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Excreted Metabolites of 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane in the Mouse and Hamster

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Swiss mice and Syrian golden hamsters were fed 250 μg/g of dietary DDT for up to 4 months and urinary and fecal metabolites were investigated. Both species excreted the base labile glucuronide of bis(*p*-chlorophenyl)acetic acid (DDA) as the principal urinary metabolite. The more stable glycine and alanine conjugates of DDA were also found. The mouse differed from the hamster in excreting DDE in the urine. The excretion of DDE increased during the course of the experiment until in the fourth month it was nearly as prominent as DDA. Endogenously formed cinnamoylglycine was also excreted by the mouse in amounts that increased upon DDT administration. The feces of both species contained DDD and DDT. Fecal excretion was not an important route for the elimination of polar DDT metabolites in these animals.

A comparative study of the urinary and fecal metabolites of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) in the hamster and the mouse is of interest since these species respond differently to both acute and chronic administration of the compound. The LD_{50} in Swiss mice is about 300 mg/kg while in hamsters it is greater than 2000 mg/kg, solubility limitations making an accurate assessment difficult (Gingell and Wallcave, 1974; Agthe *et al.*, 1970). In chronic feeding studies DDT was a liver tumorigen in several mouse strains (reviewed by Terracini *et al.*, 1973) but the Syrian golden hamster was resistant (Agthe *et al.*, 1970).

We have attempted to identify urinary and fecal DDT metabolites in these species and, in long-term feeding studies, to determine whether there were qualitative or quantitative time-dependent changes in the metabolites. These studies were continued for 16 days in animals fed 100 μg/g of dietary DDT and for about 4 months in mice and 3 months in hamsters fed 250 μg/g. Additional information concerning the total number of conjugated forms of excreted metabolites was obtained from acute oral administration of ^{14}C-labeled DDT.

There are no reports to our knowledge on the identification of urinary or fecal DDT metabolites in the hamster. Mouse urine was investigated in some detail by Apple (1968) who reported bis(*p*-chlorophenyl)acetic acid (DDA) as the principal excreted metabolite.

EXPERIMENTAL SECTION

Chemicals. DDT of >99.0% purity (glc check) was obtained from Geigy Agricultural Chemicals, Ardsley, N. Y. Randomly ring-^{14}C-labeled DDT (64 μCi/mg) was obtained from the Radiochemical Centre, Amersham, Bucks, England. DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene], DDD [1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethane], and DDA were purchased from Pfaltz and Bauer, Flushing, N. Y. Bovine liver β-glucuronidase was purchased from Sigma Chemical Co., St. Louis, Mo.

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The glycine and alanine conjugates of DDA were made in about 40% yields by the following procedure. Crude bis(*p*-chlorophenyl)acetyl chloride was prepared by refluxing 0.005 mol (1.4 g) of DDA and 3 ml of thionyl chloride for 2 hr and removing excess of the latter *in vacuo* at 50°. The acid chloride was added in three portions with vigorous shaking to an ice-cold solution of 0.006 mol of the amino acid in 10 ml of 7% (w/v) NaOH. After acidification of the solution with concentrated HCl the precipitated DDA conjugate was dried on filter paper, extracted with small portions of ether to remove unreacted DDA, and recrystallized from benzene–ethyl acetate (9:1). Methyl esters of the conjugates were made by reaction with ethereal diazomethane and recrystallized from benzene.

These conjugates have not previously been reported. They had the following characteristics (analyses by Micro-Tech Labs., Inc., Skokie, Ill.): *N*-bis(*p*-chlorophenyl)acetylglycine (DDA-Gly), colorless needles, mp 153° (*Anal.* Calcd for $C_{16}H_{13}O_3NCl_2$: C, 56.82; H, 3.88; N, 4.14; Cl, 20.96. Found: C, 56.88; H, 3.85; N, 3.96; Cl, 21.06); methyl ester, mp 159°; *N*-bis(*p*-chlorophenyl)-acetyl-DL-alanine (DDA-Ala), colorless platelets, mp 163° (*Anal.* Calcd for $C_{17}H_{15}O_3NCl_2$: C, 57.98; H, 4.29; N, 3.98; Cl, 20.14. Found: C, 58.10; H, 4.27; N, 3.89; Cl, 19.92); methyl ester, mp 135°.

Cinnamoylglycine was prepared similarly, mp 191° (lit. 193°). It has been described by Dakin (1909). The methyl ester, made by reaction with diazomethane, had mp 89°.

Preparation of Diets. Stick food was required for mice and pellets for hamsters. Sticks containing DDT were prepared by spraying a 1% acetone solution of DDT onto 1 kg of powdered diet (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.). After mixing on a roller, a hot 5% gelatin solution was added, a paste formed, and the mixture extruded into sticks using a cake decorating implement. The sticks were dried at room temperature and had a gelatin content of about 4%. Sticks not containing DDT were made for control purposes. DDT was added to the hamster diet by dropping the acetone solution onto Wayne Lab-Blox pellets and allowing the solvent to evaporate.

Since all the pellets were eaten in these experiments nonuniformities in DDT distribution were of little consequence.

Sticks were similarly made from a semisynthetic diet containing casein (20%), corn starch (59%), corn oil (10%), fiber (5%), mineral mix (4%), and vitamin mix (2%). Starch was purchased from CPC Industrial Division, Englewood Cliffs, N. J., and corn oil locally. The other components were obtained from Nutritional Biochemicals, Cleveland, Ohio.

The DDT content of the diets was checked by extracting a crushed 2.0-g sample with acetone for 6 hr in a Sohxlet apparatus and analyzing by glc. Deviations from nominal values did not exceed 5% and no significant alteration was noted on storage.

Collection of Urine and Feces (Long-Term Feeding). Random bred male Swiss albino mice and male Syrian golden hamsters from our colonies were 8–10 weeks old at the start of DDT feeding. Four animals of each species were used for each feeding group and the same animals were under test throughout the course of the experiment. In the 250-μg/g tests urine and feces were collected over a 24-hr period at weekly intervals. In the 100-μg/g studies urine and feces were collected and analyzed every 48 hr. During the collection period mice were housed in glass metabolic cages ("Metabowl," Jencons Ltd., Hemel Hempstead, England) and hamsters in stainless steel metabolic cages (Hoeltge Division of ESC, Cincinnati, Ohio). When not collecting, the animals were housed in plastic cages on corn-cob bedding ("Bed-o'cobs," Anderson Cob Mills, Maumee, Ohio). Control animals were maintained under the same conditions. All animals received food and water *ad libitum*. Urine and feces collections were pooled and kept frozen until analyzed.

Collection of Urine and Feces (Acute Administration). For autoradiography and counting experiments, another set of animals, 10–20 weeks old, was used. These animals were dosed *via* stomach tube with an olive oil solution of ^{14}C-labeled DDT. The specific activity of the DDT was adjusted so that each animal received 25 mg/kg of compound and approximately 50 μCi/kg of radioactivity. Urine and feces were collected over 5 days using the metabolic cages mentioned above. The urine was collected under toluene, and urine and feces frozen until analyzed.

Urine Extraction and Cleanup. In most cases an aliquot portion of urine (usually 10 ml) was made basic by addition of 0.2 ml of 10 *M* NaOH and allowed to stand for 15 min before acidifying to about pH 2 with concentrated HCl. However, acidification of the urine immediately after thawing will preserve the very base labile DDA–glucuronide conjugate (see below). The acidified urine was extracted by shaking for 1 min with 25 ml of ether (Fisher lab grade). Centrifugation of both phases was sometimes required to break emulsion formation. The $CaCl_2$-dried ether extract was evaporated and the residue used as such for tlc separations or treated with excess ethereal diazomethane for glc analysis.

A separation of methylated extracts into three fractions containing neutral metabolites, DDA methyl ester, and methyl esters of the DDA–amino acid conjugates, respectively, was possible on Florisil. The extract was dissolved in 2 ml of ether–hexane (1:4) and passed through a 7 mm i.d. column (*e.g.*, "Chromaflex" K-420100, Kontes Glass Co.) containing 1.0 g of Florisil (Fisher Scientific Co.). The three fractions were obtained by elution in sequence with 10 ml each of hexane and ether–hexane (1:4) and 15 ml of ether.

Feces Extraction. Feces (5–15 g) were crushed and stirred for 1 hr with 50 ml of methanol. The suspension was centrifuged, and a 20-ml portion removed, diluted with 10 ml of water, and extracted with 3 × 20 ml of ether–hexane (1:4). The combined extracts were dried and evaporated. The residue was dissolved in hexane and chromatographed on 1.0 g of Florisil using 20 ml of hexane to elute. The eluate, containing essentially only neutral metabolites, was analyzed by glc. Attempts to analyze extracts for polar metabolites by glc were unsatisfactory because of the complexity of the methylated mixtures. These mixtures were separated into neutral and polar fractions as described for urine extracts.

Analysis. A Beckman GC-45 gas chromatograph with flame ionization detectors was used for analyses and for collections. In the latter instance the chromatograph was used in conjunction with a 9:1 stream splitter and a Packard Model 850 fraction collector. Six foot × 4 mm i.d. glass columns were packed with 13.3% OV-1 on 80–100 mesh Chromosorb W or with 10% OV-17 on the same support. Isothermal oven temperatures of 225 or 240°, He carrier flow of 60 ml/min, and H_2 and air flows of 40 and 300 ml/min, respectively, were used.

Thin-layer chromatography was performed on 0.25 mm silica gel G glass plates (Analtech, Inc., Newark, Del.). Polar metabolites were separated with a benzene–dioxane–acetic acid (110:30:1.5) mixture and neutral metabolites with hexane.

Mass spectra were obtained with an AEI MS-9 spectrometer using the direct probe inlet. The ion source temperature was approximately 200° and electron energy was 70 eV. The distribution of radioactivity on tlc plates was determined by autoradiography and by scanning on a Packard 7201 radiochromatogram scanner. Quantitation of the radioactivity was performed by eluting the tlc bands with methanol and counting in a Nuclear-Chicago Unilux II scintillation counter.

A spot plate color test was developed as an aid in identification of polar DDT metabolites. The application of a chromate–sulfuric acid solution by means of a stirring rod or dropper to a well-dried portion of the extract residue or chromatographic fraction produced a transient reddish-purple coloration in the presence of amino acid conjugates of DDA. Ten micrograms of DDA-Gly or DDA-Ala gave an intense coloration and 1 μg was easily detected. The test was also positive and useful for DDT, DDA, and neutral metabolites but the color was not as intense. No false positives were obtained with the urine and feces samples investigated. The chromate solution was prepared by shaking 10 mg of sodium chromate or dichromate with 10 ml of concentrated H_2SO_4 and used within 1 hr of preparation.

RESULTS

Metabolites in Urine. Autoradiography of tlc plates spotted with urine extracts from [^{14}C]DDT dosed mice and hamsters revealed some ten or more radioactive bands when developed with the benzene–dioxane–acetic acid system. The predominant band in urine acidified to about pH 2 and extracted immediately after thawing remained at the origin and accounted for nearly 80% of the excreted radioactivity, but after mild alkaline hydrolysis radioactivity at the origin decreased sharply and the predominant band was then DDA. Making the urine 6 *N* in HCl and refluxing for 18 hr eliminated nearly all radioactivity except in the DDA band.

The base labile conjugate is probably the ester glucuronide since the band at the origin disappeared and the DDA band increased when urine was incubated at pH 4.5 with bovine liver β-glucuronidase. Furthermore this band exhibited a strong positive response to the naphthoresorcinol–trichloroacetic acid reagent (Dawson *et al.*, 1969) which gives a blue coloration with uronic acids.

Conjugates of DDA with glycine and alanine were also identified in the autoradiograms. These showed clearly that the glycine conjugate was the second most prominent metabolite in the urine of each species, but one or two as yet unidentified metabolites may occur in somewhat greater quantity than DDA-Ala.

Table I. DDT Metabolites in Mouse and Hamster Urine[a]

Species	Collect. period, days	Mean wt animal during period, g	DDT consumed		Total DDA excreted[b]		DDE excreted[b]	
			Total mg	mg/kg per day	As DDT, mg	% of ingested DDT	As DDT, mg	% of ingested DDT
Sw. mouse	1–30	33.7	25.0	25.2	2.8	11.0	0.28	1.1
	31–65	37.4	29.3	22.4	3.6	12.1	0.53	1.8
	66–93	40.7	22.0	19.4	2.2	10.0	0.75	3.4
	94–121	41.5	28.8	24.7	2.5	8.7	1.65	5.7
S. G. hamster	1–30	133	86.5	21.6	5.5	6.3	Nil	
	31–65	161	77.3	13.7	4.4	5.7	Nil	
	66–93	170	63.3	13.3	3.1	4.9	Nil	

[a] Mean values of four mice or hamsters fed 250 μg/g of dietary DDT *ad libitum*. [b] Integrated values from weekly 24-hr samplings. In both species DDA-Gly accounts for about 20% and DDA-Ala for about 3% of total DDA.

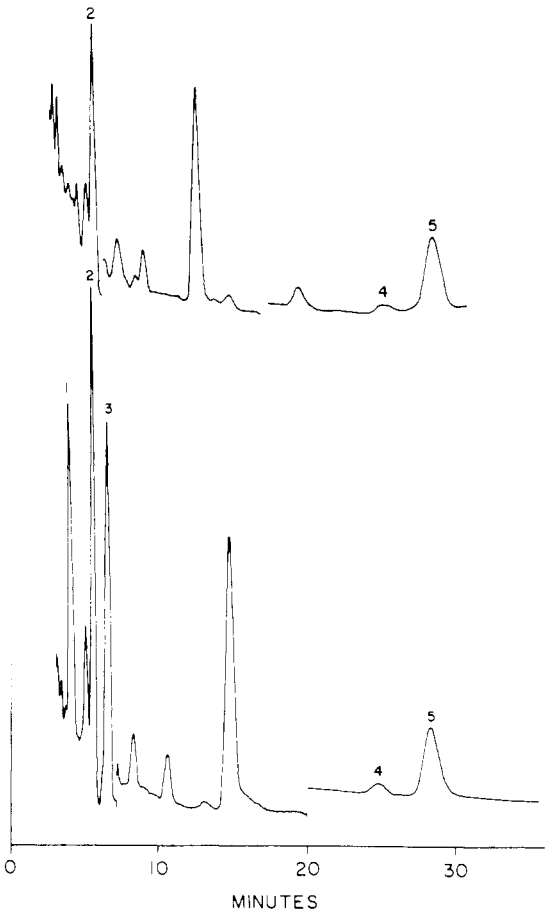

Figure 1. Chromatograms on OV-1 at 225° of methylated ether extracts of hamster urine (above) and mouse urine (below). Peak identification (× attenuation): (1) cinnamoylglycine methyl ester (×800), (2) DDA methyl ester (×1600), (3) DDE (×1600), (4) DDA-Ala methyl ester (×400), (5) DDA-Gly methyl ester (×400).

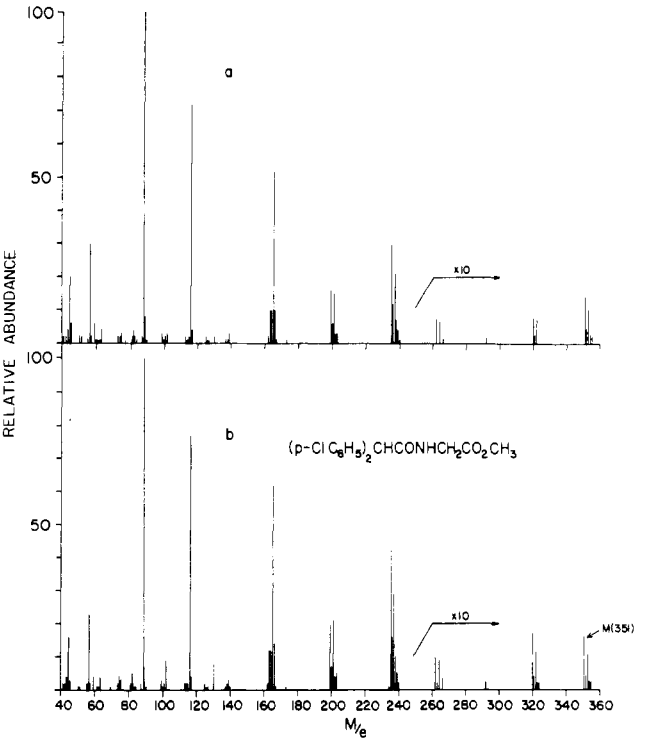

Figure 2. Mass spectrum of synthetic DDA-Gly methyl ester (below) and a substance isolated from methylated hamster urine extract (above).

Isolation of the two amino acid conjugates was achieved by gas chromatography of urine extracts obtained from the long-term feeding experiments. Cinnamoylglycine was also isolated from mouse urine by this procedure. Figure 1 shows representative chromatograms of methylated extracts of mouse and hamster urine. These chromatograms were obtained after the hamsters and mice had been fed 250 μg/g of dietary DDT for about 2 and 4 months, respectively. Evidence obtained by administering [^{14}C]DDT to animals on the DDT diet indicated that only the peaks numbered from 2 through 5 were DDT metabolites. The same metabolites were found in mice and hamsters fed 100 μg/g. The identity of DDE was established by its retention time on two glc columns (OV-1 and OV-17) and on silica gel tlc, and by its extractability from alkaline urine by hexane. The identification of DDA-Gly, DDA-Ala, and cinnamoylglycine was obtained by comparison with synthetic compounds of glc retention times and mass spectra. The latter are shown in Figures 2, 3, and 4, respectively.

The amount of DDE excreted over 48-hr periods by four mice fed 100 μg/g of dietary DDT increased from an undetectable level during the first 2 days to 0.7 μg during the 5th and 6th days, 3.9 μg during the 11th and 12th days, and 4.5 μg during the 15th and 16th days. No DDE was found in hamster urine. A second group of mice on 250 μg/g of DDT excreted approximately tenfold more DDE during this period and further collections of urine and feces were confined to mice and hamsters maintained on the higher dosage.

In Table I are comparative data for the urinary DDT metabolites. The term "total DDA" used in this table does not include as yet unidentified DDA conjugates, but at least 80% of the true DDA totals are accounted for by the identified metabolites. The data have been grouped

Table II. DDD and DDT in Mouse and Hamster Feces[a]

Species	Collect. period, days	Mean wt animal during period, g	DDT consumed		DDD excreted[b]		DDT excreted[b]	
			Total, mg	mg/kg per day	As DDT, mg	% of ingested DDT	As DDT, mg	% of ingested DDT
Sw. mouse	1–51	35.0	43.3	24.2	1.9	4.3	1.9	4.3
	52–107	40.0	52.0	23.0	2.7	5.2	2.4	4.5
S. G. hamster	1–44	143	121	19.2	2.6	2.1	1.4	1.2
	45–86	166	95	13.6	2.0	2.1	1.2	1.3

[a] Mean values of four mice or hamsters fed 250 μg/g of dietary DDT *ad libitum*. [b] Integrated values from weekly (days 1–30) and biweekly 24-hr samplings.

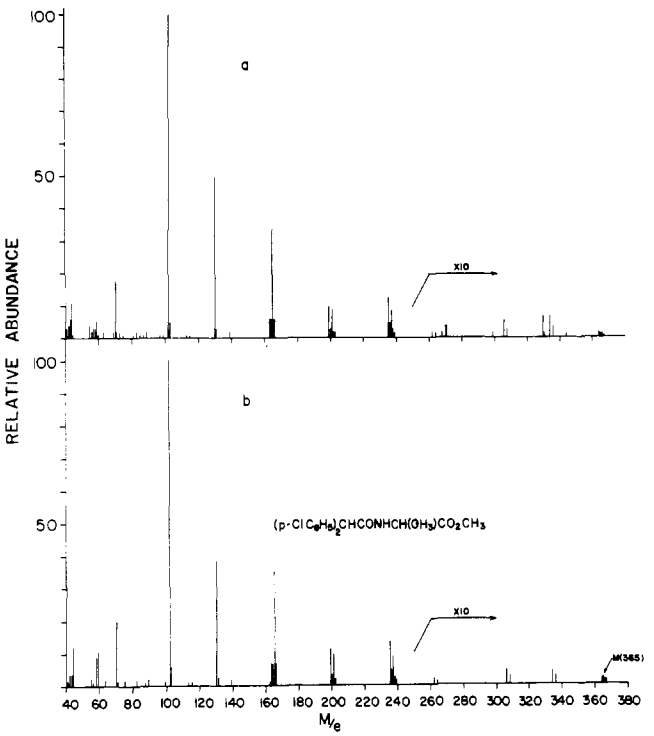

Figure 3. Mass spectrum of synthetic DDA-Ala methyl ester (below) and a substance isolated from methylated hamster urine extract (above).

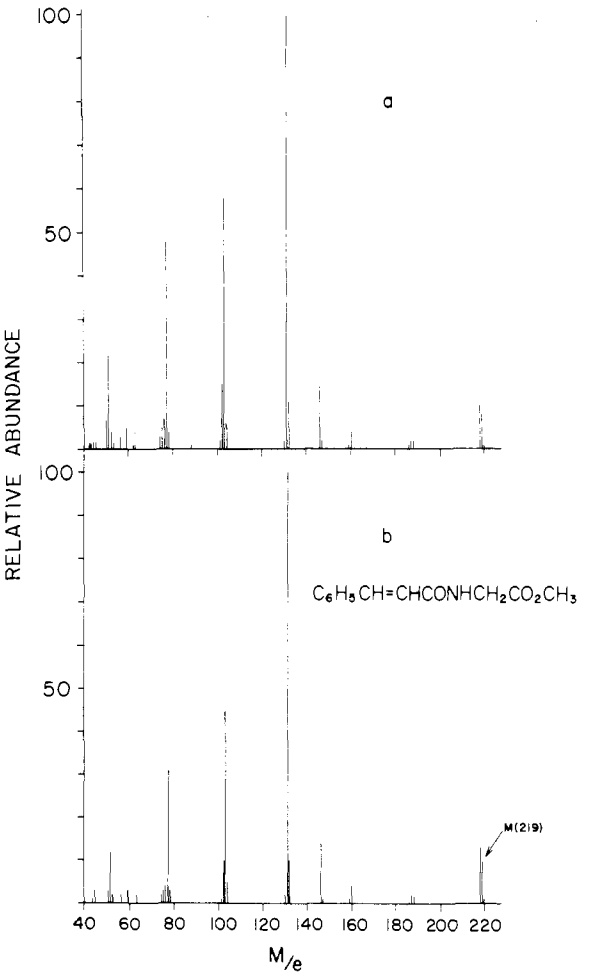

Figure 4. Mass spectrum of synthetic cinnamoylglycine methyl ester (below) and a substance isolated from methylated mouse urine extract (above).

into periods of about 1 month to show trends in excretion patterns. A linear regression analysis of all data points showed that for the periods covered the increase in the excretion of DDE with time (expressed as per cent of ingested DDT) was significant with $P < 0.005$. An increase in the ratio of DDE to total metabolites with time was also significant with $P < 0.01$. Changes in the excretion of total DDA were not significant in either mouse or hamster.

After 30 days on a DDT diet mice excreted approximately 1.5–2.0 mg/mouse per day of cinnamoylglycine. This represented an increase of three- to fivefold over the levels found in control mice.

Metabolites in Feces. The neutral metabolite DDD and unchanged DDT were found in both mouse and hamster feces. In two analyses of mouse feces a small glc peak corresponding to DDE was found but its presence could be due to contamination with urine. As shown in Table II, the excretion of DDD and DDT remained nearly constant during the feeding studies.

Only a small portion of the ingested DDT was excreted as polar metabolites. In acute studies with labeled DDT over 80% of the total fecal radioactivity in each species was accounted for by DDD and DDT (tlc chromatography using hexane) while in the long-term studies only the neutral metabolite fraction (Florisil column) gave a strong response to the chromate–sulfuric acid reagent.

DISCUSSION

No significant differences between the mouse and hamster were found with respect to DDT metabolites excreted in feces. It is likely that fecal DDD is a product of the conversion of DDT by gut bacteria. This conversion has been demonstrated in the rat (Mendel and Walton, 1966) and in a bacterium isolated from mouse intestinal flora (Barker *et al.*, 1965).

Our findings that only limited amounts of polar metabolites are excreted in mouse and hamster feces contrast with investigations in the rat where Pinto *et al.* (1965) and Jensen *et al.* (1957) reported DDA and its conjugates as the principal fecal DDT metabolites. Since DDA conju-

gates in feces are probably derived from bile, our results indicate that mice and hamsters may be poor biliary excretors compared with the rat.

Short-term experiments with labeled DDT showed that, except for relatively small amounts of neutral compounds, substantially all urinary metabolites in both species were hydrolyzable conjugates of DDA. DDA has been identified in the urine of all mammalian species examined (Apple, 1968; Hayes, 1965), but this is the first demonstration that in the fresh urine of mouse and hamster nearly all the DDA is conjugated.

The presence of cinnamoylglycine in mouse urine is an interesting species difference although not directly related to DDT metabolism since control mouse urine contained this compound in lesser amounts. Its formation was definitely related to some factor in the Wayne diet since mice on a semisynthetic diet with or without DDT did not excrete it. On the other hand this factor was probably not cinnamic acid itself (or some related compound such as cinnamaldehyde) since addition of sodium cinnamate to the drinking water of animals on the semisynthetic diet did not lead to the excretion of cinnamic acid or cinnamoylglycine in either species.

The most important difference between the species with regard to urinary DDT metabolites was the steadily increasing elimination of DDE in the mouse during the long-term feeding experiments. Apple (1968) orally administered DDT in olive oil to rodents for 5 days and collected urine for 8 days. He reported that mice, in contrast to rats and rabbits, excreted DDE as the principal neutral urinary metabolite although less than 1% of the administered DDT was recovered in this form. Our single dose administrations confirmed this small excretion in the mouse and failed to reveal any in the hamster. After 2 weeks on dietary DDT, however, the mouse was already excreting over 1% of ingested DDT as DDE, and at the termination of the experiments nearly as much DDE as DDA was found.

Biotransformation to DDE is probably not of importance in explaining the acute toxicity of DDT in the mouse since the metabolite is much less toxic than DDT itself (LD_{50} > 1600 mg/kg). Acute toxicity differences in comparison with the hamster may be due to differences in the blood–brain barrier to DDT in these species (Gingell and Wallcave, 1974).

Although the significance of urinary DDE excretion in the mouse is not certain, it is of interest to note that DDE is found in much higher concentrations in the livers of mice fed 250 μg/g of dietary DDT than in corresponding hamster livers (Gingell and Wallcave, 1974). Tomatis *et al.* (1971) reported significant amounts of DDE in mouse kidney but there are no comparative data for the hamster. Furthermore, it has been reported by Tomatis *et al.* (1974) that DDE fed at levels of 250 μg/g in the diet led to a high incidence and early appearance of liver tumors. The authors suggest that the carcinogenic effect of DDT on mouse liver may be a consequence of its conversion to DDE. Our findings of high levels of DDE in mouse liver and urine together with low levels (or none) in the hamster are consistent with such speculations.

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COMMUNICATIONS

Carotenoids of Yellow and Red Lutescent Tomatoes

The carotenoids of yellow and red lutescent tomato fruits were characterized at several stages of ripeness. The yellow lutescent tomatoes accumulated mostly neurosporene, β-carotene, and lutein during ripening, reaching a final carotenoid level of approximately 40 μg/g dry wt of fruit. The red lutescent tomatoes developed a composition of carotenoids, resembled the normal red tomatoes, and reached a carotenoid level of about 900 μg/g dry wt of fruit. The lutescent gene does not seem to alter drastically the carotenoid composition of the tomato fruits.

The red lutescent tomatoes were mentioned by Mackinney (1958) and used by Simpson and his coworkers (Raymundo *et al.*, 1970; Villegas *et al.*, 1972) in studies of carotenoid biosynthesis. Characteristically, the lutescent gene in tomatoes expresses itself by a premature yellowing of the leaves and an unusual ripening pattern of the fruits. The immature fruits contain much less chlorophyll than normal tomatoes and the fruit turns almost completely white at the mature stage. Several days may elapse before the synthesis of lycopene occurs.

The yellow lutescent tomatoes possess the same characteristics of the red lutescent tomatoes except that the