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SHORT COMMUNICATIONS

Metabolism *in vitro* of 5,5-diphenylhydantoin*

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THE METABOLISM of 5,5-diphenylhydantoin (DPH) in rats, rhesus monkeys and man leads to the formation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH)[1] and 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DiOH).[2] Recently Kutt and Verebley[3] reported on a system *in vitro* that is capable of transforming DPH to HPPH. Their system utilized a 9000 *g* supernatant fraction of liver homogenates fortified with NAD, NADP and ATP. Although they reported that HPPH was produced in the assay system, their kinetic calculations were based on the disappearance of substrate and not on the appearance of product. This communication presents a method for assaying the formation of both HPPH and DiOH *in vitro* and gives some preliminary data obtained using the method.

Several female Sprague–Dawley rats (140–160 g) were sacrificed by decapitation and their livers rapidly removed. The livers were weighed, minced and homogenized in cold 0·1 M phosphate buffer (pH 7·4) using a glass mortar fitted with a Teflon pestle. The resulting homogenates (25%, w/v) were pooled and aliquots of the pooled homogenates were centrifuged at 9000 *g* (~ 3°) for 30 min in a Sorval RC-2B centrifuge. A milky white layer forms on top of the 9000 *g* supernatant (9000 *g* S), which reportedly[3] contains an inhibitor of the microsomal mixed-oxidase system. The milky layer was carefully removed with minimum mixing and stored frozen. Most of the remaining 9000 *g* S was distributed into several containers and frozen. This material supplied enzyme for much of the work to be presented. The rest of the 9000 *g* S was centrifuged again, but at 105,000 *g*, in a Beckman L3-40 ultracentrifuge for 60 min (~ 3°). The resulting microsomal pellets were washed once with 10 ml of phosphate buffer and then resuspended in a volume of the same buffer equal to the volume of material used to prepare the microsomes. The resuspended microsomes and the 105,000 *g* supernatant were frozen until needed. The protein concentration of each fraction was determined by the method of Lowry *et al.*[4] using crystalline bovine serum albumin as standard.

An NADPH-generating system similar to the one described by Guarino *et al.*[5] was used in our assays. In order to study the dependency of product formation on the concentration of cofactors, we varied the amounts of NADP and NAD added to the system. The 4-^{14}C-DPH substrate was purchased from New England Nuclear (sp. act. 4·65 mc/m-mole). The concentration of substrate in the final reaction mixture varied from 1×10^{-5} to 5×10^{-4} M. The substrate was dissolved in methanol prior to use and the appropriate amount of methanol substrate was placed in each incubation flask and dried. Two ml of the NADP-generating system was added to each flask and the flasks were brought to 37° in a Dubnoff metabolic incubator. One ml of the liver fraction, pre-warmed to 37°, was added to the flasks and, after a very brief mixing, 1·0 ml of the mixture was removed and placed in 3 ml of cold ethyl acetate. This aliquot was used as the zero time sample. The cold ethyl acetate stopped the enzyme reaction and initiated the process of extracting DPH and its metabolites. The ethyl acetate incubation mixture was shaken for 15 min, briefly centrifuged, and the ethylacetate layer transferred to another culture tube and dried. The extraction process was repeated five times and the ethyl acetate layers of each tube were pooled. The extraction series removes better than 99 per cent of the radioactive counts from the aqueous phase. The remaining enzyme–substrate mixture was incubated with shaking, in air, for 15 min at which time another 1·0-ml aliquot was removed and carried through the extraction process as described.

After the extracts were dried, 50 μl methanol was carefully added to each tube and 10 μl of the methanol residue mixture was spotted on F-254 Silica gel thin-layer chromatography plates (Brinkmann Company) along with radioactive standards. Separation of DPH and its metabolites was afforded by a benzene–methanol–acetic acid (45 : 8 : 4) solvent system. After the run (~ 1·5 hr), the plates were dried and placed in contact with Eastman Kodak BB-54 Medical X-Ray Film and stored in cassettes for 24–196 hr. The developed films were used to locate the radioactive materials and to search for other metabolites. The radioactive spots on the plates were scraped into scintillation vials containing Bray's solution[6] and counted in a liquid scintillation counter.

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As can be seen in Fig. 1, the solvent system used gives excellent separation of DPH, HPPH and DiOH. The R_f values for the respective materials in our system were 0·45, 0·32, 0·14. The plates also clearly show that DPH is much more actively metabolized by the 9000 *g* S liver fraction than by either the initial homogenate or the microsomal fraction. Setting the sum of the radioactive counts of HPPH and DiOH produced by the 9000 *g* S fraction as 100 per cent, the microsomal fraction was around 45 per cent and the initial homogenate 10 per cent as active as the 9000 *g* S fraction. These findings agree quite well with the results obtained by Kutt and Verebly[3] using similar fractions. They proposed that the lipid layer formed on top of the 9000 *g* S fraction contained an inhibitor of the microsomal mixed-oxidase enzymes. We found that addition of small amounts of this material to the 9000 *g* S fraction did reduce the enzymic activity of the system. Addition of stored 105,000 *g* supernatant fraction to resuspended microsomal pellets did not enhance the ability of the system to metabolize DPH, nor did it change the ratio of HPPH to DiOH formed.

One question that came immediately to mind was the possibility that, since two principal metabolites of DPH are formed, there may be two metabolic pathways for the biotransformation of DPH. We attempted to look at this possibility indirectly by studying the effects that changes in substrate and cofactor concentrations might have on the relative amount of each metabolite formed (Fig. 2). Under optimal conditions the rate of HPPH to DiOH formed in the system is approximately 5. We found that varying the substrate or the cofactor concentration, or both, did not significantly alter this ratio.

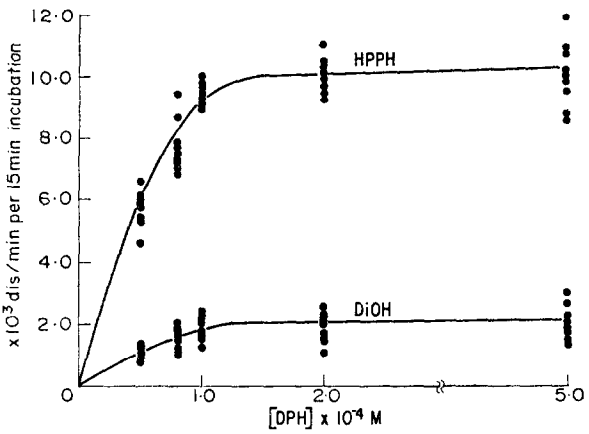

FIG. 2. Relationship between product formation and ^{14}C-DPH concentration in 9000 *g* supernatant fractions prepared from rat liver homogenates. ^{14}C-DPH concentrations = 5×10^{-5}, 8×10^{-5}, 1×10^{-4}, 2×10^{-4} and 5×10^{-4} M. Protein concentration in each incubation mixture = 10 mg/ml. Each dot represents duplicate runs. See text for abbreviations.

We also looked at this problem in terms of the effect that an inhibitor of DPH metabolism might have on the HPPH/DiOH ratio. Kutt and Verebley[3] reported that phenobarbital competitively inhibited the metabolism of DPH in their system *in vitro*. The results obtained in our study agree closely with theirs. Phenobarbital at 1×10^{-2}, 5×10^{-3} and 1×10^{-3} M was added to 9000 *g* S fractions containing 2×10^{-4} M DPH. At these phenobarbital concentrations, both HPPH and DiOH synthesis were equally inhibited and the HPPH to DiOH ratios were unchanged.

The above data indicate that there is probably only one major metabolic pathway for the biotransformation of DPH. It is quite possible that the two metabolites have a common enzyme pathway, but at some point an intermediate is produced that can disproportionate into HPPH and DiOH. A choice for such an intermediate might be an aromatic epoxide. However, another problem is generated by this theory. Since during pregnancy we find that the proportion of HPPH to DiOH in the plasma and urine of rhesus monkey is markedly decreased,* the question naturally arises as to the controls of the hypothetical disproportionation step.

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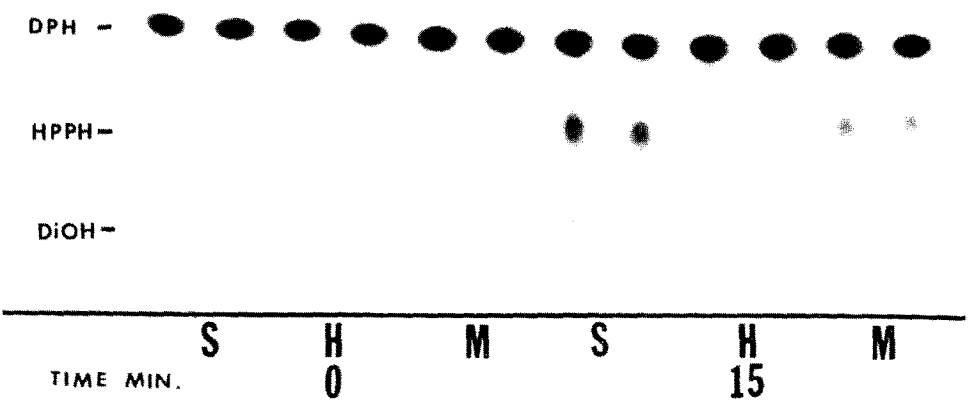

FIG. 1. Metabolism of ^{14}C-DPH by fractions prepared from rat liver homogenates. S = 9000 *g* supernatant fraction, H = total liver homogenate and M = microsomal fraction. ^{14}C-DPH concentration = 2 10^{-4} M. Complete NADPH-regenerating system added to each incubation flask. Plate exposed to X-ray film for 40 hr. See text for abbreviations.

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S-methylation of dithiocarbamates derived from amino acids

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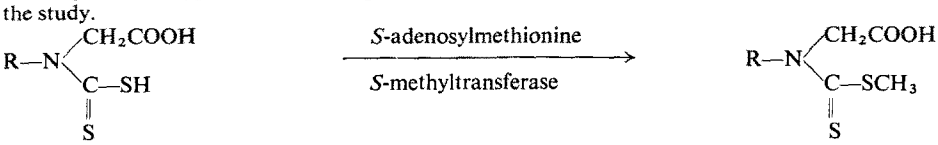
CARBON DISULFIDE is a major occupational health hazard in the viscose rayon industry.[1] Accidental poisoning with carbon disulfide can also occur during its use in pesticide formulations.[2] Toxic effects of carbon disulfide are generally ascribed to the formation of dithiocarbamic acid derivatives which then can complex metal-containing enzymes,[3] but neither the biochemical basis underlying the most prominent pathological changes of chronic exposure nor the metabolism of carbon disulfide and its derivatives is fully understood.[1] Carbon disulfide is known to react with many biochemically important substances[1] including amino acids and proteins,[4] giving dithiocarbamate derivatives. The further fate of these dithiocarbamates has not been fully elucidated. In view of our finding of the diethyldithiocarbamic acid methyl ester as a metabolite of diethyldithiocarbamate,[5] we were interested to find out whether compounds of type I (see below) could be also *S*-methylated in biological systems to compounds of type II. We chose glycine (R—H) and sarcosine (R—CH_3) dithiocarbamates for the study.



(I) (II)

S-adenosyl-L-methionine (methyl-^{14}C), referred to as SAM-^{14}C, was purchased from New England Nuclear, Boston, Mass., and the unlabeled SAM was from Sigma Chemical Co., St. Louis, Mo. The authentic *S*-methyl esters of dithiocarbamate of sarcosine and glycine, which were used as reference compounds for the study, were synthesized as described below.

Synthesis of dithiocarbamate of sarcosine (Sarc. DC) and S-methyl dithiocarbamate of sarcosine (Sarc. DCMe). The barium salt of dithiocarbamate of sarcosine, $[CH_3N(CH_2COOH)C(:S)S^-]_2 Ba^{2+}$, was prepared according to the method of Musil and Irgolic.[6] Sodium salt was generated as needed, as described below under glycine derivative.

The methyl ester of the sarcosine derivative, $CH_3N(CH_2COOH)C(:S)SCH_3$, was prepared according to the method of Rothwell and Wain.[7] The product was recrystallized from a benzene–petroleum ether mixture to give 55 per cent yield of the compound, m.p. 141–144° (lit. 144–146°); ultraviolet spectra show maxima at 248 and 278 nm with ϵ_{248} 7800 and ϵ_{278} 10,000 (in ethanol).

Synthesis of dithiocarbamate of glycine (Gly. DC) and of S-methyl dithiocarbamate of glycine (Gly. DCMe). The barium salt of dithiocarbamate of glycine, $[HN(CH_2COOH)C(:S)S^-]_2Ba^{2+}$, was prepared according to the method of Musil and Irgolic.[6] The sodium salt was then generated as described