Oxidative Metabolism of Rotenone in Mammals, Fish, and Insects and Its Relation to Selective Toxicity

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Rotenone is hydroxylated to give 8′-hydroxyrotenone, 6′,7′-dihydro-6′,7′-dihydroxyrotenone, and various rotenolones when incubated with the microsome mixed function oxidase system of mammalian liver, fish liver, and insect tissues. Microsomal enzymes from these sources are similar but not identical in respect to the CO-binding pigment (P-450) and to the sensitivity to various inhibitors. The soluble fraction of the liver homogenates enhances whereas the soluble fraction of cockroach fat body and mid-gut homogenates inhibits rotenone metabolism, the inhibition resulting from the presence in the soluble fraction of a protein with a molecular weight of 6,000 to 15,000. Components in insect homogenates also inhibit the metabolism of organophosphates by the rat liver microsome–NADPH system. The results of *in vivo* and *in vitro* studies on rotenone detoxification indicate that the effects of components in the soluble fraction possibly are related to the selective toxicity of rotenone to mammals, fish, and insects.

Rotenone is only moderately toxic to most mammals but is extremely toxic to many species of fish and insects. Rotenone inhibits the respiration of mitochondria by blocking the reduced nicotinamide adenine dinucleotide (NADH)-dehydrogenase segment of the respiratory chain, when this enzyme is derived from species that are highly susceptible or resistant to rotenone poisoning (Fukami, 1956, 1961; Fukami and Tomizawa, 1956; Lindahl and Oberg, 1961; Ernster *et al.*, 1963; Horgan *et al.*, 1968). Thus, the selective toxicity of rotenone apparently does not result from differences in the primary site of action in insects and mammals. Rather, it appears that the selectivity depends upon differences in the distribution pattern or in the detoxification rate of rotenone in various organisms. Rotenone is extensively metabolized by the rat liver and housefly microsome-reduced nicotinamide adenine dinucleotide phosphate (NADPH) system but not by systems involving other combinations of cofactors and individual subcellular fractions from rat liver. Addition of the soluble fraction of rat liver homogenate to the microsomal fraction greatly increases the extent of rotenone metabolism and of formation of unknown, water-soluble products (Fukami *et al.*, 1967). The same major organosoluble metabolites are formed by the microsomal mixed-function oxidase systems of rat liver and housefly abdomens and these products are hydroxylated derivatives resulting from attack on the isopropenyl side chain as well as at the B-C ring juncture. The products and pathways involved in rotenone metabolism are defined and illustrated in recent reports (Fukami *et al.*, 1967; Yamamoto, 1969).

This paper deals with the possibility that differences in detoxication rate or route contribute to the selective toxicity of rotenone to mammals, fish, and insects.

MATERIALS AND METHODS

Thin-Layer Chromatography (TLC). Silica gel G was used at 0.25-mm. gel thickness on 20 × 20-cm. plates. Developing solvents were benzene–methanol mixture (9 to 1) for rotenone and its metabolites, hexane–acetone mixture (4 to 1) for *O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate (diazinon) and its metabolites, and hexane–acetone mixture (5 to 1) for *O,O*-dimethyl *S*-[2-(ethylthio)-ethyl] phosphorodithioate (thiometon) and its metabolites. Radioactive materials were detected by autoradiography, and the radioactive regions of the gel were scraped from the plates into scintillation vials for direct radioactive measurement. Unlabeled rotenoids, in amounts of 10 μg. or more, were detected with phosphomolybdic acid reagent (Stahl, 1965) and thiophosphorus esters were detected with 0.5% palladium chloride in dilute hydrochloric acid solution (Bäumler and Rippstein, 1961).

Chemicals. Rotenone-5*a*-^{14}C (2.36 mCi. per mmole; Nishizawa and Casida, 1965), with a radiochemical purity of 97% or better, was held as a stock solution in absolute ethanol in the dark at 4° C. Diazinon-*O*-ethyl-^{14}C (0.68 mCi. per mmole; Sumitomo Radiochemical Co. Ltd., Osaka, Japan), with a radiochemical purity of 99%, was held as a stock solution in benzene. Thiometon-^{32}P (2.46 mCi. per mmole; Division of Entomology, University of Nagoya, Nagoya, Japan) was purified by TLC and held as a stock solution in benzene.

Unlabeled insecticides or synergists were obtained from the following sources: rotenone derivatives were from Leslie Crombie, University College of South Wales and Monmouthshire, Cardiff, U.K., or by synthesis (Fukami *et al.*, 1967); diazinon and diazoxon (the phosphate analog of diazinon) from Nippon Kayaku Co. Ltd., Tokyo, Japan; thiometon and thiometoxon (the phosphorothiolate analog of thiometon, also known as demeton-*S*-methyl) from Sankyo Co. Ltd., Tokyo, Japan; methylenedioxyphenyl compounds (piperonyl butoxide and sulfoxide), *N*-(2-ethylhexyl)-bicyclo(2.2.1)-5-heptene-2,3-dicarboximide (MGK 264), and *N*-(2-ethylhexyl)-1-isopropyl-4-methyl-bicyclo(2.2.2)-5-octene-2,3-dicarboximide (Synepirin 500) from Sumitomo Chemical Co., Osaka, Japan; 2-diethylaminoethyl 2,2-diphenylvalerate (SKF-525A) from Tsutomu Nakatsugawa, Iowa State University, Ames, Iowa. Diethylaminoethyl (DEAE) and carboxymethyl (CM) cellulose were from Brown Co., Berlin, N. H., while Sephadex G-25, G-50, and G-100 dextran were from Pharmacia, Uppsala, Sweden.

Enzyme Preparations. The tissues or organs studied were obtained from male albino rats (150 to 250 grams), male carp (1 to 1.5 kilogram, from National Fisheries Experimental Station, Tokyo, Japan), male and female American cockroaches (*Periplaneta americana L.*), horn beetle larvae (*Xylo-*

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trupes dichotomus L.), and German cockroaches (*Blattella germanica* L.). Homogenates were prepared at 20% (w. per v.) concentration and 5° C. in 0.25*M* sucrose–0.05 *M* Na_2HPO_4–KH_2PO_4–0.01*M* ethylenediaminetetraacetic acid (EDTA) medium, pH 7.4. Each homogenate was centrifuged at 600 × g for 5 minutes, and the supernatant was centrifuged at 15,000 × g for 30 minutes at 2° C. The microsome-plus-soluble fraction, that portion not sedimented at 15,000 × g, was separated into the microsomal pellet and soluble fraction by centrifugation at 105,000 × g for 1 hour at 2° C. The microsomal pellet was washed, by suspension in sucrose–phosphate–EDTA medium and centrifugation at 105,000 × g for 30 minutes at 2° C., and resuspended in sucrose–phosphate–EDTA medium to reconstitute to the original 20% homogenate equivalent. This procedure was varied in only two cases: rat liver homogenates, used in the studies on various combinations of microsome and soluble fractions from different species, were prepared at 25% (w. per v.) in 0.25*M* sucrose and the microsome fraction was reconstituted to 25% homogenate equivalent in 0.05*M* Na_2HPO_4–KH_2PO_4, pH 7.3; abdomen homogenates from the Hokota-resistant strain of houseflies (*Musca domestica* L.) were prepared at 25% (w. per v.) in 0.25*M* sucrose–0.15*M* Na_2HPO_4–KH_2PO_4 medium, pH 7.5 (Tsukamoto and Casida, 1967b). In order to obtain enough tissue to work with, the mid-gut, fat body, and muscle of cockroaches were used instead of the respective parts of the housefly abdomens.

In Vitro **Metabolism Procedures.** The incubation mixtures for most enzyme preparations, in a 2-ml. total volume in 10-ml. Erlenmeyer flasks, consisted of the labeled substrate (6 μg. of rotenone-^{14}C, 50 μg. of diazinon-^{14}C, or 100 μg. of thiometon-^{32}P), 1 ml. of 0.1*M* Na_2HPO_4–KH_2PO_4 buffer (pH 7.4), 0.5 ml. of sucrose–phosphate–EDTA medium, 0 or 2 μmoles of pyridine nucleotide cofactor, and enzyme equivalent to 100 mg. of homogenized tissue. The reaction constituents for housefly preparations were the same except that 0.25*M* sucrose–0.15*M* Na_2HPO_4–KH_2PO_4 medium (pH 7.5) was used and the final enyzme concentration was equivalent to 125 mg. of houseflies, or portion thereof, per 2.0-ml. total reaction mixture. After preparing each incubation mixture, the labeled substrate was added in ethanol to yield a final ethanol concentration of 0.1%. The flasks were shaken aerobically at 37° C. for 30 minutes in the case of thiometon or for 2 hours in the case of diazinon or rotenone. Incubation mixtures were either extracted immediately after incubation or they were frozen and kept for 24 hours or less at −20° C. prior to extraction. The radioactivity content of the incubation mixture was determined, using a 50-μl. aliquot, and the remaining portion was poured into a 15-ml. centrifuge tube, the flask was rinsed separately with 3 ml. of distilled water and 5 ml. of ether, the washes were added to the tube, the aqueous phase was extracted four times with 5-ml. portions of ether, and the combined ether extracts were adjusted to 20 ml. and dried with 5 grams of anhydrous Na_2SO_4. The percentage of total radioactivity in this "neutral ether extract" and in the aqueous phase was determined. The ether-extractable metabolites were resolved by TLC and the per cent of each product was determined by scintillation counting, as previously reported (Leeling and Casida, 1966). The aqueous phase from the rotenone incubation mixtures was further fractionated by adding 0.2 ml. of concentrated HCl to the remaining aqueous phase and extracting the acidic solution four times with 5-ml. portions of ether. After the residual ether was evaporated from the aqueous phase with a jet of air and 250 mg. of NaCl were added, the aqueous phase was extracted four times with 5-ml. portions of *n*-butanol. The radiocarbon content of the "acid ether extract" and the "*n*-butanol extract" was determined as before. (Over-all recoveries by this analytical procedure were essentially complete.)

Other Methods for *In Vitro* **Studies.** Cytochrome P-450 was measured with the Cary Model 14 recording spectrophotometer by suspending the microsomal pellets in 4.1 ml. of 0.1*M* Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, and determination of the CO-difference spectra between 390 and 620 mμ at 20° C., according to Omura and Sato (1964), using the following treatment schedule; reducing agent (2 mg. of sodium dithionite or 2 μmoles of NADPH) was added to each of two cuvettes containing the microsomal preparation and CO was bubbled, for 10 minutes, into one of the cuvettes while in the sample compartment. The total nitrogen content was determined by the micro-Kjeldahl method. (The total nitrogen content of each microsomal preparation, expressed as milligrams of nitrogen per milliliter, was: 0.59 for rat liver, 0.06 for carp liver, 0.35 for cockroach fat body, and 0.41 for cockroach mid-gut.)

The soluble fraction of homogenates of certain insect organs was further separated into component parts by gel filtration or cellulose column chromatography. Sephadex G-25 or G-50 columns (1.2 × 25 cm.) and a Sephadex G-100 column (2.4 × 50 cm.) were used at 5° C. after equilibration with 0.25*M* sucrose or 0.25*M* sucrose–0.05*M* Na_2HPO_4–KH_2PO_4–0.01*M* EDTA medium (pH 7.4). The procedure used with the G-100 column and the buffered medium generally was that of Andrews (1964), and 5-ml. fractions were collected at a flow rate of 30 ml. per hour. Prior to chromatography on DEAE or CM cellulose columns [2.0 × 46 cm.; prepared according to Moore and Lee (1960) without using air pressure], 40 ml. of the supernatant fraction was dialyzed through cellophane overnight at 4° C. against 0.005*M* tris-HCl buffer, pH 7.0. The columns were developed, at 5° to 10° C., with tris-HCl buffer gradients, as follows: I, 0.005*M* at pH 7.0, 200 ml.; II, 0.005*M* at pH 7.0 to 0.02*M* at pH 7.0, 200 ml.; III, 0.02*M* at pH 7.0 to 0.09*M* at pH 7.0, 200 ml.; IV, 0.09*M* at pH 7.0 to 0.12*M* at pH 7.0, 200 ml.; V, 0.12*M* at pH 7.0 to 0.33*M* at pH 7.0, 280 ml.; VI, 0.33*M* at pH 7.0 to 0.33*M* at pH 8.0, 200 ml.; VII, 0.33*M* at pH 8.0 to 1.0*M* NaCl in 0.33*M* tris-HCl buffer at pH 8.0, 280 ml. Twenty-milliliter eluate fractions were collected at approximately 10-minute intervals and the protein content was determined by the absorbance at 280 mμ.

The inhibitory potency of authentic rotenoids or of rotenone-^{14}C metabolites on mitochondrial respiration was determined manometrically with the Warburg apparatus at 37° C. using pyruvate or L-glutamate, respectively, as the substrate (Fukami, 1961; Ernster *et al.*, 1963). Mitochondria were prepared from the liver of male rats by homogenizing the liver at 10% (w. per v.) in sucrose–phosphate–EDTA medium, filtering through three layers of surgical gauze, and centrifuging at 600 × g for 5 minutes. The supernatant was centrifuged at 10,000 × g for 10 minutes to sediment the mitochondrial fraction which was resuspended in a solution consisting of 0.9% KCl, 0.05*M* Na_2HPO_4–KH_2PO_4, and 0.05*M* $MgCl_2$, at pH 7.4. The pyruvate oxidation system, in a volume of 2 ml., consisted of 30 μ*M* phosphate buffer (pH 7.4), 5 μ*M* ATP, 4 μ*M* NaF, 1 μ*M* NAD, 15 μ*M* $MgCl_2$, 0.018 μ*M* cytochrome *c*, 1 μ*M* succinate, 10 μ*M* pyruvate, and the mitochondrial suspension (10 to 15 mg. of protein). The L-glutamate oxidation system, in a volume of 3.2 ml., consisted of 50 μ*M* phosphate buffer (pH 7.4), 1 μ*M* ATP,

5 μM $MgCl_2$, 0.01 μM cytochrome *c*, 20 μM L-glutamate, and 1 ml. of the 10% equivalent mitochondrial suspension.

In Vivo **Metabolism Conditions.** Male albino mice (18 grams) were treated orally by stomach tube with 12 μg. of rotenone-^{14}C in 50 μl. of dimethyl sulfoxide solution, a dose that did not produce any toxic symptoms. The total radiocarbon contents of expired air, urine, feces, and tissues were determined. Urine was analyzed by the extraction and TLC procedures used for the analysis of enzyme preparations. In certain cases, rotenone and metabolites in the brain, kidney, liver, and small intestine were extracted for analysis 4 hours after treatment. Tissue analyses involved several extractions by homogenization in either cold acetone or water. In both cases, the extracts were analyzed by the procedure used for enzyme preparations, which was applied directly, in the case of the water extracts, or after evaporation of the acetone, addition of water, and extraction with ether, in the other case.

Small male carp (3 grams) were allowed to consume rotenone-^{14}C added in small food balls to their aqueous environment. (Rotenone was not introduced directly into the water because this would allow entry through the gills as well as orally.) One gram of baker's yeast powder was mixed with 0.1 ml. of water and 24 or 48 μg. of rotenone-^{14}C in 100 or 200 μl. of ethanol and the mixture was separated into 5 portions to form the small balls. Each portion was provided to 5 fish in concrete pots containing 17 liters of water. After the carp had consumed the ball, they were transferred into a glass chamber containing 5 liters of water at 17° C. in the laboratory, and, after 4 and 24 hours, specimens were collected and several tissues were removed for determination of rotenone metabolites. (Toxic symptoms were not evident during the experiment.) After removal of the carp, the water from the glass containers was evaporated on a rotary evaporator at 50° C. The metabolites in the tissues and in the residual water were determined by the procedure used for the analysis of products in mice.

Male American cockroaches were individually injected with 4 μg. of rotenone-^{14}C in 15 μl. of ethanol solution. (This dose resulted in paralysis and knockdown of the insects but they remained responsive to stimuli.) After 24 hours, the cockroaches were extracted and analyzed by a procedure similar to that used with mouse tissues.

RESULTS

Metabolism of Rotenone-^{14}C by Microsome and Microsome-plus-Soluble Fractions of Tissue Homogenates. The microsome fraction of liver is higher in rotenone-metabolizing activity than the respective fraction of brain, kidney, or small intestine of rats or carp (Table I). Activity-enhancement by NADPH is restricted to the microsome fraction of the liver of rats, mice, and carp and of the carp small intestine. The rotenone metabolites formed by the carp liver microsome–NADPH system cochromatograph with those previously reported for the rat liver microsome–NADPH system (Fukami *et al.*, 1967). (Metabolites from rat tissues other than the liver were not subjected to cochromatography studies; so, the structural assignments were based only on approximate *Rf* values which may vary with interfering extractives with the different types of tissues.) Housefly abdomen homogenates or the respective microsome fraction metabolize rotenone in the presence but not in the absence of NADPH, whereas homogenates of whole flies or of heads plus thoraces are almost inactive, even in the presence of NADPH (Fukami *et al.*, 1967; Tsukamoto *et al.*, 1967b). Cockroach fat body, but not other tissues, metabolizes rotenone to some extent when fortified with NADPH. Rotenolone II derivatives appear in significant amounts with the housefly abdomen microsomes (Fukami *et al.*, 1967), but not with mammalian or cockroach enzyme preparations (Table I). Other metabolites from cockroach fat body are identical with the major metabolites from rat liver based on comparisons of R_f values and cochromatography. Rotenone metabolism by the mixed function oxidases of cockroach fat body is much less extensive, at equivalent tissue levels, than by the oxidases from mammalian or fish liver (Table I).

The effect on rotenone metabolism of adding the soluble fraction to the microsome–NADPH system depends on the tissue: a marked increase in rotenone metabolism and/or production of water-soluble metabolites results with each liver preparation and with the carp small intestine preparation; inhibition of rotenone metabolism results with cockroach fat body preparations; no effect is noted with other tissue or organ preparations. Surprisingly, the production of water-soluble metabolites with the carp liver microsome-plus-soluble fraction does not require NADPH fortification (Table I).

The component of the rat liver soluble fraction responsible for conversion of the ether-extractable rotenone metabolites to more polar (*n*-butanol-extractable) derivatives probably is an enzyme because, when added to the microsome–NADPH system and subsequently incubated for 5 hours, the activity of the soluble fraction is evident with 5 μl. but increases with 50 μl. or, particularly, with 500 μl. of this fraction; activity is destroyed on heating at 100° C. for 3 minutes but there is little or no loss in activity on dialysis in a cellophane tube for 24 hours at 2° C. against 0.25M sucrose or on passage through a Sephadex G-50 column and recovery of the protein-containing eluate fractions. Conversion of rotenone to water-soluble metabolites by the microsome system requires the simultaneous presence of NADPH, to initiate the detoxification, and of the soluble fraction, to convert the intermediate metabolites (ether-extractable) to more polar derivatives (*n*-butanol-extractable). This conclusion is based on the results obtained with rat liver preparations and by intercomparison of the effect of the following incubation conditions on the production of water-soluble metabolites, taking into account the instability of the microsomal enzymes and, particularly, of NADPH in the presence of the soluble fraction during the incubation period: 2 hours incubation with the microsome–NADPH system, not active; 2 hours incubation with the microsome-boiled soluble-NADPH system, not active; 2 hours incubation with the microsome-soluble-NADPH system, very active; 1 hour incubation with the microsome-NADPH system (during which time rotenone metabolism is essentially complete) followed by 1 hour incubation after addition of the soluble fraction, not active; 1 hour incubation with the microsome fraction followed by 1 hour incubation after addition of NADPH and the soluble fraction, active; incubation for 1 hour with the soluble fraction and NADPH followed by 1 hour incubation after addition of the microsome fraction, active.

The enhancing activity of the soluble fraction on rotenone metabolism is not a phenomenon restricted to the rat liver preparations (Table I and Table II). The rat liver or carp liver soluble fraction increases the extent of rotenone metabolism and/or formation of water-soluble metabolites by the rat liver microsome–NADPH, carp liver microsome–NADPH, housefly abdomen microsome–NADPH, and cockroach fat body microsome–NADPH systems. However, the soluble

Table I. Metabolism of Rotenone-^{14}C by Microsome Fraction Prepared from Certain Tissues of Rats, Mice, Carp, Cockroaches, and Houseflies in Presence and Absence of NADPH and/or Respective Soluble Fraction

Tissue	Added Incubation Constituent(s)		Radiocarbon Recovered as Indicated Product, %							
	NADPH[a]	Soluble fraction[a]	Rotenone	Rotenolone I	8′-Hydroxyrotenone	8′-Hydroxyrotenolone I	6′,7′-Dihydro-6′,7′-dihydroxyrotenone	6′,7′-Dihydro-6′,7′-dihydroxyrotenolone I	Rotenolone II derivatives[b]	Water-soluble products
Rat										
Brain	−	−	80	15	0	0	3	1	0	1
	+	−	80	13	0	0	5	0	0	2
	−	+	82	12	0	0	2	1	0	3
	+	+	84	10	0	0	2	1	0	3
Kidney	−	−	72	19	0	0	3	2	0	4
	+	−	66	21	0	0	6	3	1	3
	−	+	67	20	0	0	7	1	0	5
	+	+	77	12	0	0	4	2	0	5
Liver	−	−	74	15	3	0	1	2	1	4
	+	−	15	7	11	3	28	8	5	23
	−	+	75	11	2	2	0	0	0	10
	+	+	0	0	0	1	2	2	2	93
Small intestine	−	−	63	31	0	0	3	0	0	3
	+	−	79	15	0	0	3	0	0	3
	−	+	67	26	0	0	2	0	0	5
	+	+	44	44	0	0	6	2	0	4
Mouse										
Liver	−	−	88	7	1	0	2	1	0	1
	+	−	6	3	3	7	40	8	7	26
	−	+	88	6	1	2	0	0	0	3
	+	+	6	1	0	1	8	7	1	76
Carp										
Brain	−	−	75	11	3	0	2	2	2	5
	+	−	72	14	5	0	2	2	0	5
Kidney	−	−	47	43	6	1	1	0	0	2
	+	−	63	21	7	0	4	3	0	2
Liver	−	−	25	31	11	4	8	3	14	4
	+	−	6	8	14	5	23	7	11	26
	−	+	1	1	2	4	11	4	6	71
	+	+	1	1	2	1	13	3	5	74
Small intestine	−	−	64	27	2	0	1	1	0	5
	+	−	47	9	15	1	13	2	0	13
	−	+	21	43	2	0	9	11	0	14
	+	+	14	3	20	3	19	10	0	31
Cockroach										
Fat body	−	−	74	16	2	1	1	0	0	6
	+	−	60	13	5	1	14	1	0	6
	−	+	88	6	2	1	1	0	0	2
	+	+	88	6	1	1	1	1	0	2
Mid-gut	−	−	75	19	1	1	1	0	1	2
	+	−	75	18	2	1	1	0	0	3
	−	+	81	8	1	2	1	1	0	6
	+	+	88	8	1	1	1	0	0	1
Muscle	−	−	79	16	1	1	1	0	0	2
	+	−	82	8	1	2	4	1	0	2
	−	+	88	8	1	1	0	0	1	1
	+	+	86	7	1	1	1	0	1	3
Housefly										
Abdomen	−	−	82	9	0	1	1	0	3	4
	+	−	34	8	7	2	28	2	7	12
	−	+	52	21	0	0	1	1	0	25
	+	+	34	7	6	2	14	7	7	23

[a] + = present; − = absent.
[b] Consists almost entirely of material with the R_f value of 6′,7′-dihydro-6′,7′-dihydroxyrotenolone II with the following exceptions: never more than 0.1% as rotenolone II or 8′-hydroxyrotenolone II except for 0.3% 8′-hydroxyrotenolone II with the microsome-NADPH system of cockroach muscle, 2 and 3% rotenolone II with the microsome-NADPH and microsome-soluble systems of housefly abdomen, respectively, and 0.5% 8′-hydroxyrotenolone II with the microsome-soluble-NADPH system of housefly abdomen.

Table II. Metabolism of Rotenone-^{14}C by Various Combinations of Microsome and Soluble Fractions Prepared from Homogenates of Rat Liver, Carp Liver, Housefly Abdomen, Cockroach Mid-Gut, Cockroach Fat Body, and Other Sources

Incubation Constituents			Radiocarbon Recovered as Indicated Product, %			
Microsome fraction	Soluble fraction	Cofactor	Rotenone	Rotenolone I	Other ether-soluble metabolites	Water-soluble products
Combinations of Fractions Derived from Rat Liver and Housefly Abdomen						
Rat liver	None	NADPH	3	4	86	7
None	Rat liver	NADPH	54	13	8	25
Rat liver	Rat liver	NADPH	1	2	21	76
Fly abd.	None	NADPH	32	18[a]	38	12
None	Fly abd.	NADPH	52	28[a]	12	8
Fly abd.	Fly abd.	NADPH	53	23[a]	14	10
Rat liver	Fly abd.	NADPH	3	3[a]	69	25
Fly abd.	Rat liver	NADPH	24	8[a]	25	43
Combinations of Fractions Derived from Rat Liver, Carp Liver, Cockroach Mid-Gut, Dialyzed Cockroach Mid-Gut, and Cockroach Fat Body						
Rat liver	None	NADPH	2	3	61	34
Rat liver	Rat liver	NADPH	0	0	8	92
Rat liver	Roach gut	NADPH	63	15	4	18
Rat liver	Dial. roach gut	NADPH	66	18	7	9
Rat liver	Fat body	NADPH	33	2	17	48
Carp liver	None	NADPH	6	8	60	26
Carp liver	Carp liver	NADPH	1	0	25	74
Carp liver	Roach gut	NADPH	60	14	4	22
Carp liver	Roach fat body	NADPH	4	1	14	81
Roach gut	None	NADPH	63	24	10	3
Roach gut	Roach gut	NADPH	61	26	9	4
Roach fat body	None	NADPH	55	13	31	1
Roach fat body	Roach fat body	NADPH	68	16	7	9
Roach fat body	Roach gut	NADPH	59	23	10	8
Roach fat body	Carp liver	NADPH	14	9	46	31
Roach fat body	Rat liver	NADPH	35	17	28	20
Rat liver	Rat liver	NADH	9	8	62	21
Rat liver	Roach gut	NADH	52	26	3	19
Rat liver	Dial. roach gut	NADH	66	23	5	6
Combinations of Fractions Derived from Rat Liver, Dialyzed Rat Kidney, Whole Bodies of German Cockroaches, and Mid-Gut of Horn Beetle Larvae						
Rat liver	Rat liver	NADH	21	5	48	26
Rat liver	Whole roach	NADH	61	4	5	30
Rat liver	Beetle gut	NADH	19	4	42	35
Rat liver	Dial. rat kidney	NADH	47	8	33	12

[a] Including some rotenolone II.

fraction of housefly abdomen homogenates fails to increase greatly the water-soluble rotenone derivatives formed by either the rat liver or housefly microsome–NADPH systems.

Effect of Inhibitors on Metabolism of Rotenone, Diazinon, and Thiometon by Microsome–NADPH or –NADH Systems. The inhibition pattern of cytochrome *c* and of several inhibitors of drug metabolism (piperonyl butoxide, sulfoxide, MGK 264, Synepirin 500, and SKF-525A) is similar for the microsome–NADPH system from rat liver, carp liver, and cockroach fat body (Table III). Certain of these compounds are also effective inhibitors for the rat liver microsome-plus-soluble-NADPH system.

Endogenous inhibitors are also present in some of the enzyme preparations. For example, the soluble fraction from cockroach fat body and mid-gut homogenates inhibits the amount of rotenone metabolism and conversion to water-soluble metabolites by either the rat or carp liver microsome–NADPH systems (Table II). An exception to this relationship is the combination of carp liver microsomes and the cockroach fat body soluble fraction. Based on a survey for inhibitory materials in various tissue homogenate fractions, made by addition of these preparations to the rat liver microsome–NADPH or –NADH systems, potent inhibitors are present in the soluble fraction of cockroach mid-gut and fat body homogenates, and in whole adult German cockroach homogenate, and less potent inhibitors or no inhibitors are

Table III. Effect of Inhibitors on Rotenone-^{14}C Metabolism by Enzyme Systems Prepared from Rat Liver, Carp Liver, and Cockroach Fat Body

Inhibitor[a]	Radiocarbon Recovered as Indicated Product, %: Rotenone	Rotenolone I	Other ether-soluble metabolites	Water-soluble products
Rat Liver Microsome–NADPH System				
None	15	7	56	23
Piperonyl butoxide	69	17	7	7
Sulfoxide	69	18	10	3
Synepirin 500	68	10	14	8
MGK 264	79	12	6	3
SKF-525A	61	15	12	12
Cytochrome *c*	49	29	15	7
Rat Liver Microsome-plus-Soluble–NADPH System				
None (no NADPH)	75	11	4	10
None (with NADPH)	16	4	16	64
Piperonyl butoxide	80	11	6	3
SKF-525A	75	8	6	11
Cytochrome *c*	67	16	6	11
Carp Liver Microsome–NADPH System				
None	6	8	60	26
Piperonyl butoxide	50	14	28	8
Sulfoxide	61	15	22	2
MGK 264	50	16	29	5
SKF-525A	60	18	18	4
Cytochrome *c*	40	37	18	5
Cockroach Fat Body Microsome[b]–NADPH System				
None	23	13	55	9
Piperonyl butoxide	79	13	7	1
Sulfoxide	80	13	5	2
Synepirin 500	72	12	11	5
MGK 264	80	12	7	1
SKF-525A	58	18	17	7
Cytochrome *c*	48	37	10	5

[a] Concentration of inhibitors is $5 \times 10^{-4}M$ except that for piperonyl butoxide and cytochrome *c* is $5 \times 10^{-5}M$. The enzyme preparation is preincubated for 10 minutes with the inhibitor before NADPH is added. [b] Fraction prepared from 40% (w./v.) homogenate equivalent.

present in the soluble fraction of housefly homogenates, horn beetle larval mid-gut homogenate, or the dialyzed rat kidney homogenate (Table II). Based on a search for potential inhibitory materials that might be masked by other components in the soluble fraction, there is not any evidence to indicate that the soluble fractions from homogenates of horn beetle larval mid-gut, whole housefly homogenates, and rat kidney and liver homogenates contain potent inhibitory materials, before or after chromatography of these soluble fractions on DEAE cellulose.

The inhibitor(s) in the soluble fraction of cockroach mid-gut homogenate are not dialyzable (24 hours at 4° C. against $0.05M$ Na_2HPO_4–KH_2PO_4–$0.01M$ EDTA, pH 7.4, or $0.005M$ tris-HCl, pH 7.0), are not retained on Sephadex G-25 when chromatographed in phosphate-EDTA buffer and, when the dialyzed material is used, are absorbed by the DEAE cellulose but not by the CM cellulose columns. Highly inhibitory materials eluting from the DEAE cellulose column (fractions 24 to 42, Figure 1) precipitate, along with the protein, at 70 to 80% saturation with $(NH_4)_2SO_4$, and protein coagulation and loss of inhibitory activity result on heating the protein-containing inhibitor fractions, prior to $(NH_4)_2SO_4$-treatment, for 7 minutes at 90° C. Chromatography of the undialyzed mid-gut soluble fraction on Sephadex G-100 with phosphate-EDTA buffer elutes the inhibitor(s) in fractions 32 to 38 (Figure 2), a region corresponding to a molecular weight of 6,000 to 15,000 based on the chromatographic characteristics of known proteins (Andrews, 1964; Siegel and Mondy, 1966).

The inhibitory effect of the cockroach mid-gut soluble fraction is not restricted to rotenone metabolism because it is also inhibitory to the oxidation of diazinon and thiometon (Table IV). On the basis of another experiment, not tabulated here, the mid-gut soluble fraction increases rather than inhibits the glutathione-dependent degradation of diazinon and thiometon by the rat liver soluble fraction, under the general procedure of Fukami and Shishido (1966).

Carbon Monoxide-Binding Pigment of Microsomes from Rat Liver, Carp Liver, Cockroach Mid-Gut, and Cockroach Fat Body. Difference spectra on the microsomal pigments, treated by first reducing with either dithionite or NADPH and then by bubbling CO through the microsomal suspension

Table IV. Effect of Endogenous Inhibitor(s) in Soluble Fraction of Cockroach Mid-Gut Homogenate on Metabolism of Diazinon-*O*-Ethyl-^{14}C and Thiometon-^{32}P by Rat Liver Microsomes

Incubation Constituents: Cofactor	Cockroach mid-gut soluble fraction, ml.	Radioactivity Recovered as Indicated Product, %: Known compounds or unidentified products in neutral ether extract				Water-soluble products
Diazinon-*O*-Ethyl-^{14}C						
	R_f	Diazinon 0.76	A 0.52	Diazoxon 0.35	B 0.19	
None	0	99	0	0	0	1
NADPH	0	68	4	9	1	18
NADPH	0.5	99	0	0	0	1
Thiometon-^{32}P						
	R_f	Thiometon 0.70	Thiometoxon 0.21	A 0.17	B 0.06	
None	0	93	0	0	2	5
NADPH	0	13	3	1	73	10
NADPH	0.5	56	0	0	41	3
NADH	0	41	1	0	53	5
NADH	0.5	79	0	0	18	3

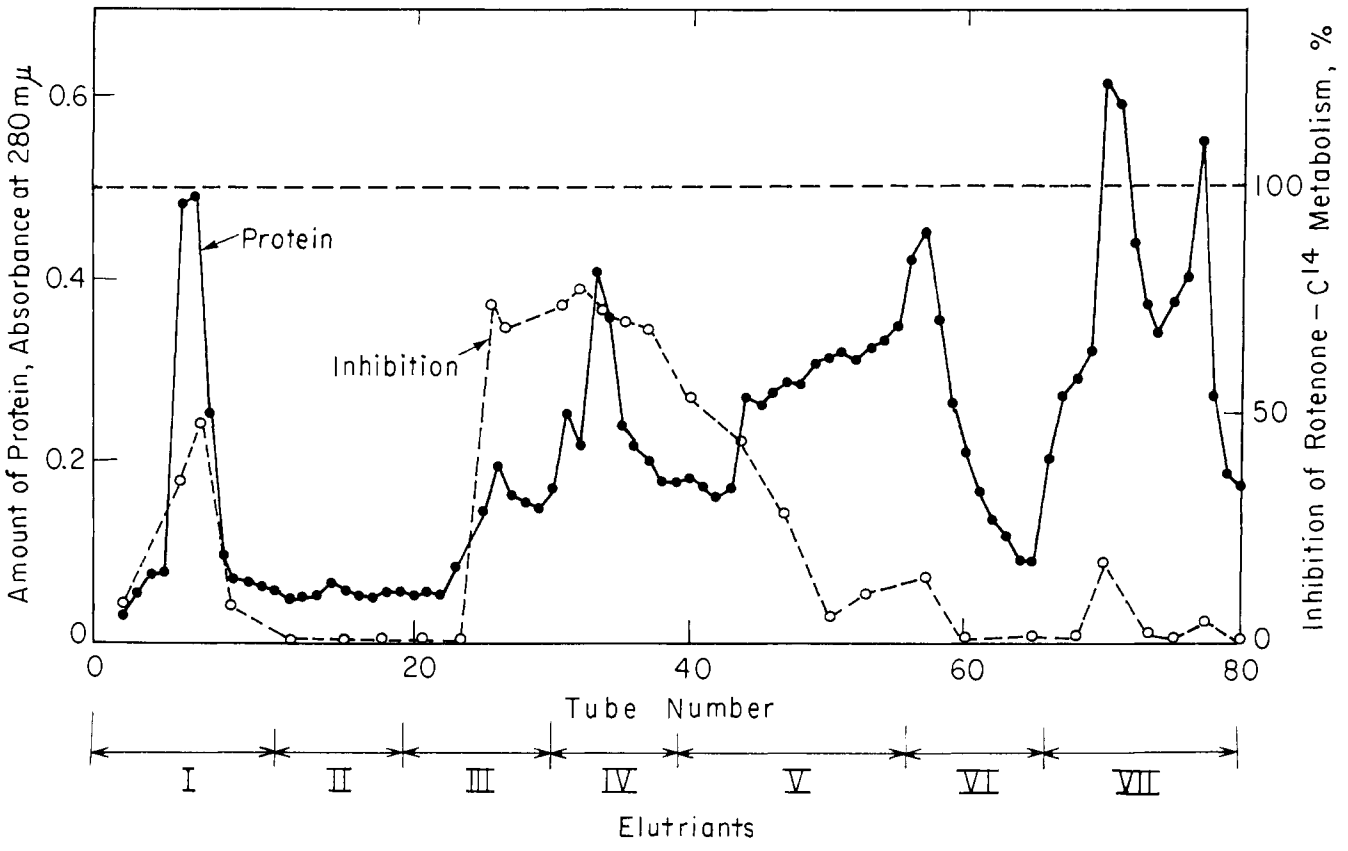

Figure 1. Chromatographic distribution of protein of cockroach mid-gut soluble fraction on a DEAE cellulose column, and of regions with activity for inhibition of rotenone-^{14}C metabolism by rat liver microsome-NADH system

Table V. Metabolites of Rotenone-^{14}C in Various Organs or in Excreta Following Oral Administration to Mice, Rats, and Carp and Injection into Male American Cockroaches

Organ or Excreta	Sampling Time or Interval, Hours	No. of Animals or Samples	Radiocarbon Recovered in Relation to Administered Dose, %	Radiocarbon Recovered from Organ or Excreta as Indicated Product, %									
				Rotenone	Rotenolone		8′-Hydroxy-rotenone	8′-Hydroxy-rotenolone		6′,7′-Dihydro-6′,7′-dihydroxy-rotenone	6′,7′-Dihydro-6′,7′-dihydroxy-rotenolone		Water-soluble products
					I	II		I	II		I	II	
Mouse													
Liver	4	4	4.4	10	>—3—<		>—6—<			>—49—<			32
Small intestine	4	4	21.6	16	>—4—<		>—4—<			>—39—<			37
Urine	0–22	4	19.5	1	>—0—<		>—0—<			>—17—<			82
Rat													
Urine	0–24	2	20.0	0	0	0	1	0	0	9	7	4	79
Carp													
Brain	4	10	0.4	7	6	3	4	4	2	6	5	7	56
Liver	4	10	2.2	8	11	0	3	4	0	7	4	3	60
Rectum	4	10	1.6	5	7	0	1	0	0	3	3	4	77
Small intestine	4	10	6.2	14	3	1	1	1	1	5	5	3	66
Liver	24	10	0.8	1	2	0	3	0	0	5	3	1	85
Rectum	24	10	6.6	1	0	1	2	0	0	1	1	1	93
Small intestine	24	10	1.9	1	1	0	1	0	0	2	1	2	92
Residual tissues	24	10	2.0	12	23	0	7	0	0	7	5	1	45
Water	24	5	10.8	2	5	0	1	0	0	1	0	12	79
Cockroach													
Fat body	24	12	2.5	51	29	0	2	0	0	4	3	3	8
Mid-gut	24	12	19.1	53	19	0	2	0	0	5	5	1	15
Nerve	24	12	1.0	54	27	0	3	0	0	4	2	2	8
Residual tissues	24	12	53.6	43	23	0	4	1	0	3	3	1	22
Feces	24	12	2.9	73	14	0	1	2	0	1	1	3	5

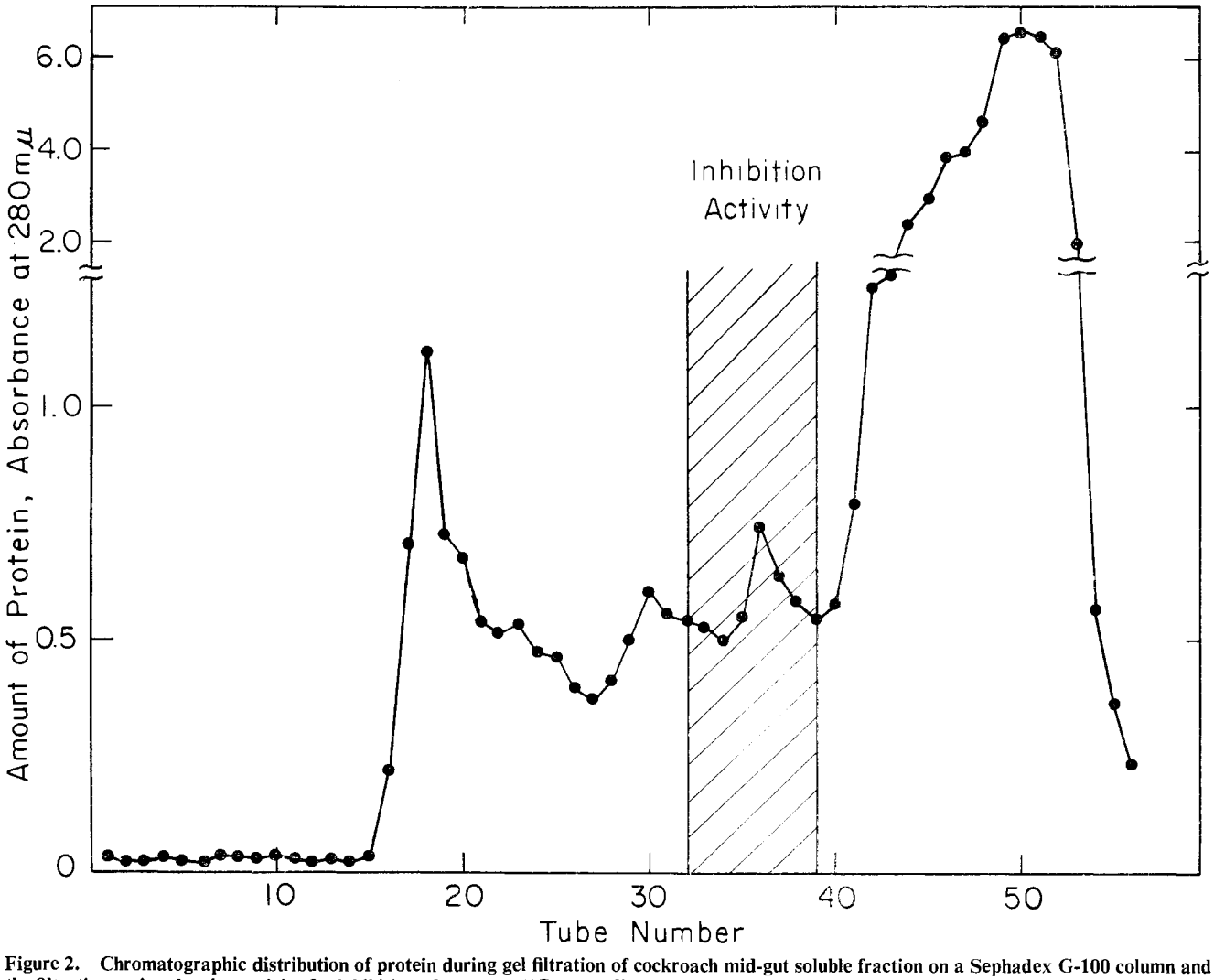

Figure 2. Chromatographic distribution of protein during gel filtration of cockroach mid-gut soluble fraction on a Sephadex G-100 column and the filtration region showing activity for inhibition of rotenone-^{14}C metabolism by rat liver microsome-NADH system

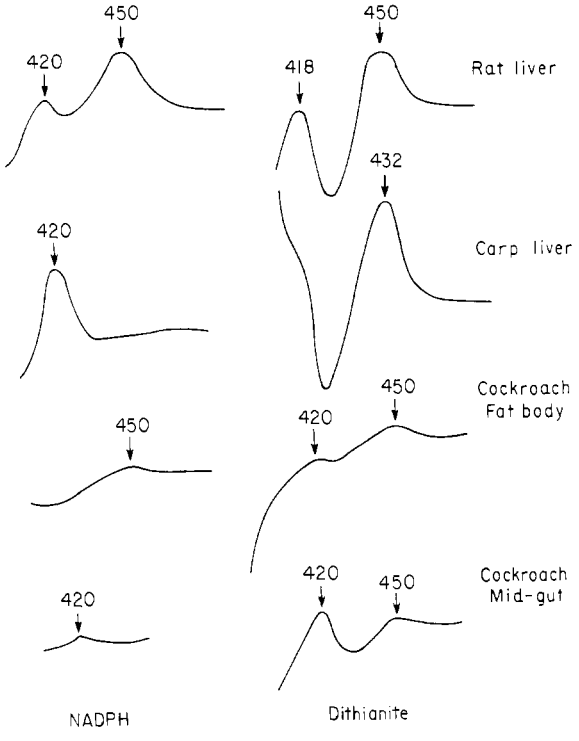

Figure 3. CO-Difference spectra of microsomal pigments from rat liver, carp liver, cockroach fat body, and cockroach mid-gut in the presence of NADPH or sodium dithionite

in buffer (Omura and Sato, 1964), are given in Figure 3. With liver microsomes reduced either with dithionite or NADPH, the CO treatment yields sharp peaks at 450, 418, and 420 mμ, as expected, but a single peak appears with carp liver microsomes at 420 mμ on reduction with NADPH and at 432 mμ on reduction with dithionite. The presence of cytochrome P-450 in cockroach fat body and mid-gut microsomes is indicated by partial to complete disappearance of the 450-mμ peak and formation of a 420-mμ peak on CO treatment of the microsomal fraction as reduced with NADPH. Quantitative results, in terms of the amount of cytochrome determined in the presence of NADPH relative to microsomal protein, show that the amount of P-450 in the fat body microsomes is approximately one-seventh that found in rat liver microsomes but that the amount in mid-gut microsomes is negligible.

Metabolism of Rotenone-^{14}C by Living Mice, Rats, Carp, and Cockroaches. Four hours after oral administration of rotenone-^{14}C to mice, the percentage recovery of the administered radiocarbon in the combined ether and water extracts of various tissues is as follows: brain, 0.04; kidney, 0.6; liver, 4.4; and small intestine, 21.6. Dihydrodihydroxyrotenone and its rotenolone are the major metabolites in the liver and small intestine. Urine collected during the first 22 hours following rotenone-^{14}C administration contains 19.5% of the administered radiocarbon which fractionates as shown in Table V. Of the 82% of water-soluble metabolites,

15% go into the acid ether extract, 61% into the *n*-butanol extract, and 5% remain in the aqueous phase.

Twenty-four hours after oral administration of rotenone-^{14}C to rats, the accumulated urine contains 20% of the administered radiocarbon and consists of products that fractionate in much the same manner as those products found in mouse urine (Table V). Dihydrodihydroxyrotenone is the major ether-extractable metabolite in rat urine, and most of the water-soluble metabolites are recovered in the *n*-butanol extract. The results for the neutral ether extracts of liver and small intestine are not given in Table V because the products found by TLC analysis were not confirmed by cochromatography; however, the pattern of metabolites is similar to that found for the liver microsome-soluble-NADPH system.

Twenty-four hours after introducing rotenone-^{14}C-food balls into water containing young carp, the water (containing excreta) consisted both of water-soluble products and a material chromatographing in the position of dihydrodihydroxyrotenolone II; however, the identification of this rotenolone is not certain because of the lack of authentic material for comparison. The metabolites in the neutral ether extracts of tissues are similar to those formed by the liver microsome-soluble-NADPH system as well as those formed *in vivo* by mice and rats; the presence of dihydrodihydroxyrotenone is based on cochromatography (Table V).

Each of the tissues and feces analyzed from male American cockroaches, injected with rotenone-^{14}C, contains rotenolone I and dihydrodihydroxyrotenone (identified by cochromatography), as well as other products similar to or the same as those formed by the fat body microsome-NADPH or microsome-soluble-NADPH enzyme systems (Table I and Table V).

Biological Activity of Rotenone Metabolites. Rotenone and 8′-hydroxyrotenone are more toxic to mice than rotenolone I; these compounds are much more toxic than rotenolone II or dihydrodihydroxyrotenone when each compound is administered intraperitoneally (Fukami *et al.*, 1967); and rotenone is a more potent inhibitor of the NADH oxidase activity of insect and mammalian mitochondria than the rotenolones (Fukami *et al.*, 1959; Horgan *et al.*, 1968). When authentic samples of the rotenoids are assayed for inhibition of rat liver mitochondrial respiration with pyruvate as the substrate, the levels necessary to inhibit O_2-uptake by 50%, in terms of micromoles of rotenoid per gram of protein, are as follows: rotenone, 33; 8′-hydroxyrotenone, 300; dihydrodihydroxyrotenone, 1600; rotenolone I (6aβ, 12aβ, 5′β isomer), 170; rotenolone I (6aα, 12aα, 5′β isomer), 1540; rotenolone II (6aβ, 12aα, 5′β isomer), >3500.

The inhibitory potency for NADH oxidase activity, as assayed with rat liver mitochondria and L-glutamate, is reduced 4-fold or more for both the whole incubation mixture and the ether-soluble fraction as a result of NADPH fortification when rotenone-^{14}C is incubated with the rat liver microsome-plus-soluble system and equivalent radiocarbon levels are used for the comparisons.

DISCUSSION

Rotenone is metabolized in living rats and houseflies, and in the microsome mixed function oxidase systems of rat liver and housefly abdomens, by hydroxylation of the 12a-position of the B-C ring juncture, yielding rotenolones, by oxidation of the isopropenyl side chain, yielding 8′-hydroxyrotenone and 6′,7′-dihydro-6′,7′-dihydroxyrotenone, and by formation of unidentified water-soluble metabolites (Fukami *et al.*, 1967; Yamamoto, 1969). The same or similar products are formed in living mice, carp, and cockroaches, and on incubation with mixed function oxidases of mouse liver, carp liver, carp small intestine, and cockroach fat body. The metabolic pathway for rotenone is the same for each of the animals studied, and the mixed function oxidases initiate the detoxication reactions and limit the persistence of rotenone. These reactions appear to be detoxification mechanisms, as shown by direct assays of authentic rotenoids for potency as NADH oxidase inhibitors and as toxicants for mice, and by assays of metabolite mixtures containing the unidentified products for potency as NADH oxidase inhibitors. In view of the high structural specificity of rotenoids, in regard to toxicity and NADH oxidase inhibition (Martin, 1946; Dann *et al.*, 1954; Fukami *et al.*, 1959; Burgos-Gonzalez and Redfearn, 1965), it is likely that structural modifications in the rotenone molecule during metabolism will reduce, or at least change, the biological activity.

The microsomal mixed function oxidases active in rotenone metabolism differ with species in activity per unit of tissue but possibly not in activity per unit of cytochrome P-450. The vertebrate livers are more active than the cockroach fat body, but they have a higher content of cytochrome P-450. Lower activity of fish liver than of mammalian liver has also been observed with other substrates (Buhler and Rasmusson, 1968). The presence of cytochrome P-450 has previously been noted in endoplasmic reticulum from fish liver, including carp, and from insects at concentrations below that found for rat liver (Chan *et al.*, 1967; Ray, 1967; Omura *et al.*, 1968). Variations in the difference spectra noted for microsomal pigments (endoplasmic reticulum) from different species and organs possibly are related to the instability of the cytochrome P-450 or to changes in the binding to the lipoprotein complex during preparation for analysis. Housefly microsomes differ somewhat from pig liver microsomes in sensitivity to CO, sensitivity to methylenedioxyphenyl compounds, and pH optima (Lewis *et al.*, 1967; Ray, 1967); also, fish liver mixed function oxidases have a lower temperature optimum than the corresponding mammalian system (Buhler and Rasmusson, 1968). Methylenedioxyphenyl compounds and certain other chemicals are similar in potency for inhibition of rotenone metabolism by rat liver microsome-NADPH and microsome-soluble-NADPH systems, and by microsome-NADPH systems from carp liver and cockroach fat body. The increased toxicity of rotenone to Mexican bean beetles and to house mosquito larvae, in the presence of piperonyl butoxide (Brannon, 1947), egonol (Matsubara, 1953), and sesame oil (Simanton, 1949), probably results from inhibition by these compounds of rotenone detoxification by mixed function oxidases.

The soluble fraction of mammalian liver homogenates increases the extent of rotenone metabolism and, particularly, the conversion to unidentified water-soluble metabolites. The active component, which appears to be a protein and may be an enzyme, converts the rotenone hydroxylation products initially formed by the mixed function oxidases to water-soluble products which probably are conjugates. Water-soluble metabolites are formed extensively in vertebrate tissues and liver-microsome-soluble-NADPH systems but not in the cockroach tissues or cockroach fat body microsome-soluble-NADPH system. The factor involved in conversion of rotenone to water-soluble products is lacking in the soluble fraction of housefly abdomens, horn beetle mid-gut, cockroach mid-gut, and cockroach fat body homogenates. Species differences in the rate of conversion of rotenone to hydroxylation products and, subsequently, to more polar

derivatives, due to components of the soluble fraction, possibly contribute to the lower toxicity of rotenone to mammals than to insects or fish.

Natural inhibitors of mixed function oxidases occur in certain tissue homogenates, particularly from insect sources. Previous studies on these natural inhibitory substances from insects show that they limit the activity and apparent stability of the enzyme systems, are localized in certain body regions and occur, to the least extent, in housefly abdomens (Tsukamoto and Casida, 1967b), are concentrated in or liberated primarily from the nuclei-plus-debris fraction (Nakatsugawa and Dahm, 1965; Chakraborty *et al.*, 1967; Tsukamoto and Casida, 1967b), are also present in the soluble fraction (Nakatsugawa and Dahm, 1965), are relatively heat stable (Matthews and Hodgson, 1966; Chakraborty *et al.*, 1967; Tsukamoto and Casida, 1967b), become less effective on enzyme dialysis (Matthews and Hodgson, 1966), on dilution (Schonbrod and Terriere, 1966), and in the presence of bovine serum albumin (Tsukamoto and Casida, 1967a), and, in part, possibly consist of neutral organic compounds (Tsukamoto and Casida, 1967a). A heat-stable inhibitor of lipid peroxidation also occurs in housefly homogenates and microsomes (Lewis *et al.*, 1967). These properties are not appropriate for a single type of inhibitory substance; so, many different types of natural inhibitors probably are involved. The present studies further define the nature of one of these inhibitors; namely the one in the soluble fraction of cockroach mid-gut homogenates, as a protein which perhaps is an enyzme with a molecular weight in the range of 6,000 to 15,000. It is not known whether comparable polypeptide- or protein inhibitors occur in other insects or function *in vivo* to control the activity of the mixed function oxidases. Therefore, these are areas of interest for future investigations in the realm of selective toxicity mechanisms.

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