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## Digitoxigenin metabolism by rat liver microsomes and its induction by phenobarbital*

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DIGOXIN AND digitoxin metabolism includes hydrolysis and epimerization,[1,2] conjugation[3] and hydroxylation.[4–7] Although the steps in this metabolic sequence have been demonstrated in a qualitative sense, the significance of their quantitative aspects is still under investigation. Considering the continuing large proportion of digitalis patients' toxic manifestations,[8,9] this study on some quantitative aspects of digitoxigenin metabolism using rat liver microsomes was undertaken. Digitoxigenin was used as substrate for initial study to help minimize the potential number of metabolic products. The effect of pretreatment with phenobarbital and phenylbutazone was also tested, since many therapeutic and toxic agents have now been implicated as influencing digitalis metabolism.[10–13]

Randomly labeled digitoxigenin was tritiated by the New England Nuclear Corp. and purified in this laboratory by preparative thin-layer chromatography on Quantagram PLQ1 Silica gel plates (Quantum Industries, Fairfield, N.J.) using methylene dichloride–methanol–formamide (90:9:1) and repeated crystallizations from ethanol–water. After preparative chromatography and three crystallizations, the melting point was identical to non-radioactive commercial digitoxigenin. A final small amount of radioactive contaminant was removed by a second preparative thin-layer chromatography step and crystallizations four and five indicated radiochemical purity of 19·6 mCi/m-mole.

Adult male Sprague–Dawley rats from Simonsen Laboratories weighing 120–210 g were starved overnight prior to incubation studies and drug-treated animals were tested 24 hr after their last treatment. Phenobarbital-treated animals received 75 mg/kg in water (5 ml/kg) administered intraperitoneally daily for 5 days. Phenylbutazone-treated animals received 100 mg/kg in corn oil (5 ml/kg) intraperitoneally on the same schedule. Control animals received either water or corn oil.

Washed microsomes were prepared as previously described[14] and diluted with isotonic alkaline KCl. Microsomal protein content was determined by the method of Lowry *et al.*[15] with bovine serum albumin (Sigma) serving as the standard. The total incubation volume was 5 ml and usually consisted of 2 mg of microsomal protein/ml with 0·5 M Tris buffer (pH 7·4 at 37°), $2{\cdot}5 \times 10^{-3}$ M glucose 6 phosphate as the sodium salt, $2 \times 10^{-3}$ M NADP as the sodium salt, tritiated digitoxigenin at $0{\cdot}25 \times 10^{-3}$ M to $2 \times 10^{-3}$ M. Glucose 6-phosphate dehydrogenase was added at 4 units/incubate. Unless noted otherwise, incubations under oxygen were performed for 15 min at 37° in a Dubnoff metabolic shaking incubator with 100 oscillations/min.

The extraction scheme was similar to that used earlier,[16] with the chloroform extract being evaporated to dryness and dissolved in 1 ml of equal volumes of methanol and chloroform. A 100-$\mu$l aliquot was counted by liquid scintillation spectrometry[16,17] and another 100-$\mu$l aliquot was subjected to chromatography for two developments on Silica gel using methylene dichloride–methanol–formamide. The plates were subsequently scanned with a Packard radiochromatogram scanner and the area under each peak was determined with a DuPont 310 special purpose analog computer. Three peaks were consistently observed in addition to the main digitoxigenin peak. The per cent of total area for each component curve plus the aliquot counted directly were used to compute the amounts in each peak as digitoxigenin equivalents. Recoveries in the chloroform extracts always exceeded 90 per cent of the radiolabeled digitoxigenin substrate used. Although absolute quantitation of metabolites was not assured, kinetic constants were estimated by the procedure of Wilkinson[18] as programmed by Cleland[19] to conform to the equation $v = VA/(K + A)$. Drug comparisons were evaluated by a completely random design analysis of variance and significant differences at $P < 0{\cdot}05$ level were determined by Duncan's new multiple range test.[20]

Early experiments indicated that digitoxigenin metabolism was contingent upon the concurrent presence of glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase. The velocity of total product formation (i.e. total of the three non-digitoxigenin peaks noted on the thin-layer chromatograms) for the complete system was linear over a 30-min period. Total product formation also increased proportionally with microsomal protein up to 4 mg of protein/ml of incubate.

Using 2 mg of microsomal protein/ml and a 15-min incubation period, the pooled apparent $K_m$ for total product formation in four experiments was $0{\cdot}16 \pm 0{\cdot}09 \times 10^{-3}$ M (mean ± S. D.). The apparent $V_{max}$ for the same experiments was 0·12 ± 0·01 nmoles/mg of microsomal protein/min.

The data from digitoxigenin metabolism using microsomal preparations from rats pretreated for

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TABLE 1. EFFECT OF DRUG PRETREATMENT ON DIGITOXIGENIN METABOLISM BY RAT LIVER MICROSOMES

| Treatment* | (1 mM) Digitoxigenin† | (0·5 mM) Digitoxigenin† |
|---|---|---|
| Corn oil control | 50 ± 17 | 52 ± 9 |
| Phenylbutazone (100 mg/kg) | 59 ± 12 | 46 ± 5 |
| Water control | 58 ± 22 | 45 ± 9 |
| Phenobarbital (75 mg/kg) | 106 ± 12‡ | 90 ± 5‡ |

* Daily intraperitoneal administration for 5 days with four rats used per treatment.

† Data for both substrate concentrations expressed as millimicromoles of products formed per milligram of microsomal protein per 15-min incubation.

‡ Significantly different from other three treatments at $P < 0{\cdot}05$.

5 days with phenobarbital, phenylbutazone or appropriate vehicle controls are shown in Table 1. Phenobarbital significantly increased the enzymatic specific activity of digitoxigenin metabolism, whereas the phenylbutazone pretreatment and the two vehicle control groups did not differ from one another.

It should be noted that the experiments reported herein and those recently reported by Stohs *et al.*[21] complement one another in various ways. They identified 3-dehydrodigitoxigenin and 3-epidigitoxigenin as products when the mixed function oxidase system was studied. Although the studies reported herein were used for the first estimation of Michaelis–Menten parameters for a digitalis compound using mammalian tissue, the small amount of product formation precluded identification like that performed by Stohs *et al.*[21] However, the radiochromatographic peaks indicated $R_f$ values akin to those reported by Stohs *et al.*[21] for 3-dehydrodigitoxigenin and 3-epidigitoxigenin in almost identical solvent systems. The third peak observed in the present studies and the unidentified polar metabolites of Stohs *et al.*[21] may represent hydroxylated product(s).

Since toxic doses of digoxin have been reported to cause increases in hepatic smooth endoplasmic reticulum and microsomal cytochrome $P_{450}$[22] and agents such as carbon tetrachloride,[10] phenobarbital,[11] phenylbutazone and diphenylhydantoin,[13] and spironolactone[12,23,24] have been implicated as influencing digitalis metabolism or toxicity, the role of drug metabolic interactions with digitalis has assumed increasing interest. The data of Table 1 indicate that presumed mechanisms may or may not hold true for certain of these interactions and that more direct evidence should be sought before relying too heavily on such presumptions.

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## Role of L-ascorbic acid on detoxification of histamine

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IT HAS been reported by Longenecker *et al.*[1] and Conney and Burns[2] that administration of a number of drugs, quite unrelated in structure and pharmacological action, stimulated synthesis of L-ascorbic acid in rats. Results obtained in this laboratory indicate that the enhanced synthesis of L-ascorbic acid might be related to an induced formation of histamine in the system.[3] Experiments using phenobarbitone, chlorpromazine and meprobamate revealed that the stimulation of L-ascorbic acid synthesis was accompanied by a concomitant increase (about 4–6-times) in urinary excretion of histamine. This enhanced urinary excretion of histamine is probably due to an elevated histamine level in the system due to induced histamine formation[4] or to inhibition of *N*-methylation of histamine by the above mentioned drugs[5] or both. We considered that the production of excess L-ascorbic acid in

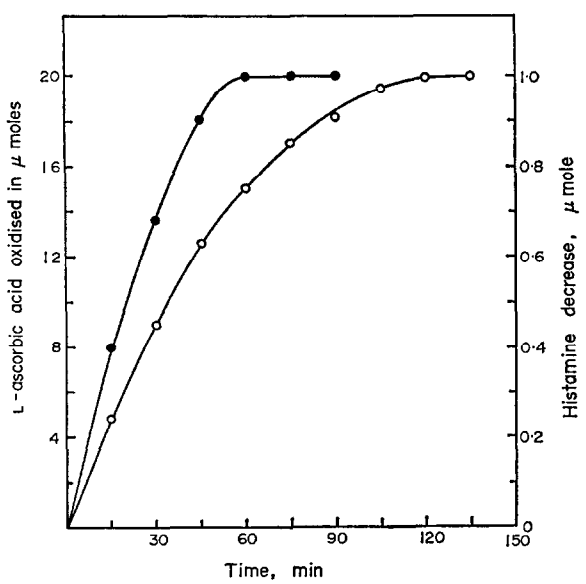


FIG. 1. Oxidation of L-ascorbic acid and biotransformation of histamine. The concentrations of L-ascorbic acid, $Cu^{2+}$ and histamine and other conditions were same as Table 1. ●—●, oxidation of L-ascorbic acid; ○—○, decrease of histamine.