DDT Metabolites in Feces and Bile of Rats

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A study of DDT-derived products occurring in rat feces and bile, following the ingestion of DDT, shows that the major products are complexed forms of DDA in both bile and feces. Uncomplexed DDA is found in the bile, and insignificant amounts of DDE are present in both bile and feces. DDT-C^{14} was used to demonstrate that the Schechter-Haller method fails to account, quantitatively, for these metabolites. Biliary excretion is responsible for almost all of the DDT metabolites in feces. Although bile and fecal metabolites have certain similarities, they are not necessarily identical.

An exhaustive review of literature on the fate of DDT in animals has been included in Hayes' work (*2*) on the pharmacology and toxicology of DDT. This source shows that DDT, DDE [1,1 - dichloro - 2,2 - bis(*p* - chlorophenyl)-ethylene], and *p,p'*-DDA [bis(*p*-chlorophenyl)acetic acid] are the only compounds identified so far in animal feces after oral ingestion of DDT.

Only a limited amount of information is available on the excretion of DDT and its metabolites by way of the bile. Smith and Stohlman (*7*) reported DDT in the bile of rabbits dosed orally with DDT. An organic chloride method of analysis was used and the results were calculated as DDT. Stiff and Castillo (*8*) essentially repeated this work and obtained similar results; however, no DDT was found when they examined the same tissues and fluids, including bile, by their xanthydrol - potassium hydroxide - pyridine colorimetric method. This discrepancy is explainable on the basis that the xanthydrol method will not give a color reaction with any of the known metabolites of DDT—i.e., DDE and DDA. Neal and von Oettingen (*4*) recognized as early as 1946 that DDT was excreted in the bile in an undetermined form which was not DDT per se. Thus it has long been recognized that DDT is not present in bile to a major extent, as such, but appears largely in a degraded or altered form.

This study was undertaken to study the nature of the metabolites in feces and bile, and their relationship to each other, and to evaluate the use of the Schechter-Haller method (*6*, *9*) as compared to radioassay.

Fecal Metabolites

Experimental Quantitative analysis of feces for DDT and DDT-derived products is complicated by three major difficulties: failure to extract all Schechter-Haller–positive or DDT-derived materials by the usual organic solvents, interference with nitration by extraneous extractives, and failure of metabolites to respond quantitatively to nitration even under severe conditions. As much effort has been devoted to the study of these analytical problems, and as the findings may be of value to the work of others who are tracing DDT and related compounds through insects and mammals, these studies will be reported in a separate paper. Any special analytical techniques employed in the present study will be indicated.

The extraction procedure which has been found so far to give the highest yield of DDT-derived products from rat feces consists of refluxing, for several hours, the dried and ground feces with acetone containing 5% acetic acid. By this procedure, 70 to 80% of the total DDT-derived products present can usually be extracted. The unextracted materials appear to be largely aqueous-alkali–soluble products which respond only very feebly to the Schechter-Haller method. The work reported here is confined primarily to the acetone-extractable products. In general, the acetone-extractable, DDT-derived products occurring in rat feces after oral ingestion of DDT consist of 25 to 50% of materials which are predominantly acidic and give a red Schechter-Haller color. The remaining material is DDT as shown by a typical blue Schechter-Haller color as well as by direct ultraviolet spectra.

Davidow Column Separation. The red Schechter-Haller–positive materials are obviously metabolic products of the orally ingested DDT and their acidic nature is evident by the fact that they can be isolated from the DDT by extraction from an ether solution with aqueous alkali and can be recovered with ether from the alkaline solution after acidification. This procedure has been used as a general method for separating the DDT and red Schechter-Haller metabolic products occurring in rat feces. Evidence that the metabolites are not DDE or DDA is given in Table I. According to these data, the fecal metabolites are retained by both the regular and modified Davidow (*1*, *9*) columns, whereas DDE is passed by both and DDA by the modified column only. However, careful examination of some samples of fecal metabolites shows that DDE may occur in amounts up to 2% of the total Schechter-Haller material present.

In another experiment, an aliquot of an extract of fecal metabolites was refluxed with 6*N* sulfuric acid for 6 hours. Treated and untreated aliquots of the metabolites were then passed through modified Davidow columns in the usual manner. The results are presented in

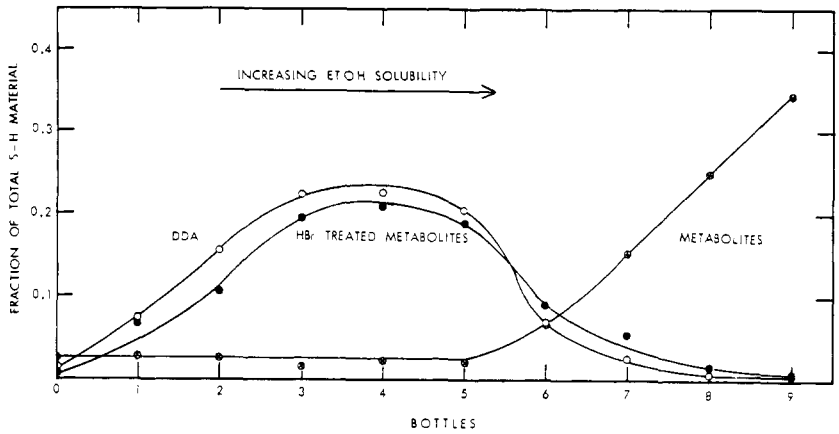

Figure 1. Distribution of DDA, fecal metabolites, and HBr-treated metabolites in iso-octane–ethyl alcohol system

Table I. Behavior of DDE, DDA, and Fecal Metabolites of DDT in Davidow Columns

Material	Micrograms	Davidow Column	Recovery, %
DDE	50	Regular[a]	101.0
		Modified[b]	100.0
DDA	50	Regular	2.2
		Modified	97.0
Metabolites	44	Regular	0.0
		Modified	0.0

[a] Fuming H_2SO_4 and concd. H_2SO_4, 1 to 1.
[b] Concd. H_2SO_4 only.

Table II. Behavior of DDA and Metabolites in Modified Davidow Column after Treatment with 6N Sulfuric Acid

Identification	DDA, γ Present	DDA, γ Recovered[a]
DDA, untreated	100	97
DDA, treated	100	90
Metabolites, untreated	60	0
Metabolites, treated	280	188

[a] After going through column. Metabolites calculated as DDA in all cases.

Table II, along with data on untreated DDA and DDA subjected to the acid treatment. The acid "hydrolysis" has little effect on the behavior of DDA in the modified Davidow column, but the metabolites are apparently partially hydrolyzed, so that they behave similarly to DDA. This suggests that the metabolites are complexed forms of DDA.

Countercurrent Distribution. As a means of further differentiating between DDA and the metabolites, countercurrent distribution techniques were investigated. The acidic constituents of feces from control rats and from rats on a DDT-fortified diet were subjected to a 10-bottle, countercurrent system using iso-octane and 95% ethyl alcohol as the solvent pair at a 6 to 1 ratio. Pure *p,p'*-DDA was added to the control sample and used as a reference. After distribution, Schechter-Haller analyses were made of the total content of each bottle. The distribution behavior of the DDA and of the metabolites is shown in Figure 1—also distribution of hydrogen bromide–treated metabolites.

The distribution of *p,p'*-DDA is totally different from that of the unknown DDT metabolites. The greater solubility of these metabolites in ethyl alcohol indicates that they are more polar than DDA. All other investigations using various countercurrent distribution systems and different solvent pairs gave essentially the same results: The fecal acidic metabolites of DDT are not simply DDA.

All attempts to isolate, purify, and characterize the extractable fecal metabolites by countercurrent distribution systems failed to give satisfactory results. Also many chromatographic methods including both paper and column techniques have been tried for this purpose without notable success. Recently, however, a partition column used by Mosbach, Zomzely, and Kendall (*3*) for separation of bile acids has been tried and found very effective in separating the acetone-extractable acidic metabolites of DDT in rat feces.

Mosbach Partition Column. A number of daily samples of feces collected from a rat which had been maintained continuously for about 9 weeks on a diet containing 400 p.p.m. of carbon-14–labeled DDT (*5*) were combined, dried, and ground. This composite sample was used for all subsequent work involving radioactive fecal materials. A summary of analyses of this sample is presented in Table III. Several points are noteworthy. By direct nitration of the dry feces using severe nitrating conditions, only 80% of the total reactive material could be accounted for by the Schechter-Haller method. If the regular nitrating conditions are used, only about 50% recovery can be realized. Also, the Schechter-Haller data on the extract are 14% low as compared to radioassay. About 40% of the red Schechter-Haller materials calculated as DDA are lost on extraction, whereas the DDT is essentially quantitatively recovered.

Published results for fecal analyses using the Schechter-Haller method are open to serious question. While it was not the primary purpose of this study to make a balance study, 95% of the total radioactive DDT ingested by the rat involved was accounted for by radioassay of the feces (68%), fat (26%), and urine (1.3%).

Radioactive acidic metabolites for studies with the Mosbach column were prepared by evaporating aliquots of acetone extracts of the sample of radioactive feces (Table III) and partitioning the oily residue between 2% aqueous sodium hydroxide and ether. The acidic metabolites were recovered from the

Table III. Schechter-Haller Analysis and Radioassay of Feces from Rat Fed C^{14}-Labeled DDT

	S-H[a], P.P.M. DDA[b]	DDT	Total as DDT	Radio Assay, P.P.M. as DDT	% Unaccounted for by S-H
Direct	490	542	1154	1440	20
Acid acetone extract	292	539	904	1046	14
Difference	198	3	250	394	
% loss on extraction	40.4	0.6	21.6	27.4	

[a] Red S-H materials calculated as DDA.
[b] A severe nitration procedure was used; regular procedure will give only about 700 p.p.m. total as DDT on direct nitration.

alkali phase by acidifying and extracting with ether. For use on the column, ether aliquots were evaporated and the residue taken up in glacial acetic acid. The modified Mosbach column was used in all subsequent work.

A glass column with an outer diameter of 1.3 cm. was packed to a height of 33 cm. with a mixture of 15 grams of Celite 545 impregnated with 12 ml. of 70% aqueous acetic acid and made into a slurry with iso-octane. The acidic metabolites were added to the column in 1 ml. of glacial acetic acid, and the column was eluted in accordance with the schedule given in Table IV. All eluents were saturated with 70% aqueous acetic acid before passing through the column.

Rate of flow used was 0.7 to 1.5 ml. per minute. Eluate fractions of 7 to 12 ml. were collected. Radioassay of the fractions were made directly by evaporating aliquots on stainless steel planchets and measuring the activity in a gas flow counter. The radioactivity *vs.* fractions collected was then plotted. Figure 2 gives a typical elution curve which exhibits five and possibly six peaks, indicating an equivalent number of different metabolites. This type of curve has been reproduced several times. Thus, the metabolites encountered in rat feces seem to be more complex than originally anticipated.

As a check on the chromatographic procedure itself, the metabolite eluted with 60% isopropyl ether was rechromatographed. The metabolite again appeared primarily in the 60% isopropyl ether indicating that the chromatographic procedure gives a true, reproducible separation of the metabolites.

The behavior of DDA itself in the acetic acid column was studied by chromatographing 200 γ of radioactive DDA along with 600 γ of nonradioactive fecal metabolites. The radioactive DDA appeared quantitatively in the iso-octane eluate. The metabolite appearing in the iso-octane fraction therefore, is either DDA or a material so similar to it that this chromatographic procedure does not differentiate between them. The latter view is supported by the data in Table I on the modified Davidow column, which show that substantially all the acidic metabolites are retained by this column, whereas DDA is not.

The data in Table II indicated that the metabolites were partially converted to DDA when refluxed with 6*N* sulfuric acid. This was studied further in an effort to determine the effect of both acid and alkaline hydrolytic treatments on the partitioning of the metabolites by the Mosbach column.

Three aliquots of acidic metabolites were subjected to the following experiment: Aliquot A received no treatment,

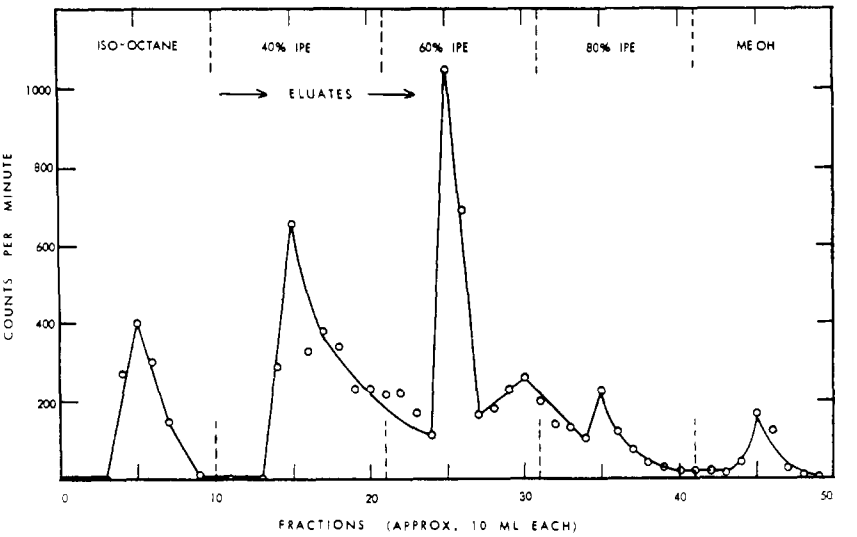

Figure 2. Separation of fecal acidic metabolites by modified Mosbach column

aliquot B was refluxed for 6 hours with 6*N* sulfuric acid, and aliquot C was refluxed with 10% sodium hydroxide for 6 hours. The treated products were recovered in ether, transferred to glacial acetic acid, and chromatographed in the modified Mosbach column (Table V). The metabolites are shown to be more sensitive to the acid treatment than to the alkaline treatment, indicating that the metabolic complexes are not esters. The acid treatment converted most of the metabolites occurring in eluates 3, 4, and 5 before treatment to a product which appears in the first eluate. The alkaline treatment had little effect, except for the apparent conversion of about 55% of the product which appeared in eluate 5 (methanol eluate), before treatment, to a product which appeared largely in eluate 2 (40% isopropyl ether), after treatment.

In another experiment, the acidic metabolites were subjected to treatment in an autoclave in the presence of 6*N* sulfuric acid. The data from this experiment are summarized in Table VI; prolonged treatment with sulfuric acid at high temperatures converts most of the materials to a product which appears in the first eluate of the column, thus behaving as DDA.

In early work with nonradioactive metabolites, some data indicated that the metabolites were effected by a Clemmensen reduction. To follow up this lead, an aliquot of radioactive acidic metabolites was subjected to the well known red phosphorus–hydriodic acid reduction reaction in acetic acid and the resulting products were chromatographed in the modified Mosbach column. An untreated equal aliquot and another equivalent aliquot, after refluxing with acetic acid, were also chromatographed. A summary of the chromatographic data on the three aliquots is presented in Table VII. A significant

Table IV. Eluents Used in Modified Mosbach Column

Eluate	Ml.	Eluent
1	120	Iso-octane
2	120	40% IPE[a] in iso-octane
3	120	60% IPE in iso-octane
4	120	80% IPE in iso-octane
5	120	Methanol

[a] IPE = isopropyl ether.

Table V. Effect of Acid and Alkali Treatment on Metabolites

	Total Radioactivity In Eluates, %		
Eluates	No treatment, A	Acid hydrolysis, B	Alk. hydrolysis, C
Iso-octane	32	65	30
40% IPE	26	22	37
60% IPE	14	6	15
80% IPE	10	5	12
MeOH	18	2	8

Table VI. Effect of Sulfuric Acid on Metabolites Autoclaved at 120° C.

	Total Radioactivity in Eluates, %			
		Autoclaved, Hours		
Eluates	No treatment	3	21	42
Iso-octane	28	63	78	83
40% IPE	23	20	18	12
60% IPE	35	11	4	5
80% IPE	12	5	0	0
MeOH	2	1	0	0

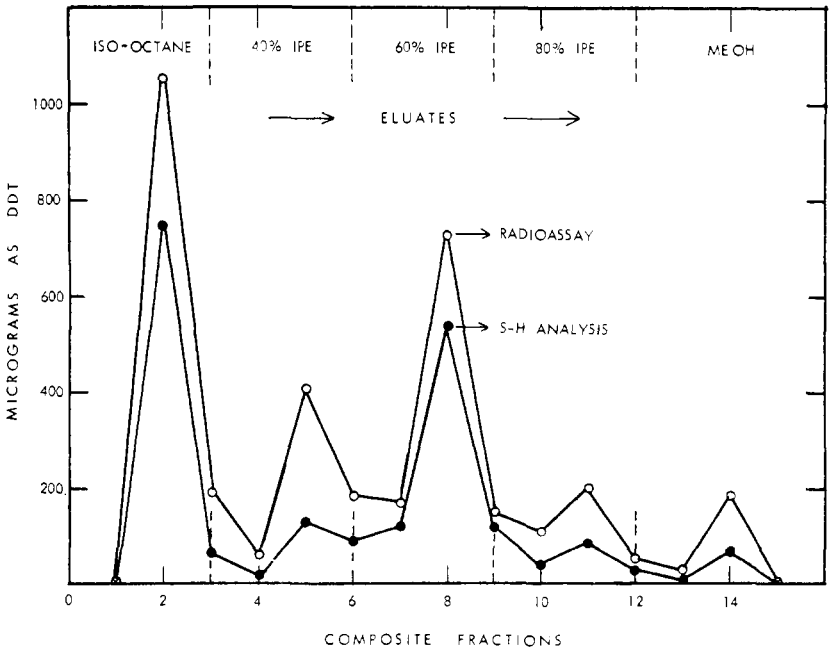

Figure 3. Radioassay and Schechter-Haller analysis of fecal metabolites separated by modified Mosbach column

effect was apparently produced on the metabolites by the red phosphorus–hydriodic acid treatment which tended to convert them to a product appearing in the first eluate. It seemed doubtful that this was actually a reduction effect, as hydriodic acid can cleave ether linkages and/or could produce an effect identical with the sulfuric acid treatments (Tables V and VI). Hence, radioactive acidic metabolites were subject to treatment with hydrobromic acid.

An aliquot of metabolites was refluxed with 48% hydrogen bromide for 5 hours. The products were extracted with ether, transferred to glacial acetic acid, and chromatographed in the modified Mosbach column. An equal aliquot of untreated metabolites was chromatographed simultaneously in the same column. The results presented in Table VIII show that the hydrobromic acid treatment is very effective in converting the metabolites to a product appearing in the first eluate.

The material obtained from the iso-octane eluate (Table VIII) from chromatographing the hydrogen bromide–treated acidic metabolites was subjected to countercurrent distribution using the iso-octane–ethyl alcohol system described earlier. Figure 1 illustrates the behavior of the hydrogen bromide–treated metabolites, untreated metabolites, and also DDA in the countercurrent system employed. The hydrogen bromide treatment has converted the metabolites to a product which behaves identically to DDA in this system—strong evidence that the acidic metabolites in rat feces are largely complexes or conjugates of DDA.

The metabolites of DDT in rat feces apparently do not respond quantitatively to the Schechter-Haller method of analysis—see Table III, for example. Accordingly, it was thought desirable to compare radioassays and Schechter-Haller analyses of the several fractions separated by the modified Mosbach column. For this purpose, a feces extract containing about 4 mg. of radioactive acidic metabolites was separated with the modified Mosbach column. Individual fractions from approximately the first, middle, and last portions of each eluate were combined into composite fractions. All composite fractions were radioassayed and analyzed by a modified Schechter-Haller method, which consisted of nitrating with 20 ml. of nitrating mixture for 2 hours. These radioassay and modified Schechter-Haller data for all composite fractions are presented in Figure 3. Composite samples representing the middle portion of each eluate were also analyzed by the regular Schechter-Haller procedure. A summary of radioassay, regular, and modified Schecther-Haller data on these composite samples is presented in Table IX.

These data well illustrate the serious error which can be introduced in assuming that the Schechter-Haller method is infallible. Thus, by the regular nitrating procedure, the Schechter-Haller analyses for all eluates average less than 50% of the radioassays. This might suggest that there is more than one metabolite present in each eluate—some of which are not Schechter-Haller positive. However, the increase in Schechter-Haller–positive material realized by increasing the severity of the nitrating conditions argues against such an interpretation. More conclusive evidence that each eluate consists principally of a single metabolite or mixture of very closely related metabolites, some of which are at least qualitatively Schechter-Haller positive, is furnished by the plot of the radioassays and modified Schechter-Haller analyses shown in Figure 3. The peaks shown in Figure 3 for both radioassay and Schechter-Haller assay would probably not parallel each other so well if there were any DDT metabolites present which were completely non–Schechter-Haller positive.

The iso-octane eluate obtained by chromatographing acidic metabolites, previously treated by autoclaving with 6*N* sulfuric acid (see Table VI), was examined radiometrically and by the modified Schechter-Haller method. The results agreed within 8%, indicating that substantially all of the acetone extractable acidic metabolic products of DDT in the feces contain the bis(chlorophenyl) moiety of DDT.

Bile Metabolites

Experimental Early analyses by the Schechter-Haller method (*6*, *9*) of small samples of bile from rats on DDT-fortified diets indicated the presence of acidic, red Schechter-Haller–positive materials, and in one case about 10% of the total Schechter-Haller–positive material was DDT. The total amounts of Schechter-Haller–posi-

Table VII. Effect of Red Phosphorus–Hydriodic Acid Reduction on Metabolites

Eluates	Total Radioactivity in Eluates, %		
	No treatment	Refluxed with acetic acid	Reduction treatment
Iso-octane	14	15	56
40% IPE	31	24	22
60% IPE	39	43	12
80% IPE	11	12	6
MeOH	5	6	4

Table VIII. Effect of Hydrobromic Acid on Metabolites

Eluates	Total Radioactivity, %	
	No treatment	Refluxed with 48% HBr
Iso-octane	28	78
40% IPE	23	18
60% IPE	35	4
80% IPE	12	0
MeOH	2	0

Table IX. Analysis of Composite Fractions from Separation of Metabolites with Mosbach Column

	Micrograms Calculated as DDT		
	Radioassay, wet combustion	Schechter-Haller	
Fraction		Regular nitration	Modified nitration
2	1055	524	751
5	405	105	134
8	730	435	542
11	200	70	86
14	186	50	65

tive material found appeared suspiciously low and the DDT metabolites were probably not responding completely to the Schechter-Haller method as had been observed in the case of fecal metabolites. The use of carbon-14–labeled DDT offered the best means of resolving this question.

In an exploratory experiment, a male white rat was treated intravenously with carbon-14–labeled DDT—radiolabel in tertiary carbon position—(5) at the rate of 17.4 mg. per kg. The dose of 3.9 mg. was applied in a gum acacia–peanut oil emulsion. Bile was collected continuously by cannulation of the bile duct, each day's collection being kept as a separate sample. The animal, given daily doses of 5 to 20 ml. of a solution containing 5% dextrose and 0.4% sodium chloride by infusion into the stomach, survived 9 days of bile collection. The daily bile samples were strongly acidified with acetic acid and extracted three times with ether. Both the water and ether phases were assayed for radioactivity after each extraction by evaporating small aliquots on planchets and counting them in a gas-flow counter. This procedure was used to indicate when all ether-soluble material had been recovered. Finally, all ether extracts were combined and radioassayed by direct counting of aliquots on planchets. The combined ether extracts were also analyzed by the Schechter-Haller method. An estimate of the DDT equivalent remaining in the water phase, after three ether extractions, was made by radioassay.

A summary of the analytical data obtained is presented in Table X. For the sake of brevity, complete radioassay data on ether and water phases are given for only four of the nine samples collected. However, complete data on the combined ether extracts and final water phase are shown for all samples. The relative activities in column 4 and 5 show that two extractions of the acidulated bile with ether essentially exhaust the bile of ether-soluble metabolites. The small amounts of activity observed in the third ether extract are undoubtedly due to incomplete separation of the phases. The activity of the water phase remains substantially constant after two extractions. Columns 6, 7, and 8 of Table X indicate the proportion of the DDT-derived material in the samples which is not extractable with ether. The average amount of radioactive material remaining in the water phase of the first six samples is 12%. In a separate experiment, 17% was found nonextractable with ether. The high recovery of radioactive material, 65% (2.55 mg.) of the amount administered (3.90 mg.), indicates the importance of bile in the excretion of these materials. The results (Table X) show again that the Schechter-Haller results are much lower than the radioassay values. Furthermore, these metabolites are acidic and give a red Schechter-Haller color.

An opportunity to obtain further information on bile metabolites occurred in connection with the studies on fecal metabolites. The bile duct of a rat on a diet containing 400 p.p.m. of carbon-14 DDT was cannulated at the end of the 9-week feeding period. Because of inadequate feeding methods, only about 10 ml. of bile was collected before the animal died on the second day of bile collection.

Exploratory analyses of this bile by both radioassay and the Schechter-Haller method again indicated that only about 50% of the total DDT-derived material present could be accounted for by the Schechter-Haller method. Moreover, the ether extractable material was largely acidic and red Schechter-Haller positive. To get some indication of the nature of the metabolites and to determine where losses occur in the Schechter-Haller analysis, duplicate aliquots of bile containing 175 γ of radioactive products calculated as DDT were acidified and extracted exhaustively with ether. An aliquot of one of the ether extracts was subjected to the regular Schechter-Haller procedure and a similar aliquot from the other extract was subjected to a modified Schechter-Haller procedure which involved severe nitrating conditions. In both cases, the radioactive products were traced by radioassay through each step of the Schechter-Haller procedures used. To provide a common basis for comparison, the data are expressed as parts per million of the original bile (Table XI).

The radioassay data presented in Table XI were obtained by oxidation of the

Table X. Radioassay and Schechter-Haller Analyses of Carbon-14–DDT Metabolites in Daily Rat Bile Samples[a]

Day after Dose	Bile Sample, Ml.	Ether Extract Number	Radioassay C.P.M./Ml. Ether	Radioassay C.P.M./Ml. Water	Radioassay P.P.M. DDT[c] Ether	Radioassay P.P.M. DDT[c] Water	% in water	S-H Analysis[b] of Ether Extracts P.P.M.	S-H Analysis[b] of Ether Extracts % of radioassay
1	11.5	1	940	270					
		2	200	240					
		3	70	250	10.0	1.3	11.5	6.6	66.0
2	10.6	1	1650	330					
		2	320	300					
		3	90	330	16.0	2.1	11.6	7.4	46.3
3	11.6	1	2480	660					
		2	290	510					
		3	130	500	27.0	2.8	9.4	12.0	44.4
4	10.6	...	...	...	45.0	7.7	14.6	20.4	45.3
5	7.1	...	...	...	62.0	9.2	12.9	26.4	42.6
6	8.3	...	...	...	68.0	9.6	12.4	24.7	36.3
7–8	4.8	...	...	...	24.0	7.9	24.8	7.0	29.2
9	1.3	...	...	...	13.0	7.7	37.0	...	...
	1.6	1	160	140					
		2	70	170					
		3	60	180	9.0	6.8	43.0	...	...

[a] Rat treated intravenously with 3.9 mg. C^{14} *p, p'* DDT (17.4 mg./kg.).
[b] All red S-H material; no DDT in evidence. Calculated as DDT.
[c] Calculated as DDT.

Table XI. Radioassays vs. Schechter-Haller Analyses of Rat Bile

Item	P.P.M. as DDT			
	Extract 1[a]		Extract 2	
	Radio-assay	S-H	Radio-assay	S-H
1 Ether extract of bile	145.0	...	147.0	...
2 Nitrated products	...	...	142.0	...
3 Benzene solution for color development	57.0	53.0[b]	83.0	90.0[c]
4 Acid phase after extraction of item 2	27.0	...	10.5	...
5 Ether extract of acidified alkali wash	48.0	...	43.0	...
6 Water phase from item 5	...	...	4.8	...
Total of 3, 4, 5, and 6	132.0	53.0	141.3	90.0

[a] Water phase contained 30 p.p.m. by radioassay and 8.7 by S-H (severe nitration).
[b] Regular nitration (1-hr. nitration with 5 ml. nitrating mixture).
[c] Severe nitration (2-hr. nitration with 20 ml. nitrating mixture).

samples to carbon dioxide, precipitation of the carbon dioxide collected in alkali as barium carbonate, and assay of filter cakes of the latter at infinite thickness (*10*). Generally this procedure agrees within 5% with Schechter-Haller analyses of known amounts of carbon-14 DDT added to extracts of various biological materials.

The data on radioassays of the duplicate ether extracts agree very well. The water phase from one extract was assayed and found to contain 30 p.p.m expressed as DDT (see footnote to Table XI). Thus, 17% of the metabolic products were not extractable with ether.

Radioassay of the nitrated products from extract 2 showed 142 p.p.m. as compared to 147 p.p.m. by direct assay of the original ether extract—little or no loss occurred in the nitration process. The radioassay of the nitrated products from extract 1 was lost during assay.

The radioassay of the completely processed benzene solutions (item 3, Table XI) used for Schechter-Haller color development agree reasonably well with the Schechter-Haller calculated values in the case of both extracts. However, both radio and Schechter-Haller assays of the completely processed benzene solutions (item 3) are very much lower than the original radioassays of the ether extracts (item 1). From item 4, extract 2, Table XI, it is noted that 10.5 p.p.m. were lost in recovering the nitrated products in benzene as this amount was left in the acid phase. Almost three times this loss occurred in the acid phase in the case of the regular nitration. Tests with DDT have shown no loss in this step of the Schechter-Haller method as used in this laboratory.

The benzene solution of the nitrated products is routinely washed with dilute alkali until the alkali phase is colorless (two or three times, usually) and then washed with water and dried with anhydrous sodium sulfate. In Table XI, the alkali wash was acidified and extracted with ether (item 5) and aliquots of the ether solution were radioassayed. The greatest losses in the Schechter-Haller procedures used for the two extracts occurred at this point. The ether extracts of the acidified alkali washes were subjected to renitration but no Schechter-Haller colors could be observed. Thus, the DDT-derived material which appears in the alkali wash is very resistant to nitration. As indicated by item 6, extract 2, the water phase from the ether extraction of the acidified alkali wash contained about 5 p.p.m. of DDT equivalent; the water phase from extract 1 was not examined.

In the case of extract 2, 141.3 p.p.m. or 96% of the 147 p.p.m. found in the original ether extract were accounted for by radioassay at each major step in the Schechter-Haller procedure. The recovery by radioassay at identical points (except for item 6) for extract 1 was also good—i.e., 91%.

DDT, DDE, and DDA in Bile

The Schechter-Haller method as employed in this laboratory is insensitive to DDT in a ratio of less than about 1 to 10 with materials like DDE and DDA which give a red color; it is uncertain beyond ratios of 1 to 5. DDE and DDA or other red Schechter-Haller acidic metabolites can be distinguished from each other only by their neutral and acidic properties or by chromatographic techniques. In general, the early Schechter-Haller analyses of bile made in this laboratory, although uncertain, indicated that small amounts of DDT may occur in bile but probably only trace amounts of DDE. The Davidow chromatographic column (*2, 9*) provides a way of demonstrating the presence of both DDT and DDE in one with considerable certainty. Accordingly, a bile sample obtained from several white rats, treated intravenously with DDT, was examined using this column. The rats were given, by way of the femoral vein, an emulsion containing 2% *p,p'*DDT, 20% peanut oil, 1.2% Asolectin (a purified soybean lecithin), 0.8% Span 20, 0.8% sodium chloride, and 0.1% sodium cholate. This emulsion formulation has been found well tolerated physiologically. Bile was collected from each animal for about 50 hours following cannulation and DDT administration. A large aliquot of the combined samples was extracted with ether and the extract was transferred to carbon tetrachloride and chromatographed in a Davidow column. Schechter-Haller analyses of the original extract for acidic constituents and of the eluate from the Davidow column are presented in Table XII along with other details of the experiment.

The acidic metabolites shown in Table XII are retained by the Davidow column, whereas the DDT and DDE are found in the eluate. A two-color analysis for DDT and DDE in the eluate is valid because the ratio of DDT to the red DDE is in an accurate range—i.e., 3.3 to 1. In general, the data in Table XII indicate that while the bulk of the products are acidic materials, some DDT and a smaller amount of DDE are no doubt present. As indicated in Tables X and XI, it is likely that the 355 γ of acidic metabolites shown in Table XII and found colorimetrically represent only about half of the actual total because of incomplete response to the Schechter-Haller method, and thus the relative amounts of DDT and DDE would be further reduced.

Similar experiments in which a single oral dose of DDT was given in peanut oil emulsion at 100 mg. per kg. to an animal whose bile duct was cannulated showed that about 3% of the total DDT-derived material found in the bile by Schechter-Haller analysis was present as DDT and about 1% was in the form of DDE. DDT and DDE do not occur in significant amounts in rat bile in the case of either intravenous or oral application of DDT.

To determine if some of the biliary DDT metabolites were present in the form of free DDA, recourse was made to

Table XII. DDT, DDE, and Acidic Metabolites in Bile from Rats Treated Intravenously with DDT[a]

Products	Total Calcd. as DDT	P.P.M. as DDT	% of Total
Acidic metabolites[b] (ether extract)	355	7.2	89
DDT } Eluate from Davidow column	33	0.68	8
DDE } Eluate from Davidow column	10	0.20	3

[a] 48.2 ml. of bile was collected from 3 rats given a total of 17 mg. of DDT (25 mg./kg.) and 1.0 ml. was obtained from a rat given 10 mg. (50 mg./kg.).
[b] An aliquot of ether extract was extracted with aqueous alkali, latter was acidulated and extracted with ether, which was then analyzed by the S-H method using regular nitration.

the modified Davidow column used for the fecal metabolites (7). An ether extract of the same bile sample for which data are shown in Table XI was prepared. Seventy per cent of the total extracted material was retained by the column. The retained material is most probably complexed DDA similar to fecal metabolites. However, the 30% that passed through represents free DDA (except for small amounts of DDT and DDE). Other experiments in which rats were given single oral doses of nonradioactive DDT at 50 mg. per kg. showed again that 25 to 35% of the metabolites found in the bile was free DDA.

Bile as a Source of Fecal Metabolites of DDT

Bile seems to be the principal source of DDT metabolites occurring in rat feces after intravenous or oral application of DDT. The most direct evidence for this is that observed with animals whose bile ducts were cannulated so that the bile was prevented from entering the intestine before giving the animals carbon-14–labeled DDT intravenously. In three instances, including the rat involved in the experiment presented in Table X, both feces and urine from the animals were collected and examined radiometrically for DDT-derived materials. In all cases, only very small amounts of radioactive material, usually well under 5% of the original dose, were found in the excreta. For example, in the case of the rat used for the data in Table X, only 0.3% of the dose was found in the feces and 2% in the urine, whereas 65% was found in the bile.

In earlier experiments in which rats were treated intravenously without cannulation of the bile duct and diversion of the bile, relatively large amounts of red Schechter-Haller material were found in the feces from these rats. Thus, with intravenous application, very little DDT-derived material appears in the feces when the bile is diverted; but, if the flow of bile to the intestine is undisturbed, large amounts of metabolites appear in the feces. This observation combined with the evidence (Table X) that an intravenous dose of DDT is largely excreted through the bile is strong support for bile as the principal source of fecal metabolites.

Discussion

Almost all of the DDT metabolites found in feces and bile are acidic in nature. Small amounts of DDE are found in both bile and feces and small amounts of DDT can be found in the bile. The relatively large amount of DDT in the feces probably represents unassimilated material.

By using carbon-14–labeled DDT, it has been found that serious losses of DDT-derived material may occur in the extraction and colorimetric estimation by the Schechter-Haller method in both feces and bile. As much as 25% of the metabolites are not extractable by organic solvents. The Schechter-Haller method gives quantitative results for the analysis of DDT, DDE, and DDA. However, in the determination of the unknown acidic materials found in both feces and bile, serious discrepancies occur when compared to radioassay. Increasing the severity of nitrating conditions increases the colorimetric recovery somewhat, indicating that they are resistant to nitration rather than being totally unresponsive. The bis(chlorophenyl) moiety is, therefore, probably intact, and the resistance to nitration is due to an added constituent.

The fecal acidic metabolites differ from free DDA in a number of respects. The acidic metabolites are retained by a modified Davidow column, whereas DDA is not. Countercurrent distribution gives a different distribution pattern for the metabolites than for DDA. Furthermore, these metabolites can be separated, by a chromatographic procedure, into a number of fractions, which vary in their quantitative response to the Schechter-Haller method, whereas DDA gives quantitative results.

The experimental data presented are not sufficient to fully elucidate the chemical nature of these metabolites. Studies indicate, however, that they consist primarily of complexed DDA. Thus, after treatment with acids, a large proportion of the metabolites are converted to a material which is similar in its behavior to DDA. This material is no longer retained by the modified Davidow column and its distribution in the countercurrent system employed is similar to that of DDA. The amount of conversion is proportional to severity of conditions during the acid treatment. This is shown by the shift of peaks in the chromatographic pattern toward the DDA peak. Furthermore, analysis of the hydrolyzed material found in the DDA peak showed agreement between the Schechter-Haller and radiometric method within 8%.

In other experiments, not presented, the hydrolyzed product had an ultraviolet spectrum essentially identical with that of DDA. That these complexes are not glucuronic acid esters is indicated by their stability to alkaline hydrolysis. Moreover, qualitative tests failed to show the presence of glucuronic acid. Their stability to alkaline hydrolysis and instability to acid hydrolysis suggests an amide linkage such as that represented by hippuric acid, an amide conjugate of benzoic acid and glycine, which is produced when animals are fed benzoic acid. On the other hand, the behavior of the metabolites in the Mosbach column suggests a possible complexing of DDA with bile acids or related compounds, as separation is achieved in a manner similar to that of the cholanic acids for which this column was originally developed.

The bile is apparently the principal source of DDT metabolites found in feces. Sixty-five per cent of a dose of DDT given intravenously was found in the bile collected by cannulation of the bile duct. Furthermore, practically no metabolites are found in the feces of cannulated animals, but when the bile flow is unhindered, relatively large fecal amounts are found.

The fecal and bile acid metabolites resemble each other, in that both consist partially of materials that cannot be extracted by organic solvents; both are acidic and both fail to give quantitative results by the Schechter-Haller method. In addition, the bile metabolites can be fractionated by the same chromatographic system used for fecal metabolites. However, as bile appears to contain appreciable amounts of free DDA, whereas no significant amounts have been found in the feces, the bile metabolites are not necessarily identical. Bile metabolites may be subject to modifications after entering the intestinal tract.

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