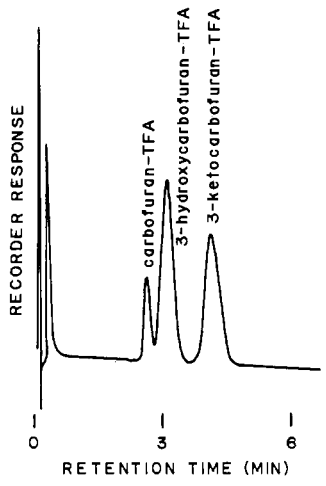

Figure 5. Gas chromatogram of mixture of TFA derivatives of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran, 10 ng of each injected, using column 4 at 195 °C and electron-capture detector

identical, as illustrated in Table I for carbaryl and carbofuran. Similar observations have been made previously with homologous perfluoroacylated amines (Vanden Heuvel *et al.*, 1964). Relative electron-capture response increased only twofold for carbaryl and fivefold for carbofuran on passing from the trifluoroacetyl to the heptafluorobutyryl derivatives. Increases of several thousandfold have been reported for homologous series of perfluoroacylated aliphatic amines (Clarke *et al.*, 1966).

Analysis of technical products can be done rapidly and conveniently by the direct derivatization procedure, as illustrated in Figure 4 for technical Bux insecticide. Bux is a mixture of six major structural isomers, the *ortho*-, *meta*-, and *para*-2- and 3-pentyl-substituted phenyl methylcarbamates. Resolution of the individual isomers by direct gas chromatography is not satisfactory; improved resolution was effected, however, by chromatography of the TFA derivatives.

Hydroxy metabolites of methylcarbamates are readily converted to nonpolar *N*- and *O*-trifluoroacetylated derivatives with trifluoroacetic anhydride. Thus, determination of metabolites by this method appears to offer some real advantages worth pursuing further. The electron-capture gas chromatogram of carbofuran and its hydroxy and keto metabolites (Figure 5) serves to illustrate this point. In addition to the convenient peak shape and retention time, the trifluoroacetylated hydroxy metabolite offers greater sensitivity to electron-capture, owing apparently to the presence of two trifluoroacetyl groups.

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A Glc Assay for Microsomal Thioether Oxidation

p-Chlorothioanisole has been investigated as a substrate for microsomal thioether oxidation. In both mouse and housefly microsomal preparations, it is oxidized to *p*-chlorophenyl methylsulfinyl ether. The ease and accuracy of the assay procedure suggest that *p*-chlorothioanisole may have wide application for the measure of thioether oxidation.

In vitro assays for microsomal thioether S-oxidation commonly use chlorpromazine as a substrate (Salzman and Brodie, 1956; Hart and Fouts, 1965; Rubin *et al.*, 1964). Chlorpromazine may, however, also undergo side-chain oxidation, ring hydroxylation, and/or N-oxidation. Furthermore, chlorpromazine is neither a simple nor a readily available substrate. Since insects both activate (Metcalf *et al.*, 1957, 1963, 1966) and detoxify (Kapoor *et al.*, 1970) insecticides through thioether oxidation, a simple substrate may have more utility for insecticide studies in both insects and higher animals.

We present here an assay method encompassing a readily available substrate whose metabolites are limited to thioether oxidation products.

METHODS AND MATERIALS

Model Metabolites. *p*-Chlorothioanisole, mp 17–19 °C, was obtained from Matheson Coleman and Bell, Norwood,

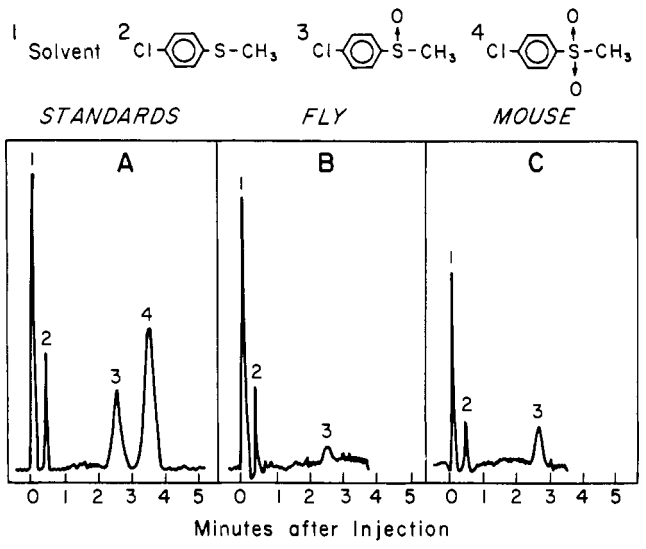

Figure 1. Glc chromatograph of *p*-chlorothioanisole and oxidation products. The solvent is hexane

Ohio. *p*-Chlorophenyl methylsulfinyl ether, mp 37–39°C, and *p*-chlorophenyl methylsulfonyl ether, mp 92°C, were synthesized by oxidation with H_2O_2 and performic acid, respectively. The products were characterized by ir and nmr spectrometry and were >99% pure by glc assay.

Gas Chromatography. A Packard Gas Chromatograph equipped with a tritium electron-capture detector was used for gas chromatography. A 2 ft × 1/8-in. i.d. coiled glass column packed with 3% Poly-A 103 on Gas Chrom Q (100–120 mesh) (Applied Science Labs., Inc., State College, Pa.) proved best for separation of products. The column was conditioned at 200°C, 100 $cm^3 N_2$/min for 48 hr. Temperatures were as follows: injection port, 230°C; detector, 190°C; column, 160°C (isothermal); and outlet, 210°C. The carrier gas was nitrogen; flow rate 100 cm^3/min.

Retention times for the sulfoxide and sulfone of *p*-chlorothioanisole were 2.5 and 3.6 min, respectively. The *p*-chlorothioanisole starting material came off immediately after the solvent (Figure 1). Peak height *vs.* width at half-height was used for quantitative analysis.

Enzyme Assays. Six white male mice (CF-1 strain, Carworth, Portage, Mich.), 8-weeks-old, were killed by decapitation. The liver was removed and 0.5 g wet weight was ground in 4.5 ml of 1.15% KCl–0.25% nicotinamide buffer in a glass homogenizer. The homogenate was spun at 10,000 × *g* for 10 min in a Sorvall RC-2B centrifuge and the supernate was spun at 100,000 × *g* for 1 hr in a Beckman Model L-2 ultracentrifuge. The Biuret protein test was run on the 10,000 × *g* supernate (0.5 ml of 10% desoxycholate, 0.5 ml of supernate, 5.0 ml of Biuret reagent). The 100,000 × *g* pellet was resuspended in 4.5 ml of sodium phosphate buffer (0.1 *M*, pH 7.8) by sonification. A Branson Model S125 sonifier equipped with a horn, 0.5 in. at the tip, was used for sonification at 20,000 Hz using centrifuge tubes in ice. This is probably the easiest way to resuspend a microsomal preparation. In separate experiments in this lab, sonification only nominally reduces the level of enzyme activity; 10% at the maximum. Livers were handled individually. Abdomens from 50 female R_{sp} houseflies 6-days-old were used for the fly assays. The routine was the same except that the abdomens were ground in 4.5 ml of sodium phosphate buffer, 0.1 *M*, pH 7.8.

Table I. Thioether Oxidation by Mouse Liver and Housefly Abdomen Microsomes

Animal	Protein conc, mg/ml	Product μmoles of sulfoxide per assay	Specific activity, mole/mg of protein/hr (×10^{-8})
Mouse 1	13.0	0.114	8.76
2	14.8	0.090	6.08
3	14.0	0.111	7.92
4	13.56	0.112	8.26
5	13.50	0.114	8.44
6	12.80	0.071	5.54
			Avg 7.50 ± 1.08
Housefly	2.20	0.071	6.45

A modified system of LaDu *et al.* (1955) was used for enzyme assays. Reagents were as follows: 0.2 cm^3 of $NaPO_4$ buffer, pH 7.8; 0.3 cm^3 of NADP (10 mg/cm^3) (Sigma); 0.2 cm^3 of G-6-P (10 mg/cm^3) (Sigma); 0.1 cm^3 of $MgCl_2$ (76 mg/cm^3); 0.1 cm^3 of G-6-P dehydrogenase (10 units/cm^3) (Torula, Sigma); 0.1 cm^3 of nicotinamide (61 mg/cm^3); 0.2 cm^3 of 100,000 × *g* suspension (Mouse); 1.0 cm^3 of 100,000 × *g* suspension (housefly); 0.1 cm^3 of *p*-chlorothioanisole (30 μmol/cm^3 95% EtOH); and H_2O to 2.5 ml. Mouse assays were incubated in 25-ml Erlenmeyer flasks for 0.5 hr at 37°C in a Dubnoff metabolic shaker. Fly assays were incubated at 27°C. Sulfoxide production was proportional to enzyme concentration at up to four times that used here for the mouse and up to 1.4 times for the fly.

Extraction of Products. The reaction was stopped by placing the flasks on ice, adding 3.0 ml of reagent grade ethyl acetate, and shaking. The layers were separated by spinning in 12-ml conical centrifuge tubes in a clinical centrifuge for 3 min. The ethyl acetate layer was removed with a pipette, evaporated at 30°C in a rotary evaporator, and the residue redissolved in 3.0 ml of spectrograde hexane for glc analysis. This method consistently gave 90% recovery of standards in pH 7.8 buffer.

RESULTS AND DISCUSSION

The results of the enzyme assays are given in Table I. Figure 1 is a typical chromatograph of standards and actual mouse and fly assays. Microsomal preparations from mouse liver oxidized *p*-chlorothioanisole to the sulfoxide analog at the rate of 7.5 (±1.08) × 10^{-8} mole/mg protein/hr. The rate for microsomal preparations from housefly abdomens was 6.44 × 10^{-8} mole/mg protein/hr. Neither *p*-chlorophenyl methylsulfonyl ether (sulfone) nor *p*-chlorothiophenol were produced under the conditions used for these assays.

On the other hand, chlorpromazine is metabolized *in vitro* by rat and human liver microsomes to many metabolites (17 or more) due to S-oxidation, demethylation, ring hydroxylation, and N-oxidation (Becket and Hewick, 1967; Coccia and Westerfeld, 1967).

Our easily obtainable and reproducible results suggest that *p*-chlorothioanisole is a superior substrate for the estimation of thioether oxidation.

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Carotenoids in Citrus: Their Accumulation Induced by Ethylene

Application of ethylene to citrus fruit after harvest enhanced external orange and red color by inducing accumulation of specific carotenoid pigments. Cryptoxanthin, β-citraurin, and to a lesser extent, violaxanthin, accumulated in the flavedo of Robinson and other citrus cultivars following treatment with ethylene or 2-chloroethylphosphonic acid (ethephon).

Ethylene has long been used to reduce the chlorophyll content of citrus peel following harvest. More recently, preharvest application of ethylene supplying materials to citrus resulted in hastening degreening on the tree (Young *et al.*, 1970). Although some early work by Baier (1932) indicated that ethylene could also improve orange pigmentation of the fruit, the work of Miller *et al.* (1940) generally has been accepted and often quoted, "Carotenoids in the peel showed no significant changes as a result of ethylene treatment."

We found, however, that postharvest ethylene application to certain citrus cultivars resulted in substantial increase of carotenoid pigments and consequently improved fruit color. In the cultivars studied, the pigments that increased most were those that contributed most to the orange and red colors, *i.e.*, cryptoxanthin (orange) and β-citraurin (reddish orange) (Figure 1). *Trans*- and *cis*-violaxanthin (yellow) increased to a lesser extent.

Subsequent to the initial preparation of this manuscript, Daito and Hirose (1970) reported enhanced carotenoid pigmentation following ethephon treatment. Our results were qualitatively similar to theirs. However, we have provided in more detail the changes in individual pigments resulting from ethylene treatment and have related them to external fruit appearance.

EXPERIMENTAL

Ethylene was applied to fruit of several cultivars, including Robinson, Orlando, Temple, Pineapple, and Hamlin. Although responses varied, ethylene application caused increased carotenoid accumulation in all tested cultivars. Since the Robinson gave the most marked response and was studied in greatest detail, most of the results reported here will be based on work with Robinson fruit.

Fruit were placed in 20-l. jars at room temperature and ethylene was added to give a concentration of 10 ppm. Ethylene was added on three consecutive days. The jars were flushed with air prior to each ethylene addition and then closed for 24 hr. After the third day, the jars were maintained at room temperature with a loose plastic cover to reduce moisture loss.

External fruit color was measured with a Hunter Color and Color Difference Meter. With this reflectance instrument, "a" values are on a green to red scale and "b" values on a blue to yellow scale. The a/b ratio increased with increasing orange or red color and was well correlated with visual differences among the fruit and with the USDA citrus fruit color standards (Harding and Sunday, 1953). This ratio was used in quantitative color evaluation.

Carotenoid pigments in the peel samples were extracted, saponified, and then tentatively identified and quantitatively determined using high pressure liquid chromatography (Stewart and Wheaton, 1971). The identity of the pigments was confirmed by studies of crystalline carotenoids obtained from the peel of several hundred kilograms of Robinson incubated with 10 ppm of ethylene for 3 days and then held at room temperature until a red color developed. Cryptoxanthin was identified by cochromatography with crystalline material from Dancy tangerines, egg yolk, yellow papaya, and balsam apple using the tlc method of Hager and Meyer-Bertenrath (1966), zinc carbonate tlc, and liquid chromatography (Stewart and Wheaton, 1971). Visible absorption peaks in hexane were at 477, 449, and 426 (sh). The mass spectrum showed the parent peak at *m/e* 552.4315; calculated for $C_{40}H_{56}O$ 552.4330. β-Citraurin was identified by cochromatography using the methods given above with a crystalline sample from Dancy tangerine. Visible absorption maxima in hexane were at 480, 453, and 428 (sh). There were no well-defined peaks in ethanol. A strong peak in the infrared spectrum was observed at 1665 (conjugated carbonyl). The mass spectrum showed the parent peak at *m/e* 432.3067; calculated for $C_{30}H_{40}O_2$ 432.3027.

RESULTS AND DISCUSSION

Robinsons harvested in October and placed in 10 ppm of ethylene for 3 days degreened in 1 week. During the following 2 weeks, the fruit changed from yellow to orange and then red. Control fruit which received no ethylene degreened