Metabolism of [^{14}C]Hydroprene (Ethyl 3,7,11-Trimethyl-2,4-dodecadienoate) by Microsomal Oxidases and Esterases from Three Species of Diptera

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Both oxidative and hydrolytic enzymes attack the juvenile hormone analogue, hydroprene (ethyl 3,7,11-trimethyl-2,4-dodecadienoate) in house flies, flesh flies, and blow flies. Microsomal oxidases isolated from the three species metabolized the parent compound as well as the carboxylic acid liberated by microsomal esterases. The first products of oxidation appear to be epoxides formed at either the 2,3 or 4,5 double bond positions and these are converted, on further exposure to the enzymes, to unresolved polar products. One of the latter may be the diol acid formed by hydration of the epoxide. The same products occur in vivo when the three species are treated with hyroprene. The substrate is oxidized more rapidly by microsomes of resistant house flies than by susceptible strains but there is little difference in their respective esterase activity. Phenobarbital in the diet of adult house flies causes a fourfold increase in the oxidative metabolism of hydroprene but has no effect on the activity of the esterase system.

The juvenile hormone analogues of the alkyl 3,7,11-trimethyl-2,4-dodecadienoate type are known to be metabolized by insect esterases and microsomal oxidases (Terriere and Yu, 1973; Yu and Terriere, 1975a; Terriere and Yu, 1977; and Weirich and Wren, 1973) but the site and extent of the attack by the oxidases is not fully understood. One of these compounds, methoprene, which contains a methoxy group in the C-11 position, is metabolized in vivo by the house fly and mosquito (Quistad et al., 1975), and by the yellow mealworm and large milkweed bug (Solomon and Metcalf, 1974) to its hydroxy ester. Presumably, this results from the action of a microsomal *O*-demethylase. However, those analogues such as hydroprene which do not contain the C-11 methoxy group are also metabolized by an NADPH-requiring system (Terriere and Yu, 1973; Yu and Terriere, 1975a; Terriere and Yu, 1977). It seems likely, therefore, that further oxidation by these enzymes occurs at one or both of the 2,4 double bond positions as has been reported in the case of methoprene (Quistad et al., 1975). Further studies of these two metabolic systems in house flies, blow flies, and flesh flies are reported here.

MATERIALS AND METHODS

Insects. Laboratory reared blow flies (*Phormia regina* (Meigen)), flesh flies (*Sarcophaga bullata* Parker), and house flies (*Musca domestica* L.) were used. The experiments with house flies involved four strains: the insecticide-susceptible CSMA and SRS strains with low microsomal oxidase activity and the insecticide resistant Isolan-B and Fc strains with high microsomal oxidase activity. Rearing methods have been described elsewhere (Terriere and Yu, 1976a; Terriere and Yu, 1976b). Except where noted all experiments were with 7-day-old female adults.

Chemicals. 5-[^{14}C]Hydroprene (ethyl (2*E*,4*E*)-3,7,11-trimethyl-2,4-dodecadienoate), sp act. 58 mCi/mM, unlabeled hydroprene, and ZR-525 (3,7,11-trimethyl-2,4-dodecadienoic acid) were supplied by the Zoecon Corp., Palo Alto, Calif.

In Vitro Experiments. Microsomes were prepared from adult abdomens by established procedures (Yu and Terriere, 1975a). The in vitro incubation medium and conditions used for studying the metabolism of hydroprene were essentially the same as those reported previously (Terriere and Yu, 1973). The 5-mL incubation mixture contained microsomes equivalent to ten abdomens (approximately 1.2 mg of protein); 0.1 M sodium phosphate buffer, pH 7.5; 10 μg of cold hydroprene together with 0.17 μg (80 000 dpm) of [^{14}C]hydroprene in 0.01 mL of methyl Cellosolve and 10 mg of bovine serum albumin. When microsomal esterase activity was being measured, the mixture contained no added NADPH. An NADPH-generating system was added when microsomal oxidase activity was to be determined and the extent of this activity was estimated by difference. The incubations were carried out in an atmosphere of air with shaking for 3 h. All incubations were in duplicate.

At the completion of incubation the mixtures were saturated with ammonium sulfate and extracted once with 10 mL and twice with 5 mL of a 2:1 mixture of ethyl ether–ethanol. This procedure recovered all of the added radioactivity. The extracts were combined and dried over sodium sulfate. Aliquots were then evaporated to approximately 0.2 mL and applied on silica gel G thin-layer chromatoplates (0.25 mm thick, 5 cm × 20 cm). The chromatoplates were developed unidimensionally with hexane–ethyl acetate (100:15), then with benzene–ethyl acetate–acetic acid (100:30:3) (Quistad et al., 1975). The radioactive metabolites and unreacted hydroprene were detected by passage through a radiometric scanning device (Vanguard automatic chromatogram scanner, Model 885). The zones of radioactivity were scraped from the plates, placed in scintillation vials with 12 mL of scintillation mixture, and counted in a Nuclear-Chicago liquid scintillation counter. Recovery of added radioactivity carried through the complete procedure was 75%.

In Vivo Experiments. Groups of five female adults were topically treated on the dorsal abdomen with [^{14}C]hydroprene in 1 μL of acetone. The treated flies were then kept for 2 h in a capped scintillation vial. After treatment, each group of flies was first rinsed with 12.5 mL of hexane and then homogenized in 10 mL of ethyl ether–ethanol (2:1). The homogenate was retained and the homogenizer rinsed once with 5 mL of the solvent mixture. The combined extracts were centrifuged to precipitate the tissues. The supernatant was decanted into a flask and the residue extracted once with 5 mL of the solvent mixture.

Aliquots of the combined extracts as well as the body rinse and container rinse (excreta) were dried over anhydrous sodium sulfate, evaporated to 0.2 mL under a stream of nitrogen, and spotted on chromatoplates as before. The plates were developed in the same solvent systems as above, scanned, and quantified by liquid scintillation counting. These procedures resulted in the recovery of 93% of the administered radioactivity.

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Characterization of Metabolites. Five zones were observed on the radiochromatograms when microsomes were incubated with [^{14}C]hydroprene in the presence of NADPH. These zones were numbered according to their increasing R_f value. Zones containing metabolites II, III, IV were scraped from the plates and extracted three times with 5 mL of ethyl acetate. The zone containing metabolite I was extracted twice with 5 mL of ethyl ether–ethanol (2:1) and once with 5 mL of ethyl acetate. Aliquots of the extracts containing approximately 20 000 dpm were evaporated to dryness under nitrogen and used in the following characterizations.

Enzymatic Conversion. The metabolite in each zone was incubated with house fly microsomes to seek evidence of further metabolism. The 5 mL of incubation mixture contained microsomes equivalent to 20 abdomens; 0.1 M sodium phosphate buffer, pH 7.5; 20 000 dpm of the metabolite; 10 mg of bovine serum albumen; and a NADPH-generating system (when microsomal oxidation was being studied). The incubations were carried out with shaking at 34 °C in an atmosphere of air for 3 h. The products were extracted and analyzed by TLC as above.

The material under zone I (in vivo experiments) was exposed to sulfatase (from limpets) and β-glucosidase (from almonds) to test for conjugation as described previously (Yu and Terriere, 1975b). The reaction mixture was then saturated with ammonium sulfate and extracted twice with 5 mL of ethyl ether–ethanol (2:1). The combined extracts were analyzed by TLC.

Esterification. Each metabolite was tested for the presence of an esterifiable group by treating with methanol–BF_3 (Supelco Inc., Bellefonte, Pa.) (Terriere and Yu, 1977). The reaction products were examined by TLC as before.

Bromination. Each metabolite was tested for the presence of a double bond by treating for 30 min with 2 mL of 10% bromine in carbon tetrachloride. The mixture was then evaporated to dryness under nitrogen. The residue was dissolved in ethyl ether–ethanol (2:1) and analyzed by TLC.

Oxidation. Metabolite III was tested for the presence of an aldehyde group by heating with 1 mL of Benedict's solution for 2 min on a boiling water bath. The mixture was then extracted twice with 2 mL of ethyl ether–ethanol (2:1) and the combined extracts were analyzed by TLC. All of the metabolites (except those under zone I) including hydroprene were treated with 0.15 g of *m*-chloroperoxybenzoic acid (MCPBA) in 8 mL of methylene chloride at 0 °C for 1–3 h with gentle shaking. The mixture was washed with 10 mL of aqueous sodium sulfite and evaporated to 0.2 mL under nitrogen. The concentrate was analyzed by TLC.

Hydration. The metabolites were treated with 5 mL of 0.05 N sulfuric acid in 50% aqueous tetrahydrofuran for 18 h at room temperature with occasional swirling. The mixture was saturated with ammonium sulfate and extracted twice with 5 mL of ethyl ether–ethanol (2:1). The combined extracts were analyzed by TLC.

Cochromatography. Metabolite IV was cochromatographed by TLC with unlabeled ZR-525 (hydroprene acid) using the methods described earlier. ZR-525 was detected by spraying the plate with a bromocresol purple solution (Terriere and Yu, 1977). The R_f value obtained was compared with that of metabolite IV from the TLC plates.

The methyl ester of metabolite IV was also characterized by gas chromatography (GLC) on two different columns and its behavior compared with that of the methyl ester of ZR-525. The conditions for the electron-capture instrument Aerograph Model 204B were: column, 5 ft × $^1/_8$ in. glass, packed with a 1:1 mixture of 5% DC 11 and 5% QF 1 on 100/120 mesh High Performance Chromosorb W; temperatures, column, 190 °C, injector port, 225 °C, detector, 235 °C; and nitrogen gas flow rate, 14.5 mL/min. Retention time was 1.3 min. Conditions for the flame ionization instrument Aerograph Model 1200 were: column, 5 ft × $^1/_8$ in. aluminum packed with SE-30 on 100/120 mesh Uraraport 30; temperature, column, 180 °C, injector port, 205 °C, detector, 230 °C; and nitrogen gas flow rate, 27 mL/min. Retention time in this instrument was 5.0 min.

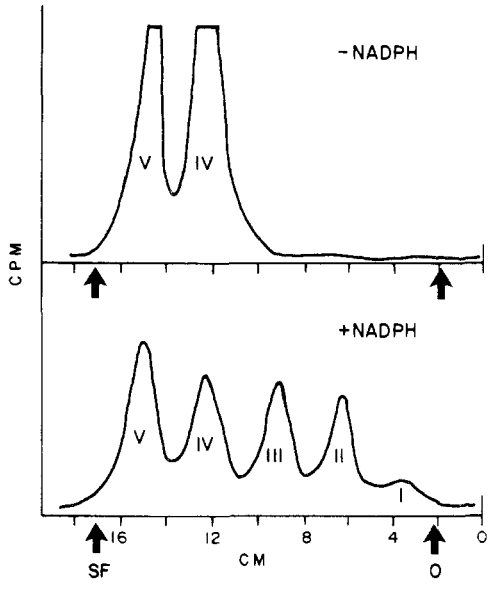

Figure 1. Typical thin-layer radiochromatogram of metabolites produced when house fly microsomes are incubated with [^{14}C]hydroprene with and without added NADPH.

RESULTS AND DISCUSSION

Evidence that at least two types of microsomal enzymes are involved in the in vitro metabolism of hydroprene is presented in the thin-layer chromatogram shown in Figure 1. The single metabolite produced in the absence of NADPH was shown, by cochromatography with an authentic sample in both TLC and GLC systems to be the carboxylic acid of hydroprene. Other tests, discussed later, confirmed this identification. The enzyme involved in this case, therefore, is an esterase.

When an NADPH-generating system was included in the incubations, three additional peaks appeared, indicating that hydroprene, and perhaps the corresponding acid, were also substrates for an oxidase enzyme system. Further examination of the eluted and rechromatographed radioactivity from the various zones indicates zones II, III, IV, and V are due to single substances while zone I represented a mixture.

Some indication of the pathways followed during the metabolism of hydroprene is given by the further metabolism of the initial products during further exposure to house fly microsomes. The results of such experiments are given in Table I where the action of the esterase (minus NADPH) and of the esterase and the oxidase (plus NADPH) has been tabulated for the four radioactive zones of the original chromatoplate. The esterase has no further effect on metabolite IV (hydroprene acid) but the oxidase converts it to metabolite II. However, metabolite II is further metabolized to products migrating to the polar zone I region. This occurs even in the absence of NADPH, a result which we attribute to the enzyme epoxide hydrase which is also present in the microsomal fraction and which

Table I. Further Conversions, in Vitro, of [^{14}C]Hydroprene Metabolites by House Fly Microsomes[a]

Substrate	Product formed[b]	
	−NADPH	+NADPH
Metab I	None	None
Metab II	Metab I[c]	Metab I[c]
Metab III	Metab II	Metab I & II
Metab IV	None	Metab II

[a] Microsomes from Isolan-B house flies. [b] Results of two-three experiments. [c] Although both conditions produced metabolite I, more radioactivity was detected in the presence of NADPH.

Table II. Results of Characterizing Tests with [^{14}C]Hydroprene and Its Metabolites

Compound	Reaction and reagent	TLC results
Metab I	Bromination (Br)	1 broad, less polar zone (R_f 0.60)
	Esterification (BF_3-CH_3OH)	2 broad, less polar zones (R_f 0.43, 0.70)
Metab II	Bromination (Br)	1 less polar zone (R_f 0.67)
	Esterification (BF_3-CH_3OH)	1 less polar zone (R_f 0.37)
	Hydration (dilute H_2SO_4)	Zone I
Metab III	Bromination (Br)	1 less polar zone (R_f 0.76)
	Esterification (BF_3-CH_3OH)	No change in *Rf*
	Oxidation (Benedict soln)	No change in *Rf*
	Oxidation (MCPBA)	Zone I
Metab IV	Bromination (Br)	1 less polar zone (R_f 0.82)
	Esterification (BF_3-CH_3OH)	1 less polar zone (R_f 0.84)
	Oxidation (MCPBA)	Zones I & II
Hydroprene	Oxidation (MCPBA)	Zones I & III

does not require this cofactor (Slade et al., 1975). The product expected from the action of this enzyme would be the diol acid. The conversion of metabolite III to metabolite II by the esterase system indicates that the former contains an ester group while the latter is acidic.

Some additional information about the hydroprene metabolites was gained by performing the characterization reactions described in the Methods section. A summary of the results of these tests is given in Table II. All of the metabolites reacted with bromine to produce less polar products, indicating that at least one of the double bonds remained intact during metabolism. Metabolites II and IV and the products present in zone I all reacted with the esterification reagent to form less polar products, indicating the presence of carboxylic or other ester-forming groups.

The lack of reaction of metabolite III with this reagent supports our belief that this metabolite is not a carboxylic acid and its failure to react with Benedict's solution indicates that it is not an aldehyde. However, metabolite III is oxidized by *m*-chloroperbenzoic acid (MCPBA) to produce products migrating to the zone I region. This is consistent with the theory that metabolite III contains an epoxy group as is the result when metabolite II is treated with dilute sulfuric acid. Further evidence that metabolites II and III contain the epoxy group is provided by the results when metabolite IV (i.e., hydroprene acid) and hydroprene are treated with MCPBA to produce metabolites II and III, respectively.

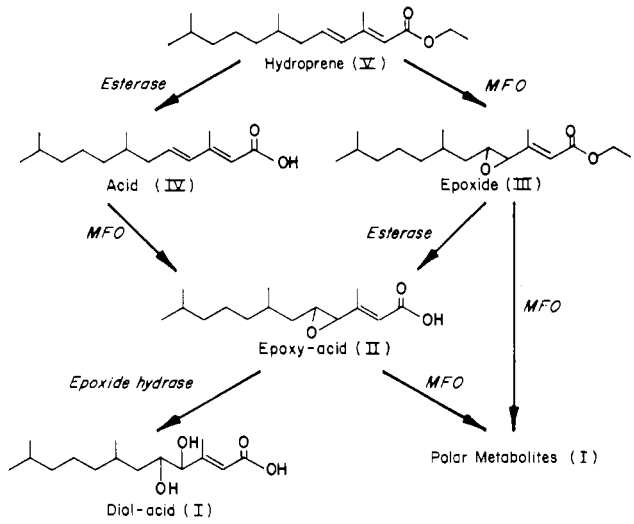

Figure 2. Proposed pathways of hydroprene metabolism by the house fly, flesh fly, and blow fly microsomal system.

An evaluation of these chemical and enzymatic conversions of the hydroprene metabolites suggests the metabolic pathways shown in Figure 2. All of the facts support the identities assigned to metabolites II, III, and IV although there is a possibility that the epoxide ring of metabolites II and III is in the 2,3 rather than the 4,5 position. However, it is likely that such compounds would be highly unstable and would not have persisted through the various experiments.

There is less evidence in support of the identity of the diol acid (metabolite I, Figure 2), the increased polarity resulting from the acid-catalyzed hydration of metabolite II and the NADPH-independent metabolism of metabolite II to more polar products. As for the other products in zone I of the chromatograms, it is suggested that these result from the rupture of the 4,5 or of the 2,3 double bond following their initial epoxidation. If the initial attack is at the 4,5 position as we assumed, these metabolites would be citronellal or citronellic acid corresponding to similar products detected when house fly larvae were treated with methoprene (Quistad et al., 1975).

Effect of Inhibitors. Further evidence that two enzyme systems are involved in the in vitro metabolism of hydroprene is seen in our tests with various inhibitors, Table III.

At 10^{-4} M piperonyl butoxide, a well-known inhibitor of microsomal oxidases, significantly inhibited the formation of hydroprene acid, i.e., it inhibited the esterase. It also strongly inhibited the microsomal oxidase, decreasing the amounts of the oxidation products.

Carbon monoxide inhibited the microsomal oxidases but had no inhibitory effect on the esterase. In fact, the amount of the acid produced was slightly higher than the control. This is probably due to inhibition of the microsomal oxidases resulting in more substrate available for the esterase. Paraoxon at 10^{-4} M inhibited the esterase as expected and phenylmercuric acetate at 10^{-4} M inhibited both enzymes. The latter is of interest because this compound is also an inhibitor of glutathione *S*-transferase in insects (Shishido et al., 1972).

Effect of Phenobarbital. Table IV shows that the oxidative metabolism of hydroprene can be increased as much as fourfold by feeding the flies a diet containing 1% sodium phenobarbital for 3 days. This compound has been found to induce the microsomal oxidases in the house fly (Yu and Terriere, 1973). The results also confirm our previous finding that the esterase is not affected by the

Table III. Effect of Inhibitors on Metabolism of [14C]Hydroprene by House Fly Microsomes[a]

Compound and concn (M)	Incubation mixture	Amount of metabolite, % of control[b]			
		Polar	Epoxy-acid	Epoxide	Acid
Piperonyl butoxide (10^{-4})	−NADPH				22
	+NADPH	0	0	43	43
CO[c]	+NADPH	0	4	7	119
Paraoxon (10^{-4})	−NADPH				8
Phenylmercuric acetate (10^{-4})	−NADPH				12
	+NADPH	0	0	0	16

[a] Microsomes from Isolan-B house flies. [b] Average of two determinations. [c] Bubbled gently for 1 min prior to incubation.

Table IV. Induction of [14C]Hydroprene Metabolism in Microsomes Prepared from House Flies Fed Phenobarbital

Incubation mixture	Amount of metabolite, % of control[a]			
	Polar	Epoxy-acid	Epoxide	Acid
−NADPH				102
+NADPH	424	264	154	81

[a] 1-day-old female flies (CSMA strain) were fed a diet containing 1% of sodium phenobarbital for 3 days. Average of two determinations.

phenobarbital treatment (Terriere and Yu, 1973).

Species Variations. Table V shows the metabolism of [14C]hydroprene by microsomes from two susceptible and two resistant house fly strains, and from blow flies and flesh flies. The pattern of metabolism was the same in all three species. The comparative esterase activity was house fly > flesh fly > blow fly, with the CSMA house flies being most active. In the case of microsomal oxidases, the oxidase activity in the two susceptible house fly strains was much lower than in the two resistant strains. The flesh flies had a higher oxidase activity than the blow flies.

Metabolism in Vivo. The results of these experiments, Table VI, show that, in vivo as well as in vitro, both enzymes attack hydroprene. The epoxide could not be detected in the excreta after any of the treatments but since these tests were of short duration (2 h), its excretion should not be ruled out.

Although not shown by the data of Table VI, there was one difference between the in vitro and in vivo results, evidence that conjugates were present in the polar metabolites. This was gained from the use of the enzymes sulfatase and β-glucosidase which appeared to convert part of the polar materials from body extract or excreta to hydroprene acid. From the amount of radioactivity recovered in the body rinse fraction 2 h after topical treatment, it is apparent that hydroprene is rather slow in its penetration of the cuticles of the three species studied.

Quistad et al. (1975) reported that house fly and mosquito larvae are able to isomerize the 2*E* form of methoprene, which also contains the 2,4-diene structure, to the 2*Z* form. We saw no evidence of this in the case of hydroprene. It is likely that the 2*Z* isomer, if present, would have been detected during the gas chromatography

Table V. Comparison of [14C]Hydroprene Metabolism by Microsomes from House Flies, Flesh Flies, and Blow Flies

Species	Incubation mixture	% of recovered radioactivity[a]				
		Polar	Epoxy-acid	Epoxide	Acid	Hydroprene
House fly						
SRS	−NADPH	0	0	0	44.8	55.3
	+NADPH	4.2	14.4	15.0	26.3	40.2
CSMA	−NADPH	0	0	0	56.2	43.8
	+NADPH	0.9	15.7	16.9	40.4	26.1
Isolan-B	−NADPH	0	0	0	42.6	57.5
	+NADPH	17.1	26.9	22.5	15.4	18.1
Fc	−NADPH	0	0	0	38.2	61.8
	+NADPH	8.7	17.2	17.7	25.3	31.3
Flesh fly	−NADPH	0	0	0	24.5	75.5
	+NADPH	8.0	12.7	21.5	20.5	37.2
Blow fly	−NADPH	0	0	0	9.6	90.4
	+NADPH	1.3	12.6	7.2	8.0	70.4

[a] Average of two determinations.

Table VI. Distribution of Radioactivity 2 h after Topical Application of [14C]Hydroprene to House Flies, Flesh Flies, and Blow Flies

Species	Distribution	% of recovered radioactivity/fly[a]				
		Polar	Epoxy-acid	Epoxide	Acid	Hydroprene
House fly (Isolan-B)[b]	Body extract	3.2	1.0	0.3	0.4	6.2
	Excreta	1.0	0.4	0	0.4	12.3
	Body rinse	0	0	0	0	74.8
Flesh fly[c]	Body extract	2.7	5.0	1.5	2.7	13.0
	Excreta	0.2	0.3	0	0.2	6.5
	Body rinse			67.9[d]		
Blow fly[c]	Body extract	0.5	0.7	0.4	0.5	7.4
	Excreta	0.2	0.2	0	0.3	9.4
	Body rinse			80.4[d]		

[a] Average of two determinations. [b] 0.27 μg of [14C]hydroprene/female adult. [c] 0.57 μg of [14C]hydroprene/female adult. [d] Not resolved.

of the methyl ester analogue of hydroprene. Aside from the difference in compounds, our experiments differed from their's in length of exposure of the insects, 2 h vs. 24 h.

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Laser Light Scattering Bioassay for Veterinary Drug Residues in Food Producing Animals. 2. Preparation Procedures and Dose-Response Studies of Drugs in Bovine Tissues

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The differential light scattering bioassay technique has been extended to bovine tissues. The method is rapid and very sensitive. Tissue sample preparation has been reduced to a simple and rapid procedure in which the tissues are compressed in a gravity press and the exuded juice used directly after filtering. Selected dose–response data are presented for penicillin, chlortetracycline, streptomycin, and sulfaquinoxaline.

Bioassay methods utilizing differential light scattering (DLS) techniques have been shown to be useful for the detection and quantitation of low levels of veterinary drug residues in fluids from food producing animals (Wyatt et al., 1976b).

The present study is intended to extend these methods to tissue assays. As traditionally performed, such tissue assays are time consuming and extremely expensive (Microbiology Laboratory Guidebook, U.S. Department of Agriculture, 1976). They require extensive preparation, grinding, and extraction processes, followed by conventional agar well diffusion tests using various strains of selectivity sensitive bacteria. The Food and Drug Administration (FDA) hopes that it may eventually be possible to establish correlations between tissue drug levels and those in the associated urine and serum. This agency is currently pursuing such correlation studies along several avenues of research and our work here forms part of their program.

Prior to any formal application of our DLS techniques to the aforementioned correlation studies, it will be necessary to establish a rapid, consistent, and reliable tissue assay procedure. Accordingly, this paper presents details of a simplified tissue preparation procedure, some dose–response results for fortified tissue extracts, data on specificity in response to drug mixtures and the mathematical formalism required for quantitative interpretations.

DIFFERENTIAL LIGHT SCATTERING BIOASSAY PROCEDURES

Details of the DLS bioassay measurement procedures for the detection of drug residues are given in an earlier paper (Wyatt et al., 1976b) as well as in related articles (Wyatt et al., 1976a; Wyatt, 1973). In summary, the method consists of the following steps: (a) For each test specimen, a liquid aliquot is combined with an aliquot of an exponential phase broth culture of a highly sensitive bacterial strain and pronase; (b) a similar control liquid specimen is prepared from a drug-free source; (c) a blank consisting of the test specimen liquid combined with a bacterial-free broth and a similar blank using the control liquid specimen are prepared; (d) the mixtures are incubated for 2–3 h; (e) the mixtures are diluted (usually with isotonic saline) and allowed to equilibrate for about 30 min; and (f) the DLS patterns of the control, test, and blank specimens are recorded, compared, and a "score" calculated.

The DLS measurement itself is made following the schematic of Figure 1: The diluted specimen is placed in a cuvette which is illuminated by a fine laser beam (vertically polarized $\lambda = 632.8$ nm); a collimated detector rotates about the cuvette measuring the scattered light intensity as a function of angle; the detector signal is converted into a graphical or digital representation; changes or differences in the DLS test pattern with respect to the DLS control pattern are examined by means of a mathematical algorithm (Wyatt et al., 1976a,b) to generate a score. Most of the measurements of the ongoing program have been made using a Differential III system (Wyatt, 1975; Stull, 1973) and an algorithm designed initially for antibiotic susceptibility testing.

Tissue extract specimens are prepared by compressing a frozen 20–30-g sample for several minutes in a 5 cm diameter gravity press activated by a 10 kg mass. The resultant juice (~1 to 5 mL) is then filtered by vacuum through a Whatman No. 40 filter. Muscle juice is further processed by combining nine parts juice to one part of a 30% citrate solution. (The citrate solution is made by mixing 13.2 g of citric acid, 7.06 g of NaOH, 97 g of sodium citrate, and distilled water to make 366.7 mL. If necessary,

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