012	0.017	0.014	0.08	0.05	0.015	<0.001	0.005	0.072
10 mg/day	0.38	2.95	4.26	2.28	5.74	3.14	1.96	0.046	0.409	5.73
100 mg/day	4.0	36.7	60.8	27.2	42.2	30.9	26.6	0.351	3.47	63.1
roosters, 1-day balance study										
0.2 mg	0.001	0.005		0.003	0.04	0.02	0.002			
10 mg	0.061	0.36		0.34	2.5	0.65	0.17			
100 mg	0.55	8.9		9.4	18.9	9.7	3.9			
roosters, 14-day balance study										
0.2 mg/day	0.021	0.018		0.032	0.13	0.07	0.007			
10 mg/day	0.32	2.12		1.08	7.07	1.95	0.60			
100 mg/day	9.8	22.9		62.2	76.3	23.1	11.3			

[a] Taken from the area covering the site where muscle was sampled. [b] Less feathers, gastrointestinal tract, small samples of muscle, skin and fat, and organs indicated. [c] Values for eggshell, egg white, and egg yolk are from the last egg laid by hens on the 14-day balance study. [d] Average body weight for the hens and roosters was 1512 and 1634 g, respectively.

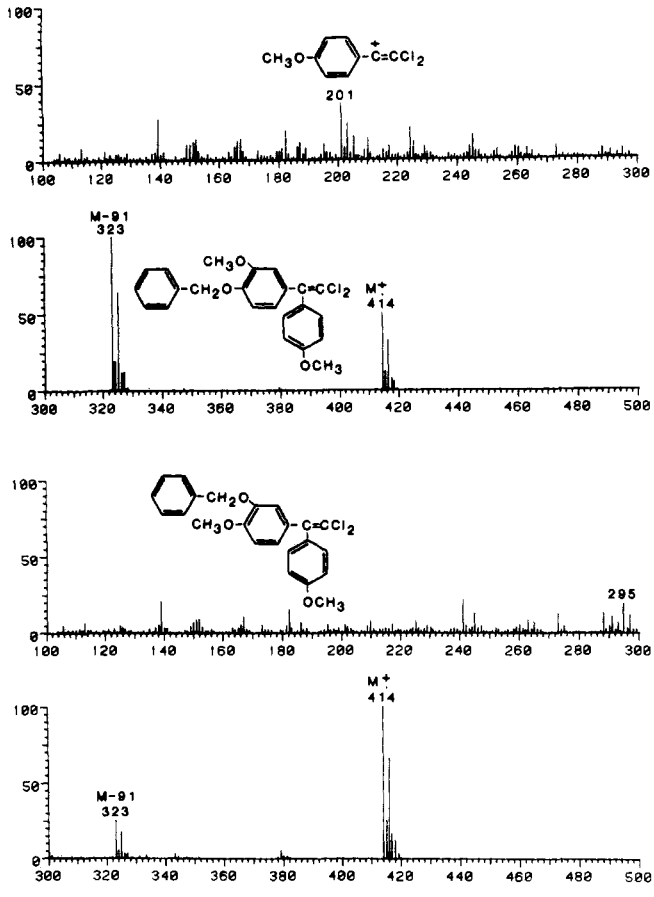


Figure 2. Mass spectra of 2-[4-(benzyloxy)-3-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene and 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)-ethene.

OCH_3), 5.10 (s, CH_2), 6.81 (dd, 6-HArOMeOBz, J = 8.31 and 2.18 Hz), 6.91 (d, 3,5-HArOMe, J = 9.18 Hz), 6.98 (d, 5-HArOMeOBz, J = 8.31 Hz), 6.99 (d, 2-HArOMeOBz, J = 2.18 Hz), 7.21 (d, 2,6-HArOMe, J = 9.18 Hz), 7.39 (m, phenyl).

RESULTS AND DISCUSSION

Percentage recovery of ^{14}C is shown in Table II, and residues in tissues and eggs, as methoxychlor equivalents in dry material, are shown in Table III. From these tables it is obvious that the results differed in some ways depending upon the sex or age, the duration of the dose, the size of the dose, or whether or not the chicken had a colostomy or bile cannulation. A higher percentage of ^{14}C was recovered in tissues from chickens dosed for 1 day compared to those dosed for 14 days (Table II). Recovery of radioactivity was greater in the carcasses and in some tissues from hens than in roosters for comparable dose levels and duration. Part of this difference can be explained by the fact that the hens contained more adipose tissue than the roosters and ^{14}C residues were relatively high in this tissue (Table III). Adipose tissue, while abundant in the abdominal area of the hens, was not present in the roosters. This is normal for White Leghorn chickens of this age.

In the 1-day balance study with hens, elimination of ^{14}C in droppings diminished with increasing size of dose, and recovery of ^{14}C in kidneys and carcasses increased with size of the dose (Table II). This effect of size of dose on recovery of ^{14}C in droppings and kidneys did not occur in any of the other five studies. While the effect of size of dose on the recovery of ^{14}C in the carcass was more obvious in the 1-day balance study with hens, this effect also occurred in the 14-day balance study with hens and in the 1- and 14-day balance studies with intact roosters.

Residues in tissues and eggs of hens dosed with 0.2 mg of methoxychlor for either 1 or 14 days did not differ (Table III). However, residues in tissues and eggs of hens dosed with 10 or 100 mg of methoxychlor for 14 days were nearly always greater than residues in tissues and eggs of hens comparably dosed for 1 day.

Tissue ^{14}C residues were lowest in muscle and highest in liver and fat (Table III). Residues in eggs were predominantly in the yolk. Yolk residues increased with time to about 1 week, when a steady state apparently was attained (Figure 3).

The percentage of ^{14}C remaining in the gastrointestinal tract of colostomized or bile-cannulated roosters was markedly higher than that of intact roosters of the same dose level and duration (Table II). The percentage of ^{14}C excreted in the urine by colostomized roosters was not appreciably affected by size of the dose, and the percentage of ^{14}C remaining in the kidneys of colostomized roosters was similar to that remaining in the kidneys of intact roosters of comparable duration. However, the percentage

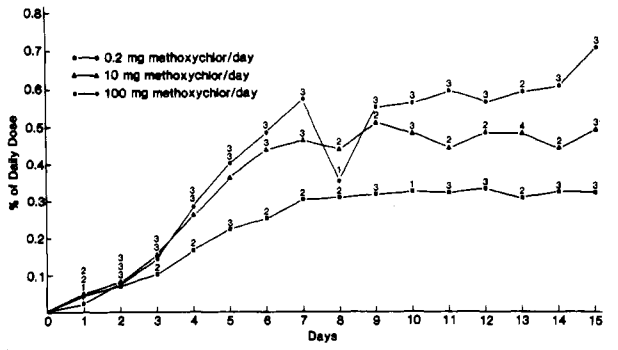


Figure 3. Recovery of ^{14}C in egg yolk from hens given [^{14}C]-methoxychlor. Indicated at each point is the number of yolks examined. The 15th-day yolk was taken from the shell gland when the hens were killed 24 h after the last dose.

of ^{14}C remaining in the livers and carcasses of colostomized roosters, although small, was about double that left in these tissues by intact roosters of comparable dose and duration. We have no explanation for this observation but believe that the values are in a normal physiological range. Also, the percentage of ^{14}C remaining in the carcasses of the colostomized roosters was smaller than that remaining in the carcasses of hens for the same dose and duration.

Bile-fistulated rooster 2, given 0.2 mg of methoxychlor in a single dose, contained 9.4% of the administered dose in the liver, 10% in the bile, and 30% in the gastrointestinal tract 24 h after dosing. Except for this rooster, recovery of ^{14}C in livers, kidneys, and carcasses of bile-fistulated roosters were comparable to those obtained from intact roosters similarly dosed. The surgical area and the interior of all surgically modified roosters appeared normal by gross observation when the roosters were killed, and the means published include rooster 2.

For colostomized roosters given 10 mg of methoxychlor, hexane extracted 31% of the ^{14}C from the feces, ethyl acetate extracted 11%, methanol extracted 28%, and water extracted 13%. Nonextractable residues were 22%. For colostomized roosters given 100 mg of methoxychlor, hexane extracted 20% of the ^{14}C from the feces, ethyl acetate extracted 7%, methanol extracted 37%, and water extracted 19%. Nonextractable residues were 18%. In all, 26 metabolites plus methoxychlor were identified (Table I). Feces contained 22 metabolites, urine contained 14, and bile contained 6. Methoxychlor was found only in the feces.

Sixteen of the metabolites (**2–6, 9–11, 13, 15–19, 21,** and **27**, Table I) found in the chicken had been identified previously in goats (Davison et al., 1982, 1983). Therefore, their MS fragmentation patterns have already been discussed (Davison et al., 1982). Metabolite **7** was not synthesized; however, the fragmentation pattern of the Me_3Si derivative of metabolite **7** (Figure 1a) was similar to that of synthetic metabolite **26** (Figure 1b), except that its molecular ion was 58 amu smaller. The Me_3Si derivative of metabolite **26** was identified by comparative MS with the Me_3Si derivative of commercially available 4,4′-dihydroxybenzophenone.

The mass spectrum of the Me_3Si derivative of metabolite **8** indicated the presence of a ring hydroxylation. The GC retention time was much shorter than that of the glucuronides, and no glucuronide related peaks were present. The remaining metabolites were identified by derivatization and interpretation of the mass spectra. All of these had fragmentation patterns characteristic of glucuronide-type metabolites (Bakke, 1976).

Six of the glucuronide-type metabolites also had undergone ring hydroxylation. Metabolite **21** was the same as metabolite **18** in the goat (Davison et al., 1982). Metabolites **20, 21, 24,** and **25** were each present as two isomers. The NMR spectra of the isomers of metabolite **20** each showed doublets at δ 6.92 and 7.63 (J = 8.75 Hz). The protons on the 3,5-positions of methoxychlor absorbed at δ 6.92 while the protons on the 2,6-positions absorbed at 7.65 (J = 8.80 Hz), suggesting that the metabolite retained an unaltered methoxyphenyl group. A glucuronide group should have shifted the 3,5-protons downfield approximately 0.15 ppm (based on comparison of 4-nitroanisole with 4-nitrophenyl glucuronide, anisole with phenyl glucuronide, and the dimethyl ether of diethylstilbestrol with diethylstilbestrol glucuronide).

Because of the difficulty in synthesizing the metabolites containing the glucuronide group, we converted them to the (benzyloxy)dimethoxy derivatives. The benzyl group was added to the free hydroxy group. The glucuronide was removed and the resulting hydroxy group was methylated. The sample used was a mixture of four metabolites: two isomers of a dichloroethane and two isomers of a trichloroethane. The reaction yielded predominantly two isomers of a dichloroethene, which were purified by GC and yielded mass spectra and GC retention times that were identical with those of 2-[4-(benzyloxy)-3-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene and 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene. Initial attempts at the synthesis of these compounds yielded products that had different mass spectra and GC retention times (the products were subsequently shown to result from migration of the benzyl groups to the ortho and/or para positions to yield benzyl-substituted phenols). The desired benzyloxy compounds could be prepared only in low yield and could be purified by HPLC. Both NMR and mass spectrometries were required to monitor the separation because the isomers yielded similar mass spectra. 2-[4-(Benzyloxy)-phenyl]-1,1-dichloro-2-(3,4-dimethoxyphenyl)ethene, 2-[4-(benzyloxy)phenyl]-1,1-dichloro-2-(2,4-dimethoxyphenyl)ethene, 2-[2-(benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene, and 2-[4-(benzyloxy)-2-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene were synthesized and found to have mass spectra that were similar to those of the derivatized metabolites. Although it is likely that metabolites **21, 24,** and **25** have the same substitution patterns as the two isomers of metabolites **20**, we were unable to establish this because of the limited amounts of metabolite available.

Metabolite **14** comprised about 38% of the ^{14}C in bile of roosters given 100 mg of methoxychlor. All other metabolites were present as mixtures in more than one fraction during cleanup so that precise quantitation was not possible. Metabolite **2** was present in the ^{14}C dosing material. However, this compound along with its demethylated and conjugated analogues was isolated from feces, bile, and urine in an amount far exceeding that which could have come from the dosing material. Therefore, metabolite **2** was a true metabolite. For roosters given 10 mg of methoxychlor, identified metabolites accounted for about 64% of the ^{14}C in feces, 70% of the ^{14}C in bile, and 54% of the ^{14}C in urine. For roosters given 100 mg of methoxychlor, identified metabolites accounted for about 57% of the ^{14}C in feces, 52% of the ^{14}C in bile, and 63% of the ^{14}C in urine.

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Registry No. 1, 72-43-5; 2, 7388-31-0; 3, 2132-70-9; 4, 28463-03-8; 5, 79648-83-2; 6, 75938-34-0; 7, 61002-54-8; 9, 2971-36-0; 10, 13005-40-8; 11, 14868-03-2; 12, 90047-64-6; 13, 79639-27-3; 14, 90047-65-7; 15, 79639-28-4; 16, 79639-30-8; 17, 79639-32-0; 18, 79639-31-9; 19, 79639-33-1; 20 isomer I, 90047-66-8; 20 isomer II, 90047-67-9; 26, 611-99-4; 27, 79639-29-5; 2-[4-(benzyloxy)-3-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene, 90047-68-0; 4-(benzyloxy)-3-methoxybenzaldehyde, 2426-87-1; (trichloromethyl)lithium, 2146-66-9; 1-[4-(benzyloxy)-3-methoxyphenyl]-2,2,2-trichloroethanol, 90047-69-1; anisole, 100-66-3; 2-[4-(benzyloxy)-3-methyoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane, 90047-70-4; 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene, 90047-71-5; 3-(benzyloxy)-4-methoxybenzaldehyde, 6346-05-0; 1-[3-(benzyloxy)-4-methoxyphenyl]-2,2,2-trichloroethanol, 90047-72-6; 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane, 90047-73-7.

Supplementary Material Available: A list of the solvents used and 6 pages of diagrams showing in detail the isolation of the metabolites from feces, urine, and bile (7 pages). Ordering information is given on any current masthead page.

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A Rapid Automated Procedure for the Determination of Trypsin Inhibitor Activity in Soy Products and Common Foodstuffs

Bonnie A. Charpentier* and Dale E. Lemmel

An automated method for the determination of trypsin inhibitor activity has been developed based on an improved procedure using the synthetic substrate benzoylarginine-*p*-nitroanilide. The method has been found to be rapid and reproducible and has been applied to a variety of foodstuffs including eggs, cheese, beef, several vegetables, and soy products. Results show that trypsin inhibitor activity decreases dramatically during the processing of soy flour to food products. Activities found in the foods analyzed have been compared on fresh weight, dry weight, and protein content bases. The advantages and limitations of the method are discussed.

Trypsin inhibitors are widely distributed in nature, occurring in a large variety of both plant and animal components of the human diet (Liener and Kakade, 1980; Belitz et al., 1982; Doell et al., 1981). Much work has been done to characterize various trypsin inhibitors and to study their physiological effects in animals and humans. Interest has centered in particular upon trypsin inhibitors present in soybeans, a valuable source of protein for both animals and humans. Soy protease inhibitors have been of interest because of their antinutritional effects and interference with protein digestion (Hill et al., 1982; Krogdahl and Holm, 1981; Liener, 1981; Satterlee et al., 1979). They have also been shown to cause pancreatic enlargement in chicks (Chernick et al., 1948) and in mice and rats (Rackis, 1965, 1974; Yamatori and Fujita, 1976; Kakade et al., 1973). In other studies, however, using animals such as dogs, calves, pigs, and monkeys (Kakade et al., 1975; Yen et al., 1974; Struthers et al., 1983), such pancreatic effects were not produced. Heating of soy products has been shown to destroy both antinutritional and pancreatic effects (Liener, 1975, 1981; Struthers et al., 1983). Reviews by Liener and Kakade (1980) and by Rackis and Gumbmann (1981) summarize the properties and nutritional significance of known protease inhibitors.

In view of the interests noted above, the need for an accurate and widely applicable analysis for trypsin inhibitors is obvious. Various column chromatographic methods (Whitaker and Sgarbieri, 1981), affinity chromatography (Gomes et al., 1979; Chan and de Lunen, 1982; Lin et al., 1980), and electrophoresis (Lewosz et al., 1981; Sgarbieri and Witaker, 1981) have proved valuable for the isolation and characterization of diverse trypsin inhibitors; these methods, however, are not suitable for rapid quantitation of trypsin inhibitor activity. The use of a pH stat to measure tryptic proteolysis and trypsin inhibitor content of soy flours has proved difficult with some samples and has shown no advantage as yet over conventional methods (Stinson and Snyder, 1980; Hill et al., 1982).

The most widely used method for measuring trypsin inhibitor activity evolved from a procedure developed by Kunitz (1947) in which the hydrolysis of casein by trypsin was measured spectrophotometrically in the presence and absence of inhibitors. Erlanger et al. (1961) introduced the use of a synthetic substrate, benzoylarginine-*p*-nitroanilide (BAPNA), in place of casein; subsequent work by Kakade et al. (1969) showed the use of BAPNA to be preferable because of simplicity and accuracy. Kakade's work, using the definition of 1 trypsin unit (TU) to be an

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