The definitive effects of pesticides on avian liver microsomal enzymes in general have yet to be described and the literature presents conflicting findings. Although Stephen and coworkers (1971) found an increase in sleeping time in chickens, they were unable to show any consistent change in liver microsomal enzyme activity. Contrary to finding a decrease in liver microsomal activity, Abou-Donia and Menzel (1968) found that liver microsomal oxidative activity was increased in chicks hatched from eggs which had been injected with DDT. Peakall (1967, 1970) observed an increase in the rate of steroid metabolism by hepatic microsomes of pigeons or doves treated with DDT, also suggesting a stimulation of liver microsomal activity. However, reduction in liver enzyme activity has also been reported. Sell *et al.* (1971) found reduced hepatic hydroxylase activity after feeding DDT to White Leghorn hens or Japanese quail (Sell *et al.*, 1972). Gillett and Arscott (1969) also found hepatic microsomal epoxidase activity markedly depressed in quail fed DDT. These depressions of hepatic enzyme activity are in accord with the prolonged sleeping times we observed in quail fed DDT.

Although lengthened sleeping time is an indication of reduced activity of liver microsomal enzymes which detoxify pentobarbital, it is misleading to imply that pesticides which lengthen sleeping time decrease "liver microsomal enzymes." It would be unusual and unexpected if all liver microsomal enzymes responded to pesticides in the same manner. Avoidance of the simplistic use of "liver microsomal enzymes" as a single class, all reacting in an identical fashion, and consideration of specific microsomal enzymes will provide a greater understanding of the biological effects of pesticides.

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Long-Term Studies of Residue Retention and Excretion by Cows Fed a Polychlorinated Biphenyl (Aroclor 1254)

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Nine cows were fed 200 mg per day of Aroclor 1254 (PCB) for 60 days. Milk and body fat samples were obtained during and for 60 days following feeding. Concentrations of PCB in milk fat approached equilibrium after 40 days. The average concentration in milk from 40 to 60 days was 60.9 ± 4.5 μg/g fat. Concentration in body fat was 41.7 ± 11.5 μg/g at 60 days. When feeding stopped, concentration in milk fat declined 50% within 15 days. After 15 days the rate of the first-order decline in concentration was much less. The average rate constant was 0.010 day^{-1} and varied among cows from 0.005 to 0.016 day^{-1}. The variation could not be related to such parameters as milk fat production or body weight change. Decline in concentration of PCB in body fat paralleled decline in concentration of PCB in milk fat.

Considerable interest has developed in polychlorinated biphenyls (PCB), a class of industrial organochlorine compounds. After many years of use, residues of PCB's have recently been found distributed widely in environmental and food samples (Kolbye 1972; Peakall and Lincer, 1970). The most extensive uses of PCB's have been as dielectrics and plasticizers (Nisbet and Sarofim, 1972). However, the minor use of the PCB Aroclor 1254 in a silo sealant poses a significant source of PCB contamination of milk.

Aroclor 1254 is a commercial mixture of PCB's containing an average 54% chlorine, with the major components ranging from tetrachlorobiphenyl to heptachlorobiphenyl (Simons and Welti, 1971). A commercial sealant applied to the interiors of concrete stave silos contained about 10% Aroclor 1254. This source has caused PCB residues in milk exceeding the U. S. Food and Drug Administration guideline of 5 μg/g in milk fat (Kolbye, 1972).

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Residues of PCB in silage occur at high levels adjacent to the treated walls (Skrentny *et al.*, 1971). There is some migration of the residue in the silage, but residues seldom occur beyond 3 ft from the wall. Gas chromatographic examination of the residues from silage suggest little, if any, microbial or chemical change in the composition of Aroclor 1254 (Fries, 1972; Skrentny *et al.*, 1971).

We have compared the behavior of PCB and DDE residues in milk after environmental exposure (Fries *et al.*, 1972). Only the period after removal of the sources of both contaminants from the diet was studied. The rates of decline in milk concentrations of the two residues were identical. Platonow *et al.* (1971) have studied the distribution of residues in the milk of two cows that received a single dose of PCB. The average excretion in milk was 5% within 4 days. We are not aware of long-term controlled studies on the relationship of dietary intake of PCB to residue accumulation and excretion in the cow.

This study was carried out to determine milk and body fat residues while cows consumed a fixed level of Aroclor 1254 and to determine the rate of decline of milk and body fat residue levels after removing PCB from the diet.

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Table I. Days in Lactation, Weight, Intake, and Production of the Cows[a]

Cow	Days in lactation	Weight, kg	Dry matter intake, kg/day	Milk, kg/day	Fat, %	Fat, kg/day
1	211	559	16.9	17.7	4.1	0.72
2	207	536	16.3	16.3	4.1	0.62
3	206	557	15.0	11.9	4.3	0.51
4	143	537	16.5	16.3	3.9	0.72
5	161	577	15.8	14.5	4.2	0.61
6	189	526	17.9	20.6	3.8	0.78
7	89	567	17.8	22.7	3.9	0.88
8	46	496	16.8	22.9	5.6	0.79
9	48	507	17.1	21.9	3.4	0.75

[a] Days in lactation are at the start of the study. All other values are averages for the 120 days of the study.

MATERIALS AND METHODS

Cows. Nine first-lactation Holstein cows were used and their pertinent characteristics are presented in Table I. The cows were selected to provide observations at three stages of lactation and at various levels of production within a stage of lactation. However, because of the differing rates of production decline, the differences in production became less as the experiment progressed.

Cows were fed corn silage, alfalfa hay, dehydrated alfalfa pellets, and concentrate. A description of the feeding regime has been presented by Miller *et al.* (1971). There were some differences in ration composition between cows and within cows during the experiment. These differences did not affect results and only total dry matter intake is presented (Table I).

Dosing and Sampling. All cows were fed 200 mg of Aroclor 1254 per day for 60 days. Aroclor 1254 in acetone solution was pipetted on a small portion of the concentrate and the acetone was allowed to evaporate. The spiked concentrate was hand mixed with the remainder of the concentrate at the time of feeding. Significant feed refusals were not encountered. Based on the measured dry matter intake and the body weight of the animals, this dose rate was equivalent to 12.4 ± 0.9 mg/kg of dry matter or 0.37 ± 0.2 mg/kg of body weight.

Milk samples were collected at 5-day intervals during the feeding period and the following 60 days. More frequent milk samples were obtained both when feeding was initiated and when feeding was stopped. Body fat biopsy samples from the tailhead area were obtained at 30, 60, 90, and 120 days after the start of the study.

Analysis. Fat from the milk and biopsy samples was isolated and cleaned up using U. S. FDA (1968) multipesticide residue methodology. The concentration in most samples was relatively high and there were no significant interferences. Thus, it was not necessary to use more elaborate clean-up methods such as silicic acid chromatography (Armour and Burke, 1970) or dehydrochlorination (Price *et al.*, 1972).

Concentrations of PCB were determined by gas–liquid chromatography using electron capture detection. A Hewlett-Packard Model 7600A instrument equipped with ^{63}Ni electron capture detector and an electronic integrator was used. The 6 ft × ¼ in. o.d. glass column was packed with 10% DC-200 on 80 to 100 mesh Gas Chrom Q. The carrier gas was a 95% argon–5% methane mixture with a 120 ml/min flow rate. A purge gas was not used. Column, inlet, and detector temperatures were 200, 240, and 250°, respectively.

Typical chromatograms of milk fat and body fat residues, as well as an Aroclor 1254 standard, are presented in Figure 1. Under these conditions there are 15 peaks in the standard. There were only seven significant peaks in the residue samples. Their retention times were equal to the retention times of peaks 8 through 15 (except 10) in the standard. The concentration of PCB in the samples was calculated by comparing the areas of these seven peaks with the area of the corresponding peaks of Aroclor 1254 standards.

RESULTS AND DISCUSSION

Nature of Residues. The peaks with the shorter retention times (peaks 1–7) did not occur in either type of residue samples (Figure 1). The components that did occur in the residue samples (peaks 8–15) were present in approximately the same proportion as in the Aroclor 1254 standard. The relative proportions of these components did not differ significantly among sampling times, among cows, or between types of samples. For this reason data on the individual components are not presented.

Sissons and Welti (1971) have determined the composition of Aroclor 1254 and the retention indices of its components. From their work and some comparisons with individual PCB's (Figure 1), it was concluded that the major components occurring in the residue samples were pentachlorobiphenyls and hexachlorobiphenyls. The components that were metabolized and did not occur in the residue samples were tetrachlorobiphenyls and some of the pentachlorobiphenyls.

There is little information on the metabolic fate of PCB's. Block and Cornish (1969) reported the formation of 4-chloro-4'-hydroxybiphenyl and some of its conjugation products from 4-chlorobiphenyl in rabbits. It is possible that this reaction is quite general for PCB's if the appropriate positions are open for hydroxylation. The possibility of an open position would decrease as the degree of chlorination increased.

The relative electron capture responses of PCB's from tetrachlorobiphenyls through decachlorobiphenyl generally do not vary by a factor greater than 2 (Zitko *et al.*, 1971). Thus, our method of quantitation should compare reasonably well with newer methods in which all components are converted to decachlorobiphenyl.

Residues while Feeding. Milk samples obtained before the start of PCB feeding did not contain detectable PCB

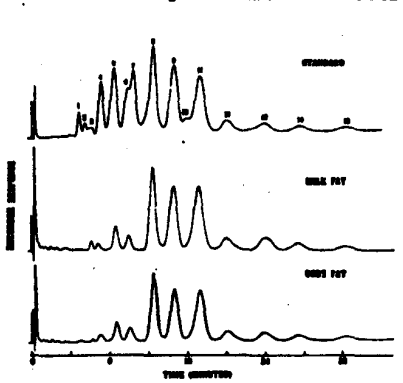

Figure 1. Typical gas chromatograms of an Aroclor 1254 standard, a milk fat residue sample, and a body fat residue sample. Retention times of peaks 1, 2, 4, 6, and 9 were equal to the retention times of the pure compounds 2,5,2',5'-tetrachlorobiphenyl, 2,3,2',5'-tetrachlorobiphenyl, 2,5,3',4'-tetrachlorobiphenyl, 2,3,4,2',5'-pentachlorobiphenyl, and 2,4,5,2',4',5'-hexachlorobiphenyl, respectively.

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residues or significant interference. Thus we concluded that the feeds and the cows were not exposed to sources of PCB other than the material fed.

The average levels of PCB in milk fat and body fat during the 60-day feeding period are presented in Figure 2. The shape of the milk fat concentration curve is typical of the curves for other chlorinated hydrocarbon compounds (Fries *et al.*, 1969). After 40 days, the level of milk fat approached equilibrium but was still rising slowly.

Concentration of PCB in body fat rose more slowly and was always lower than concentration in milk fat. The ratio of concentration in body fat to concentration in milk fat became closer (1:1) at 60 days. At equilibrium one would expect a ratio of 1:1, if the findings of Stull *et al.* (1971) with DDT can be applied to PCB.

The evidence indicates that the cows were not at equilibrium at 60 days. However, the response from the 40th to the 60th day is adequate to determine if any characteristics of the cows significantly affect levels excreted in milk or accumulated in body fat.

Average concentrations of PCB in milk fat from the 40th to 60th day were similar for all cows (Table II). The average for all cows was 60.9 ± 4.5 μg/g and the minor variations were not related to production or any of the parameters listed in Table I. Because of the small variation in concentration, the amount excreted per day was directly related to fat production. The average excretion was 42.3 mg/day, accounting for 21% of intake.

Concentrations in body fat at 60 days, averaging 41.7 ± 11.5 μg/g, were more variable than concentrations in milk fat (Table II). With all cows, the concentration in body fat was lower than the concentration in milk fat. As in the case of levels in milk fat, variation among cows cannot be explained by the parameters measured in this experiment.

The total amount of body fat, one of the major parameters that could affect body fat concentration, cannot be measured with ease. In a previous experiment with rats we have shown that the total retention of chlorinated hydrocarbons is quite constant under a given set of conditions (Fries *et al.*, 1971). Concentrations in body fat were inversely proportional to the total amount of body fat. If a similar phenomenon held true for these cows, one would expect that cows with smaller body fat pools would have higher body fat concentrations of PCB.

Residue Elimination. There was a rapid initial decline in the level of PCB in milk fat when PCB feeding stopped (Figure 3). After 15 days the rate of decline was quite slow. The shape of this curve is similar to curves that have been established for other chlorinated hydrocarbon compounds (Fries *et al.*, 1969).

The decline in concentration in milk fat is adequately

Table II. Fat Production, PCB Excretion in Milk, and PCB Accumulation in Body Fat of Cows Consuming 200 mg of PCB per Day[a]

Cow	Milk fat production, kg/day	PCB in milk fat: Concentration, μg/g	PCB in milk fat: Excretion, mg/day	Body fat, μg/g
1	0.72	59.2	42.6	34.5
2	0.61	69.3	35.6	39.0
3	0.53	57.9	30.7	30.5
4	0.72	60.1	43.3	25.3
5	0.58	64.2	37.2	54.0
6	0.77	63.8	49.1	53.2
7	0.89	56.6	50.4	37.1
8	0.70	57.6	40.3	32.3
9	0.73	70.6	51.5	60.2

[a]Milk fat production and concentrations in milk fat are averages for the 40th to 60th day of feeding. Concentrations in body fat are at the 60th day of feeding.

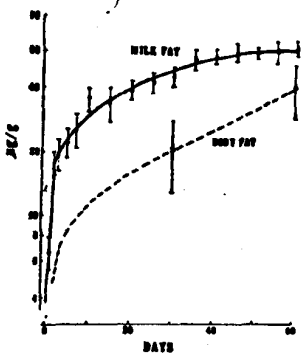

Figure 2. Concentration of PCB in milk fat and body fat of cows fed 200 mg of PCB per day. Each point is an average of nine cows ± standard deviation. The curve for body fat is extrapolated before 30 days.

described by a two-component first-order system with the equation

$$C = C_1 e^{-k_1 t} + C_2 e^{-k_2 t} \qquad (1)$$

where C is the concentration at any time, C_1 is the initial concentration of the first component, C_2 is the initial concentration of the second component, k_1 and k_2 are rate constants, and t is time in days. This is a general equation describing the concentration of a compound in the effluent of a two-compartment system. We have discussed its application to residue elimination from cows elsewhere (Fries *et al.*, 1969).

After 15 days the contribution of the first term to the total concentration is negligible and can be ignored. The values of the constants for the second term were estimated by fitting the conventional linear regression of the logarithmic form of the second term. After subtracting the

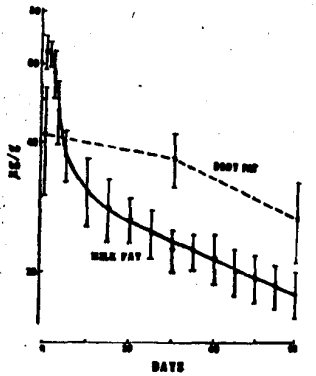

Figure 3. Concentration of PCB in milk fat and body fat of cows after feeding stopped. Each point is an average of nine cows ± standard deviation.

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Table III. Decline in Concentration of PCB Milk Fat after Feeding Stopped

Cow	Initial concentration: Milk fat, μg/g	Initial concentration: Body fat, μg/g	Milk fat production, kg/day	Weight change,[a] kg/day	Initial decline,[b] %	Rate,[c] day⁻¹
1	59.2	34.5	0.61	0.22	57	0.011
2	58.3	39.0	0.60	0.42	55	0.005
3	57.9	39.5	0.44	0.73	55	0.007
4	60.1	25.3	0.67	0.29	64	0.008
5	64.2	54.0	0.56	0.56	54	0.008
6	69.5	53.2	0.69	0.31	52	0.010
7	56.6	37.1	0.61	0.07	58	0.010
8	67.6	52.3	0.72	0.07	58	0.006
9	70.0	60.2	0.70	0.31	49	0.015

[a] 15 to 60 days post-feeding. [b] 100 [(concentration at 0 days − concentration at 15 days) (concentration at 0 days)]. [c] k_2 in eq 1 calculated for 15 to 60 days post-feeding.

$$\ln C = \ln C_2 - k_2 t \qquad (2)$$

values for the second term from the overall concentration, it was possible to estimate the constants of the first term. The equation estimated using the averages of all cows was

$$C = 30.6\, e^{-0.33t} + 32.3\, e^{-0.010t} \qquad (3)$$

With individual cows, it is not possible to precisely evaluate the first term of eq 1 because of the limited number of observations. In practice this is not of great importance. The level of PCB in milk fat of all cows declined about 5 in the first 15 days, and there was little variation among cows (Table III). This is in agreement with our field studies (Fries *et al.*, 1972).

Unlike the initial decline in concentration, the rate of decline for the slower compartment is quite variable among cows (Table III). We have also observed this variation in previous studies (Fries *et al.*, 1972). These differences are of practical importance. If two cows start at the same level, the time required to return to the FDA guideline is inversely proportional to the magnitude of the rate constant during the second phase. In this study the range between the extreme cows was a factor of 3. Even wider ranges were observed in the field studies.

Despite selecting cows in varying stages of lactation and of varying production levels, it was not possible to establish causes of variation among cows. It can be assumed that the only significant source of PCB for this compartment is the body fat stores. This is supported by the decline in concentration in body fat that paralleled the decline in concentration in milk fat (Figure 3). The close positive relationship between concentrations of chlorinated hydrocarbons in milk fat and body fat is presented elsewhere (Fries and Marrow, 1972).

If the model is appropriate, there are several factors that could influence the rate of decline in concentration in milk fat. Cows with higher fat production would tend to excrete more PCB, and this could be expected to increase the rate of decline. However, there was no significant relationship between the rates of decline and the amount of fat produced (Table III).

A second factor that could affect the rate is a change in body weight. All the cows were gaining weight in this study (Table III). The amount of PCB remaining in the body would be diluted by this added body fat. Thus, one would expect the dilution as indicated by weight gain to enhance the rate of decline in milk fat concentration. However, there was no consistent relationship between these two factors (Table III).

The third factor, not evaluated in this study, is the total amount of body fat. A given rate of milk fat production would clear a larger fraction of body fat if a cow has a small amount of body fat than if she has a large amount of fat. Some indication of the amount of body fat can be obtained from the concentration of PCB in the body fat at 60 days feeding if it is assumed that all cows absorb PCB with the same efficiency. The concentration would be inversely related to the amount of body fat and the most rapid decline would occur with the cows with the highest initial concentration in body fat. However, the overall relationship between initial concentration in body fat and rate was not consistent (Table III).

Table IV. Comparison of DDE and PCB Accumulation and Excretion by Cows[a]

Parameter	DDE	PCB
Milk fat level, 20 days (μg/g)	0.21 ± 0.04	0.20 ± 0.02
Milk fat level, 60 days (μg/g)	0.29 ± 0.05	0.32 ± 0.03
Body fat level, 60 days (μg/g)	0.23 ± 0.02	0.21 ± 0.06
C_1 (μg/g)	0.12	0.15
k_1 (day⁻¹)	0.31	0.32
C_2 (μg/g)	0.17	0.16
k_2 (day⁻¹)	0.013	0.010

[a] DDE values are from Fries *et al.* (1969). Intakes are normalized to 1 mg/day. C_1, k_1, C_2, and k_2 are the constants of eq 1.

The rate of decline is probably controlled by a combination of the above and/or other factors rather than a single factor. With the small number of animals involved, it was not possible to do a more elaborate multiple factor analysis of the data.

The length of time required to restore a cow or a herd to a level below the FDA guideline of 5 μg/g of milk fat depends not only on the rate constant but also on the initial concentration in the milk fat. In the worst case, if the initial concentration was 10 μg/g (twice the guideline) and rate was 0.005 day⁻¹, the time required would be 139 days. An additional 139 days would be required for each doubling of the initial concentration. A more reasonable estimate could be made by using the average rate for all cows (0.010 day⁻¹). In this case, 69 days would be required to reduce the concentration by half.

Similarity to DDE. In earlier work (Fries *et al.*, 1972), we suggested that residue behavior of Aroclor 1254 was similar to DDE, the most persistent degradation product of DDT. The conclusion was based on the similar rates of elimination after the cows had been removed to clean feed. The results of this study confirm and extend the conclusion.

We have conducted experiments with DDE under conditions similar to this experiment (Fries *et al.*, 1969). The parameters of the DDE experiments are in close agreement with the parameters of this PCB experiment when all values are normalized to an equal intake (Table IV). The close agreement of the two sets of parameters is remarkable considering that PCB's are a multiple residue and that many systematic errors may have been introduced by our method of quantitation. The result supports the conclusion that the relative areas of the peaks considered did not vary significantly among samples. Systematic errors in estimation of the absolute PCB values would not affect the parameters listed in Table IV.

DDE is among the organochlorine compounds most resistant to metabolic degradation. The agreement of the two sets of parameters suggests that the PCB's present in the residues are also among the most resistant compounds to metabolic degradation.

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Toxic Metabolites of Diazinon in Sheep

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Diazinon is oxidized in sheep to several cholinesterase-inhibiting metabolites. The structures of three of them were determined by direct spectroscopic measurement. Two are monohydroxy diazinons, and the third is a dehydration product of one of these; the three structures had already been proposed for diazinon metabolites produced by mice, when the establishment of structure was based on cochromatography with synthetic samples. Some quantitative aspects of the distribution of the compounds in sheep are reported.

Because diazinon is widely used as an agricultural insecticide in conditions where it may be ingested by mammals, its metabolism has been studied for many years. It is only recently, however, that specific structures have been put forward for some of the metabolites formed. In all these structures the pyrimidine moiety has been modified, either before or after cleavage from the diethyl phosphorothionyl group. Mucke *et al.* (1970) showed that in rats the isopropyl group of the pyrimidinol derived from diazinon was hydroxylated to give two isomeric hydroxypyrimidinones. These compounds are much less toxic than diazinon because they have lost the phosphorus-containing group. Miyasaki *et al.* (1970), in an extensive study of the metabolism of diazinon by mice, suggested structures for several urinary metabolites in which all the phosphate bonds were still intact. Identifications were based on a comparison of the chromatographic properties of the metabolites with those of synthetic compounds.

The present work is concerned with those metabolites of diazinon in sheep that are still indirect inhibitors of cholinesterase. Three of these were isolated in sufficient quantity to establish their structures by direct spectroscopic measurements. We have reported the occurrence of two of them (referred to below as I and II) in brief preliminary accounts (Machin *et al.*, 1971b, 1972).

EXPERIMENTAL SECTION

Materials. Solvents were analytical reagent grade and were distilled immediately before use. Chloroform was washed free from ethanol and dried before distillation.

Diazinon and diazoxon were gifts from Fisons, Ltd., Agrochemicals Division. Hydroxydiazinon (*O,O*-diethyl *O*-[2-hydroxypropp-2-yl]-6-methylpyrimidin-4-yl] phosphorothionate) was prepared by irradiation of diazinon (Machin *et al.*, 1971a).

Thin-layer chromatography (tlc) was on 8-in. square plates coated with silica gel G or *GF*/UV254 (Macherv, Nagel & Co.); final separations were carried out on the specially purified grade N-HR/UV254. Column chromatography was on Florisil, Johns-Manville Celite 545, and Woelm silica gel and neutral alumina.

Gas Chromatography. Residues were determined and preparative separations of the metabolites monitored on a Varian Aerograph Model 204 chromatograph with a thermionic detector or on a chromatograph (Machin and Morris, 1972) which was also used to purify samples. Columns were 3 ft or 5 ft × ⅛ in. o.d. glass, packed with 1.5–2% XE-60 or 25% SE-30 on Aeropak 30, 100–120 mesh.

Isolation of Metabolites. Two sheep were dosed by stomach tube with diazinon (1 g/kg) which produced moderate symptoms of poisoning. One was killed after 48 hr. Urine was collected from the other for 3 days and the sheep was allowed to recover. Metabolites I and II were isolated from urine collected during this 3-day period. The urine (120 ml) was diluted to 500 ml with water and extracted for 48 hr with chloroform (800 ml) in a liquid–liquid extractor. The chloroform extract was divided into portions (4 × 200 ml), each of which was concentrated to 10 ml and applied to a column of silica gel (Brockman grade II, 25 g, ¾ in. diameter). The metabolites were eluted with 1:40 methanol–chloroform (150 ml) and the eluate was concentrated, first in a rotary evaporator and then in a stream of dry nitrogen, to 0.3 ml. After tlc with 1:4 acetone–hexane as mobile solvent, the bands containing I and II were eluted separately and chromatographed twice more, first with the same solvent and then with 2:3 ethyl acetate–hexane to remove an unknown contaminant detected by its fluorescence at 360 nm. I and II were again eluted with acetone and the solvent was evaporated. Metabolite I was also separated from the tissues, as described previously (Machin *et al.*, 1971b).

Metabolite III was mainly concentrated in the fat and was extracted by macerating 200-g portions of fat three times with acetone (200 ml). After each maceration the suspension was centrifuged at 3000 rpm for 30 min at −20° and the supernatant was decanted. The extracts

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