thetic medium than in the control tubes. When the synthetic medium was supplemented with fraction IV-4 the percentage of these forms was approximately as low as in the control tubes with plasma. Differential counts made at the end of 24 hours indicated that in experiments No. 37 and No. 41 (in which 80 to 90 percent of the parasites were in the early ring stage when the experiment was started) 90 to 94 percent of parasites in plasma-free medium developed only to the mature trophic stage, whereas in the plasma-free medium supplemented with fraction IV-4, 61 to 82 percent developed to schizont and segmenter stages similar to those that developed in vitro in the presence of plasma, and in vivo. Such results suggest that a substance or substances in whole plasma and in fraction IV-4 are required for intracellular asexual development of this strain of *P. knowlesi*.

The beneficial effect of plasma on in vitro cultivation of cells and tissues was interpreted as being a nonspecific "physical" property of plasma macromolecules (*13*). Anfinsen *et al.* (*7*), in discussing the results of their experiments in which albumin was substituted for plasma, concluded that "the albumin does not appear to act as a nutritional factor but rather in a physical manner." Although no assignment of a physiological role to a substance or substances in fraction IV-4 of human plasma can be made, the role of this fraction appears to be more than a nonspecific one for the following reasons: (i) Other fractions of human plasma, gamma globulin fraction II, globulin beta fraction III, beta lipoprotein fraction III-0, albumin fraction V, and crystalline albumin (*14*) were also tested individually in the same manner as fraction IV-4, but growth and multiplication of *P. knowlesi* were as poor in these as in medium without plasma. If the effect of a plasma fraction is just a nonspecific one, such as buffer action or osmotic effects, one or more of these plasma fractions should have supported growth of the parasite. (ii) Concentration of fraction IV-4 in the medium is critical in its effect on growth and multiplication of the parasite. Fraction IV-4 was tested at concentrations of 3.5, 7.0, 15.0, and 18.5 mg per milliliter of medium, and the best result was obtained in tubes having a concentration of 15.0 mg/ml. While a satisfactory result was obtained with 7.0 mg/ml, an adverse effect on growth and multiplication of the parasite and an increase in percentage of degenerate and extracellular forms were observed with the lowest and highest concentrations of fraction IV-4. (iii) Glucose utilization during the 24-hour growth period in tubes with fraction IV-4 was approximately equal to that in tubes with plasma and 80 to 140 percent higher than in tubes without plasma or fraction IV-4. (iv) Chang *et al.* (*15*), while attempting to characterize the protein or proteins of human plasma essential for maintenance and growth of human conjunctival cells in culture, stated that "growth-promoting activity is associated chiefly with Fraction IV-4."

Results presented in Table 1 clearly demonstrate that fraction IV-4 can replace whole monkey plasma when added to the synthetic medium for cultivation of parasites. While this is significant progress toward our goal of attaining a truly chemically defined growth medium, it must be emphasized that fraction IV-4 is a complex mixture of known and unknown materials (*16, 17*).

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10. The strain used for these studies was originally isolated from a monkey (*Macaca irus*) from Malaya and was given to us in 1965 by E. H. Sadun of Walter Reed Army Institute of Research, Washington, D.C.
11. Composition of modified Ringer solution used for washing red blood cells: NaCl, 8.21 g; KCl, 0.30 g; $CaCl_2$, 0.20 g; $MgCl_2$, 0.10 g; and $H_2O$, 1000 ml.
12. Human plasma IV-4 is prepared by Cohn's method No. 6 [E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, H. L. Taylor, *J. Amer. Chem. Soc.* **68**, 459 (1946)] and was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.
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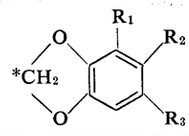
## Methylene-$C^{14}$-Dioxyphenyl Compounds: Metabolism in Relation to Their Synergistic Action

Abstract. *The methylene-$C^{14}$ group is hydroxylated yielding formate-$C^{14}$ in the microsome-reduced nicotinamide-adenine dinucleotide phosphate system in vitro and yielding expired $C^{14}O_2$ in living mice and houseflies. Methylenedioxyphenyl compounds apparently serve as alternate substrates for this enzymatic hydroxylation system of microsomes, and thus reduce the rate of metabolism and prolong the action of certain drugs and insecticide chemicals.*

The duration of action and toxicity of many drugs in mammals and of many insecticide chemicals in insects are greatly increased by joint treatment with certain compounds containing the methylenedioxyphenyl (1,3-benzodioxole) group. Such compounds prolong barbiturate-induced sleep in mammals (*1*), act as co-carcinogens with benzo-[*a*]-pyrene (*2*), and increase the toxicity of certain insecticidal chemicals (*3*). They synergize the insecticidal activity of compounds within almost all classes of insecticide chemicals [pyrethroids, chlorinated hydrocarbons, organophosphorus compounds, carbamates, and others (*4*)]. The published information on structural requirements for synergistic activity is largely restricted to that on the enhancement of the insecticidal activity of pyrethrum and carbamates. Of interest here is the finding that compounds having a planar methylenedioxyphenyl ring system are optimally synergistic to these insecticides, and that only slight reduction in activity results from incorporation of one sulfur atom or a deutero-

Table 1. Structure of methylene-$C^{14}$-dioxyphenyl compounds. The asterisk indicates the labeled carbon atom.




| Compound | Substituents | | |
|---|---|---|---|
| | $R_1$ | $R_2$ | $R_3$ |
| Safrole | H | $CH_2CH{=}CH_2$ | H |
| Dihydrosafrole | H | $CH_2CH_2CH_3$ | H |
| Myristicin | $OCH_3$ | H | $CH_2CH{=}CH_2$ |
| Sulfoxide A | H | $CH_2CH(CH_3)SOC_8H_{17}$-n | H |
| Sulfoxide B† | | | |
| Piperonyl butoxide | H | $CH_2(OCH_2CH_2)_2OC_4H_9$-n | H |

† Sulfoxide B is a diastereoisomer of sulfoxide A about sulfoxide group and asymmetric carbon of propyl group (*12*).

methylene group into the bicyclic ring system, but great reductions in activity result from other modifications. The nature of apolar substituents on the phenyl group governs the activity and specificity of the synergistic action (*5*).

Detoxification processes, involving oxidations and hydroxylations, are inhibited in vivo by methylenedioxyphenyl compounds (*1–4*); the resultant extended persistence of the toxicant in the presence of these agents appears to account for much of their action. Drugs or insecticides synergized by methylenedioxyphenyl compounds are, in large part, those metabolized by the microsome-reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) enzymatic hydroxylation system. In mammalian liver (*6*) and insect preparations (*7*, *8*) in vitro, this enzyme, or enzymes, system is inhibited by methylenedioxyphenyl compounds. The inhibition of insect microsomal systems in vitro is largely competitive (*8*), although some progressive inhibition of enzyme activity possibly takes place (*7*). There are proposals without direct experimental evidence that the synergists are substrates for the microsomal enzymes of oxidation (*7*), that they interfere with some process, such as the utilization of oxygen, which is common to a variety of oxidations (*8*), and that the electrophilic benzodioxolium ion resulting from transfer of hydride from the methylenedioxyphenyl group during oxidative metabolism reacts with a nucleophilic group in the active site of the enzyme (*9*). Methylenedioxyphenyl compounds are biologically unstable; safrole and dihydrosafrole are excreted in the form of piperonylic acid (*10*) and piperonyl butoxide is metabolized in insects to yield unidentified metabolites (*11*).

The mode of action of the methylenedioxyphenyl compounds has not been further clarified, mainly because suitable analytical methods were not available for ascertaining the fate, in vivo and in vitro, of the critical methylenedioxy portion of the molecule. Methylene-$C^{14}$-dioxyphenyl compounds are the basis for a convenient approach to this problem. The six compounds, shown in Table 1, were prepared (*12*), and their metabolism in vivo and in vitro was investigated in both a mammalian and an insect species. [Each of these compounds is active as a synergist for pyrethrum and carbaryl (1-naphthyl methylcarbamate) when assayed with houseflies (*12*).]

The fate of the six methylene-$C^{14}$-dioxyphenyl compounds was compared with that of formaldehyde-$C^{14}$, formate-$C^{14}$, and carbonate-$C^{14}$ in female houseflies (*Musca domestica* L., of the SCR strain) and male albino mice. Each labeled compound, which had an adjusted specific activity of 1 mc/mmole, was injected into flies at 170 $\mu$mole per kilogram of body weight or administered to mice by stomach tube, at 5 $\mu$mole per kilogram of body weight. Radiocarbon in the expired air (both as $C^{14}O_2$ and as the original compound), in the excreta, and in the animal (as residue) was determined 24 hours after its injection into the flies and 48 hours after its administration to the mice. Urine from the treated mice was extracted with toluene to determine the proportions of the radiocarbon soluble in water and in toluene; that soluble in toluene probably represents the original material, or, at least, a metabolite, soluble in organic solvents. Also, each methylene-$C^{14}$-dioxyphenyl compound was incubated at a concentration of $1 \times 10^{-5}M$ with homogenates of housefly abdomens, or with mouse liver homogenates, or with fractions (nuclear, mitochondrial, microsomal, and soluble) prepared from these homogenates by centrifugation (*13*). We used 60 mg of fresh fly tissue or 280 mg of mouse liver (or the equivalent weight of tissue in the case of fractions) per 2 ml of incubation mixture; incubations were for 2 hours in air with shaking at 30°C for the fly and at 38°C for the liver preparations. Most studies involved comparison of the activities of preparations with and without the addition of $NADPH_2$ (6 $\mu$mole for the fly and 1 $\mu$mole for the liver preparations); in the case of mouse liver microsomes, other cofactors were used and compared with $NADPH_2$. In each case, the loss of radiocarbon during the incubation period was determined. After incubation, the reaction mixtures were extracted with ether, and the radiocarbon content of the ether and water phases was determined. When microsomal systems of liver (with and without added $NADPH_2$) were used, the labeled products remaining in the aqueous phase were further fractionated by derivative formation. To one portion unlabeled formaldehyde was added, followed by dimedon; the formaldehyde-$C^{14}$ content was determined by counting the radioactivity in the formaldemethone derivative (*14*). To another portion unlabeled sodium formate was added, the *p*-bromophenacyl formate derivative [mp 99°C (*15*)] was formed, and the formate-$C^{14}$ content was determined. The ether extract was subjected to thin-layer chromatography (*12*), and the radioactive components were detected by autoradiography.

Studies in vivo demonstrate that extensive scission of the methylene-$C^{14}$-dioxyphenyl grouping in mice and flies results in expiration of $C^{14}O_2$ (Table 2). Within 48 hours after oral administration of methylene-$C^{14}$-dioxyphenyl compounds to mice, 61 to 76 percent of the radiocarbon is given off as $C^{14}O_2$. Comparable figures for sodium carbonate-$C^{14}$, formaldehyde-$C^{14}$, and formate-$C^{14}$, respectively, are 91, 65, and 83 percent. The percentage of radiocarbon excreted in the urine within 48 hours, as products soluble in water or toluene, varies with the compound, but is lower for materials with long, apolar side chains (sulfoxide A, sulfoxide B, and piperonyl butoxide). Radioactive carbonate, formaldehyde, and formate yield 4 to 8 percent of the radiocarbon in the urine. The amount of radiocarbon eliminated in the feces or deposited in the body 48 hours after administration does not vary greatly with the compound and is similar to that found

Table 2. Fate of the radiocarbon from methylene-$C^{14}$-dioxyphenyl compounds in mice, houseflies, and enzymatic systems.

| Compound | Radiocarbon recovered (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | In vivo | | | | | | In vitro, as $H_2O$-soluble metabolites | | | |
| | Mouse | | | | | Fly | Mouse liver microsomes | | Fly abdomen homogenates | |
| | $C^{14}O_2$ | Urine | | Feces | Body | $C^{14}O_2$ | Alone | +$NADPH_2$ | Alone | +$NADPH_2$ |
| | | $H_2O$-sol. | Toluene-sol. | | | | | | | |
| Safrole | 61 | 16 | 7 | 3 | 14 | 6 | 1 | 9 | 9 | 18 |
| Dihydrosafrole | 65 | 15 | 3 | 4 | 10 | 12 | 1 | 29 | 9 | 17 |
| Myristicin | 73 | 12 | 3 | 3 | 11 | 13 | 2 | 30 | 15 | 18 |
| Sulfoxide A | 64 | 9 | 2 | 6 | 11 | 19 | 6 | 21 | 11 | 47 |
| Sulfoxide B | 69 | 7 | 0.5 | 5 | 9 | 20 | 5 | 21 | 35 | 42 |
| Piperonyl butoxide | 76 | 6 | 0.5 | 4 | 12 | 11 | 11 | 43 | 8 | 19 |

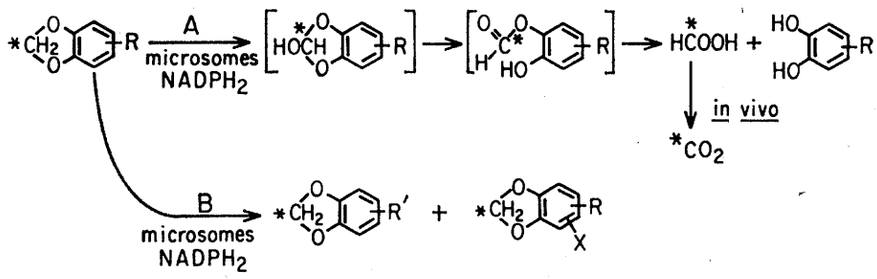



Fig. 1. Proposed pathway for metabolism of methylene-$C^{14}$-dioxyphenyl compounds. Pathway A involves initial enzymatic hydroxylation of the methylene-$C^{14}$ group, and this pathway accounts for (i) the release of formate-$C^{14}$ on scission of the hydroxymethylene-$C^{14}$-dioxyphenyl group, and (ii) the metabolism in vivo of the formate-$C^{14}$ intermediate to yield $C^{14}O_2$. (The two intermediates in this pathway have not been isolated, and are probably very unstable.) Pathway B results in modification of the ring substituent(s) (R′) or in introduction of an additional group (X) into the phenyl ring. In living mice and in the mouse liver microsomal system, pathway B represents a minor degradation route whereas, in houseflies, the significance of pathway B probably is greater because there is less extensive scission of the methylenedioxyphenyl group.

after administration of formate-$C^{14}$.

Under the conditions used, metabolism of the methylenedioxyphenyl compounds is slower and apparently involves less complete scission of the methylenedioxy grouping in houseflies than it does in mice; in fact, only 6 to 20 percent of the dose injected into flies is recovered as $C^{14}O_2$ within 24 hours. Carbon dioxide labeled with $C^{14}$ accounts for about 60 percent of the radiocarbon recovered from flies 24 hours after injection of labeled formate or formaldehyde; it accounts for 98 percent of the radiocarbon recovered from labeled carbonate. A large proportion (35 to 69 percent) of the radiocarbon injected as sulfoxide A, sulfoxide B, or piperonyl butoxide is eliminated in the feces, but only a small fraction (6 to 8 percent) is so found after administration of radioactive safrole, dihydrosafrole, or myristicin. Volatilization from flies accounts for 51 to 59 percent of the radiocarbon from safrole and dihydrosafrole respectively, and for 21 percent of that from myristicin, but none of the radiocarbon from the other synergists is lost in this way. The entire amount of material volatilized from safrole and dihydrosafrole appears to be chromatographically identical to that injected, and more than two-thirds of the volatile material, in the case of myristicin, is identical to that injected. Residual radiocarbon in the flies after 24 hours is in the same range (12 to 16 percent) as that in the mice 48 hours after the administration of safrole, dihydrosafrole, and piperonyl butoxide; on the other hand, myristicin and the sulfoxide diastereoisomers yield much more residual radiolabeled material in the fly (24 to 50 percent) than in the mouse.

Studies in vitro show that the system from the mouse liver most active in metabolizing each of the six labeled compounds is the microsome-$NADPH_2$ system and that its major metabolite is formate. In all cases, the enzyme activity is largely localized in the microsomal fraction, but, with some compounds, the activity of this fraction is enhanced by addition of the soluble fraction of the homogenate. The activity of the microsomal fraction in metabolizing each of the aromatic substrates is almost nonexistent when nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP) is added to the system, but is low when $NADH_2$ is added and high when $NADPH_2$ is added. These relationships involving the specificity of the liver fraction and the cofactor are the same as those with 4-nitro-1,2-methylenedioxybenzene when the liberation of 4-nitrocatchol is followed colorimetrically. We know that 4-nitrophenol is liberated from 4-nitromethoxybenzene (4-nitroanisole) by microsomal enzymes, formaldehyde being released probably through an unstable 4-nitrohydroxymethoxybenzene intermediate (*16*).

The activity of the microsomal system from liver, with and without added $NADPH_2$, is shown in Table 2. In these studies (as well as in similar studies with homogenates of housefly abdomens), the metabolism of myristicin and, particularly, of safrole and dihydrosafrole is more extensive than that directly indicated in Table 2 because considerable (up to 90 percent) volatilization of the substrates from the reaction occurs during the incubation period and, therefore, the amount volatilized is not available for conversion to water-soluble metabolites. During the incubation period, the radiocarbon from carbonate-$C^{14}$ is almost entirely lost, but there is not any loss of the radiocarbon from formaldehyde-$C^{14}$, formate-$C^{14}$, or from any of the methylene-$C^{14}$-dioxyphenyl compounds (except for that due to volatilization in an unmetabolized form as indicated above); therefore, it is evident that metabolism of these various compounds does not proceed in vitro to form carbonate-$C^{14}$. Formate-$C^{14}$ is quantitatively recovered intact following incubation, even when $NADPH_2$ is added. Formaldehyde-$C^{14}$ is converted during incubation and derivative formation to formate-$C^{14}$ to the extent of 17 to 19 percent whether or not $NADPH_2$ is added; the formaldehyde-$C^{14}$ recovery following incubation is 59 percent without and 41 percent with added $NADPH_2$. Formate-$C^{14}$ accounts for 76 to 100 percent of the water-soluble metabolites of the methylene-$C^{14}$-dioxyphenyl compounds; almost no formaldehyde-$C^{14}$ is found. Therefore, formate-$C^{14}$ probably is not formed by reactions having formaldehyde-$C^{14}$ as an intermediate. In each case, the radiolabeled materials in the ether ex-

tract are a mixture of the original compound and two or more labeled metabolites. The number of metabolites formed from piperonyl butoxide is at least eight.

In homogenates of housefly abdomens, the methylene-$C^{14}$-dioxyphenyl compounds are converted to water-soluble products always to a greater extent when $NADPH_2$ is added to the incubation mixtures than when it is not added (Table 2). In the cases of sulfoxide B and piperonyl butoxide, the microsomal fraction from the fly abdomens is active, when $NADPH_2$ is added, converting these compounds to water-soluble products.

The metabolism of the methylene-$C^{14}$-dioxyphenyl compounds proceeds as outlined in Fig. 1. The methylenedioxyphenyl compounds are substrates for the microsomal enzyme-$NADPH_2$ system which also metabolizes many drugs and insecticides. By serving as alternative substrates (and, therefore, as competitive inhibitors) for this system (*17*), the methylenedioxyphenyl compounds prolong the persistence of the drug or insecticide so that a lower initial dose is effective. It is not known whether any of the intermediates combine chemically with components of the microsomes, such as the active sites of the enzymes. This mechanism of hydroxylation accounts, in part, for the action of methylenedioxyphenyl compounds as insecticide synergists and as inhibitors of drug detoxification; it does not directly account for the retardation of growth (*18*), carcinogenic activity (*19*), or the production of fatty livers (*20*) by some methylenedioxyphenyl compounds.

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## Occurrence of Isoprenoid Fatty Acids in the Green River Shale

Abstract. *The most abundant free fatty acids present in a sample of shale from the Green River Formation (Eocene, about 60 × $10^6$ years) from Sulfur Creek are the acylic $C_{19}$ and $C_{20}$ isoprenoid acids. The dominance of these acids parallels the abundance of the corresponding hydrocarbons pristane and phytane.*

Four isoprenoid acids, including norphytanic (2,6,10,14-tetramethylpentadecanoic acid, $C_{19}H_{38}O_2$ and phytanic (3,7,11,15-tetramethylhexadecanoic acid, $C_{20}H_{40}O_2$), have recently been isolated from a California petroleum (*1*). The naphthenic acid fractions of crude petroleums are highly complex mixtures (*2*) and like all petroleum constituents are difficult to relate to the source rocks and hence to their biological origins. Further progress in organic geochemistry ideally necessitates study of geological situations where the extractable organic compounds are indigenous to the facies. Under such circumstances a chemical correlation with biological matter originally incorporated in the sediment is feasible. The Green River Formation (*3*) is of this type. This oil shale has been extensively studied both geologically (*4*) and chemically (*5*) and is the accumulated organic-rich sediment derived from large inland lakes of Eocene age.

A sample (200 g) of Green River Shale from the 334-m depth of a core taken from Sulfur Creek (*6*) was pulverized (to pass 200 mesh), digested with 40-percent hydrofluoric acid/hydrochloric acid (1 : 1) at room temperature for 24 hours. The residue was filtered off, washed with water, and dried. The repulverized residue (90 g) was extracted ultrasonically three times with benzene/methanol (1 : 1). The total free acids were isolated from the extract (5 g) according to the method of McCarthy and Duthie (*7*) and converted to their methyl esters (methanol/hydrochloric acid). Column and preparative thin layer chromatography afforded a pure methyl ester fraction (12 mg). Figure 1 represents a gas chromatogram of these esters on SE-30; the peaks labeled phytanic and norphytanic acid esters were enhanced when this mixture was coinjected with the authentic isoprenoid acid esters (*8*). All the labeled peaks have been identified by combined gas chromatography–mass spectrometry.

Fractions trapped from 3-percent Versamid on Gas Chrom Q were reinjected into the gas chromatography-mass spectrometry apparatus, equipped with a 6-percent SE-30 column (2 m by 4 mm; temperature, 190°C; scan time 2 seconds). Peaks which coincided on the Versamid column, that is, phytanic and $n$-$C_{17}$ and norphytanic and $n$-$C_{16}$ acid methyl esters, were well separated on the SE-30 column. The $n$-$C_{14}$→$n$-$C_{18}$, phytanic (Fig. 2) and norphytanic (Fig. 2) acid esters were identified by comparing the mass spectra so obtained with those of authentic compounds (*8*). In addition, the infrared spectra (film and in $CCl_4$ solution) of the isolated and the authentic