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IMPAIRED DRUG METABOLISM IN EXPERIMENTAL CIRRHOSIS IN THE RAT*

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Abstract—Cirrhosis was produced in the rat by chronic administration of phenobarbital and carbon tetrachloride for 10 weeks. The metabolism of three substrates, aminopyrine, hexobarbital and propranolol, has been investigated in a 9000 *g* supernatant fraction of liver homogenate and in intact hepatocytes in cirrhotic livers and compared to appropriate phenobarbital-treated controls. In the 9000 *g* supernatant preparation, the maximal velocity of metabolism for each substrate and the cytochrome P450 concentration were reduced significantly in cirrhotic livers, while total protein and DNA concentration remained unchanged. In intact hepatocytes, the V_{max} for each substrate was reduced, while the apparent K_m remained unchanged. In both *in vitro* systems, the metabolism of propranolol and of hexobarbital was influenced by cirrhosis to a greater extent than that of aminopyrine. It is concluded that the carbon tetrachloride-phenobarbital model of cirrhosis in the rat is a suitable model in which to study the effects of chronic liver disease on drug disposition.

Although it has been established that the elimination of several drugs is impaired in patients with chronic cirrhosis [1], the relationship between the pathophysiology of the disease and the mechanisms of altered hepatic drug clearance remains to be defined [2]. A better understanding of the effects of cirrhosis on drug metabolism might be facilitated by the development of an appropriate animal model. McLean *et al.* [3] first showed that phenobarbital-treated rats chronically exposed to carbon tetrachloride developed histological changes in the liver similar to those of human cirrhosis. Subsequently, Marshall and McLean [4] demonstrated that the livers from such animals had a lower cytochrome P450 content and an impaired ability to demethylate aminopyrine.

Therefore, we have developed this animal model of cirrhosis to investigate the influence of liver disease on the ability of liver homogenates and isolated hepatocytes to metabolize three model substrates chosen to have low, intermediate and high clearances *in vivo*, that is, aminopyrine, hexobarbital and propranolol respectively.

METHODS

Induction of cirrhosis. Male Sprague–Dawley rats, weighing 100–125 g, were fed Purina Chow and water *ad lib.* Cirrhosis was produced by simultaneous administration of carbon tetrachloride (CCl_4) and phenobarbital [3]. Phenobarbital was added to the drinking water at a concentration of 0.5 g/l; a fresh solution was prepared weekly. The concentration of phenobarbital in the drinking water was determined using the EMIT enzyme immunoassay (Syva, Palo Alto, CA), and it was verified that phenobarbital was stable in water for at least 1 week. CCl_4 was administered twice weekly by inhalation. Groups of six to eight rats were placed in a wooden box with a glass front (35 × 36 × 38 cm, 48 l capacity). Compressed air was bubbled through a sequence of two bottles containing CCl_4 and then into the box at a flow rate of 1.5 l/min. The box was placed in a fume hood, and air left the box by leaking through the joints. CCl_4 was blown into the box for 2 min on the first gassing, 3 min on the second and third gassing, and 5 min thereafter. The rats were then left in the cage for the same length of time as the gassing period. Control rats received only phenobarbital throughout the treatment period.

Cirrhosis was defined to be present when fibrous tissue bands completely encircled zones of hepatic parenchymal elements. The mildest form of cirrhosis recognized resembled closely the classic finely nodular, portal cirrhosis seen in humans. As the amount of fibrous tissue increased, the concomitant loss of hepatocytes and formation of regenerative nodules produced a more coarsely nodular pattern.

Histological examination of the liver was performed in all animals, and only those which showed evidence of cirrhosis (75 per cent of treated animals) were included in the cirrhotic group.

Aminopyrine half-life. [^{14}C]aminopyrine (29 μg/1.5 μCi, Amersham Searle Corp., Arlington Heights, IL) was injected into the peritoneal cavity of unanes-

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thetized rats. Rats were housed in individual air-tight restraining cages; exhaled $^{14}CO_2$ was drawn through concentrated sulfuric acid to remove water and then through a scintillation vial to collect all CO_2 during eight consecutive 15-min periods starting 60 min after drug administration. Ten milliliters of a 2:1 (v/v) methanol–ethanolamine mixture was used as a trapping agent for the CO_2. Trapped radioactivity was determined after adding 10 ml Unogel (Schwarz/Mann, Orangeburg, NY). Exhaled $^{14}CO_2$ decreased exponentially during the collection period, and the half-life was calculated by a least square regression analysis of the logarithm of the amount of $^{14}CO_2$ produced with respect to time.

Drug metabolism by liver homogenates. The metabolism of propranol, hexobarbital and aminopyrine by the 9000 *g* supernatant fraction of liver homogenates was measured as follows. Livers were excised, homogenized in a glass homogenizer with a Teflon pestle in 4 vol. of potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 9000 *g* for 15 min at 4°. The reaction mixture contained: glucose 6-phosphate (5 mM), NADP (1 mM), $MgCl_2$ (5 mM), potassium phosphate buffer, pH 7.4 (100 mM), and semicarbazide (5 mM) in the case of the aminopyrine assay. The substrate concentrations used were chosen after preliminary experiments which demonstrated that these concentrations were greater than those required to produce maximal rates of metabolism (V_{max}). Substrate concentrations were: [^{3}H]propranolol, 3.33 μM (0.5 μCi, New England Nuclear Corp. Boston, MA); [^{3}H]-hexobarbital, 1 mM (0.1 μCi, New England Nuclear Corp., Boston, MA) and aminopyrine, 8 mM (ICN Pharmaceuticals Inc., Cleveland, OH). The reaction mixture was preincubated for 10 min at 37° and the reaction begun by adding the 9000 *g* fraction: 0.2 ml of the supernatant solution was added for the propranolol assay and 1.0 ml for the hexobarbital and aminopyrine assays. The final volume was 3.0 ml, and all determinations were carried out in triplicate. Samples were incubated in a Dubnoff incubator at 37° under air for 10, 15 and 30 min for propranolol, hexobarbital and aminopyrine respectively. For the propranolol assay, the reaction was stopped with 1 ml of 2.5 N NaOH, and the disappearance of the drug measured according to Shand and Oates [5]. For the hexobarbital assay, the reaction was stopped by adding 6 ml of ice-cold 0.1 M phosphate buffer, pH 6.8, and substrate disappearance was measured according to Holcomb *et al.* [6]. The formation of formaldehyde from aminopyrine was estimated by the method of Nash [7], as modified by Cochin and Axelrod [8].

Protein content of the liver homogenate was measured according to the method of Lowry *et al.* [9] and DNA according to Burton [10]. Cytochrome P450 content of the 9000 *g* supernatant fraction was estimated by the method of Omura and Sato [11].

Drug metabolism by isolated hepatocytes. Rat liver cells were prepared according to a modification of the procedure of Berry and Friend [12]. Rats were anesthetized with ether; the livers were removed and perfused at a rate of 20 ml/min via the portal vein in an Ambec 1000 perfusion apparatus primed with 200 ml of calcium-free Krebs–Henseleit bicarbonate buffer, pH 7.4, containing 100 mg/100 ml of glucose and gassed with 95% O_2 and 5% CO_2. The first 100 ml of perfusate was discarded and 35 mg of collagenase (EC 3.4.4.19, CLS-Type I, Worthington Biochemical Corp., Freehold, NJ) added to the remaining 100 ml of perfusate in the reservoir. Perfusion was continued for 40 min in a closed, recycling system. The liver was removed, minced with scissors and transferred to a plastic beaker containing 15 mg of collagenase in 50 ml of calcium-free buffer. The mixture was incubated at 37° for 10 min in a Dubnoff incubator, gassed as above, and filtered through a nylon mesh. Cells were washed three times in Krebs Henseleit phosphate buffer, pH 7.4, containing 1% bovine serum albumin and 100 mg/100 ml of glucose without collagenase. The suspension was centrifuged for 1 min and resuspended in the same buffer. Viability of the cells was estimated by counting the percentage of cells which excluded 0.2% Trypan Blue. The concentration of cells in the suspension was determined by counting in a Buerker counting chamber. The hepatocrit was measured by centrifugation in a heparinized capillary tube.

The metabolism of propranolol and hexobarbital was determined by measuring substrate disappearance by the same methods as for the 9000 *g* supernatant assays. For propranolol, 1 ml of the cell preparation, diluted to a hepatocrit of 0.33%, was preincubated for 10 min. The reaction was begun by adding 0.1 ml of [^{3}H]propranolol (0.2 μCi) diluted with appropriate amounts of unlabeled propranolol. No cofactors were added. The mixture was incubated for 8 min in air at 37° in a Dubnoff incubator. All determinations were carried out in triplicate.

For hexobarbital, cells were diluted to a hepatocrit of 1%, 1 ml of cell suspension was preincubated for 10 min, and the reaction was begun by adding 0.1 ml of [^{3}H]hexobarbital (0.1 μCi) diluted with unlabeled drug. The mixture was incubated for 15 min under the same conditions as for propranolol. Since a substantial fraction of the substrate was metabolized during the propranolol and hexobarbital assay, an integrated form of the Michaelis Menten equation was used to derive Lineweaver–Burk plots [13].

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{\ln(S_0/S)}{S_0 - S} + \frac{1}{V_{max}} \qquad (1)$$

The metabolism of aminopyrine was assessed by measuring $^{14}CO_2$ production from [^{14}C]aminopyrine. One ml of cell preparation, diluted to form a 2% hepatocrit, was preincubated for 10 min in a 25-ml Erlenmeyer flask and the reaction was started by the addition of substrate. The flask was connected to a scintillation vial containing 10 ml of a 2:1 (v/v) methanol–ethanolamine mixture. Air was drawn through the system by a vacuum pump, so that all $^{14}CO_2$ formed during a 30-min period was trapped in the methanol–ethanolamine mixture; [^{14}C]aminopyrine incubated under the same conditions but without cells was used as a blank.

The V_{max} and apparent K_m of each individual experiment from the cirrhotic and control animals were determined by linear regression analysis of Lineweaver–Burk plots. An estimate of intrinsic clearance (Cl_{int}) was made from the relationship [14]:

$$Cl_{int} = \frac{V_{max}}{K_m} \qquad (2)$$

RESULTS

Aminopyrine half-life. The aminopyrine $T_{\frac{1}{2}}$ was used to follow the time course of impaired drug metabolism after stopping the administration of phenobarbital and CCl_4 (Fig. 1). The initially prolonged $T_{\frac{1}{2}}$ decreased by 45 per cent to achieve a stable plateau by day 7. All subsequent experiments were carried out, therefore, between 7 and 12 days after cessation of phenobarbital and carbon tetrachloride administration.

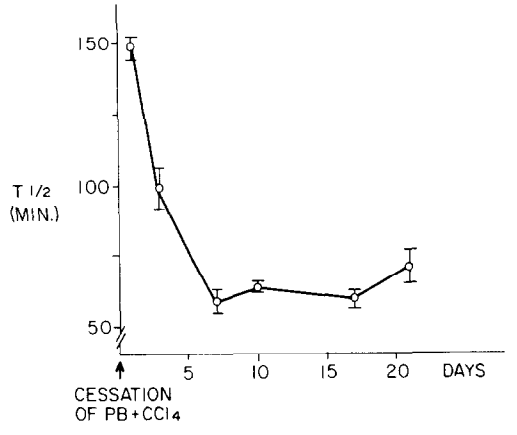

Fig. 1. The half-life of [^{14}C]aminopyrine obtained from aminopyrine breath testing in three cirrhotic rats with respect to time after discontinuing a 10-week course of phenobarbital (PB) and carbon tetrachloride (CCl_4) treatment (mean ± S. E. M.).

Aminopyrine $T_{\frac{1}{2}}$ also was measured in a larger group of cirrhotic animals on day 10 and was significantly prolonged by 38 per cent in comparison to control animals (mean ± S. E. M.: 57 ± 3 vs 44 ± 3 min respectively, $P < 0.01$).

Drug metabolism by the 9000 g *supernatant fraction.* In investigations with the 9000 *g* supernatant fraction, cirrhotic rats had a significantly reduced cytochrome P450 concentration (by approximately one third in comparison to controls), while liver weight, DNA concentration and protein concentration were not significantly different (Table 1). Cirrhosis was associated with a reduced rate of metabolism of each substrate (Table 1).

Drug metabolism by isolated hepatocytes. Light microscopy indicated that the isolated liver cell suspension consisted almost exclusively of hepatocytes. Viability was similar in cells from control (82.1 ± 2.2, mean ± S. E. M.) and cirrhotic livers (77.5 ± 2.3 mean ± S. E. M.). However, the yield of cells was usually lower in the cirrhotic livers than in controls (mean ± S. E. M.: 0.54 ± 0.09 vs 2.82 ± 0.43 g tightly packed cells/liver respectively). The DNA content of hepatocytes from cirrhotic or control animals was similar (mean ± S. E. M.: 914 ± 150 vs 887 ± 120 μg DNA/g tightly packed cells respectively). Subsequent drug metabolism studies were corrected to a constant hepatocrit.

The metabolism of aminopyrine, hexobarbital and propranolol was linear with time and hepatocyte concentration within the assay conditions. Each of the three drugs showed substrate concentration-dependent rates of metabolism consistent with Michaelis–Menten kinetics (Figs. 2–4). In control rats, similar to 9000 *g* supernatant metabolism, the V_{max} of hexobarbital was considerably greater than that of either aminopyrine or propranolol (Table 2). Marked differences in K_m values resulted in intrinsic clearance with a rank order similar to that found *in vivo* (i.e. propranolol > hexobarbital > aminopyrine). Cirrhotic rats showed no change in K_m but a reduction in V_{max} of propranolol and hexobarbital, resulting in a reduction in intrinsic clearance.

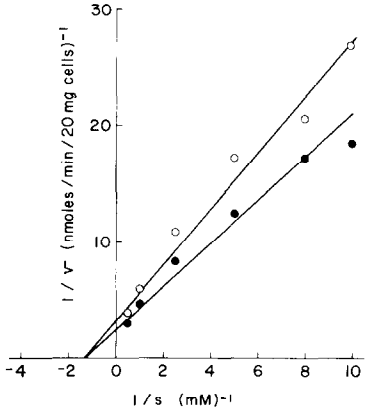

Fig. 2. Lineweaver–Burk plots of the metabolism of [^{14}C]-aminopyrine in isolated hepatocytes from control ($n = 4$, closed circles) and cirrhotic ($n = 4$, open circles) rat livers.

Table 1. Drug metabolism by the 9000 *g* supernatant fraction in control and cirrhotic rats*

	Liver wt (g)	DNA (μg/g liver)	Protein (mg/g liver)	Cytochrome P450 (nmoles/g liver)	Aminopyrine *N*-demethylase (nmoles/min·g liver^{-1})	Propranolol (nmoles/min·g liver^{-1})	Hexobarbital (nmoles/min·g liver^{-1})
Controls ($n = 6$)	18.7 ± 0.45	1212 ± 59	179 ± 2.4	43.5 ± 2.3	39.7 ± 1.9	22.4 ± 0.73	706 ± 27
Cirrhosis ($n = 6$)	19.5 ± 1.52	1188 ± 79	162 ± 8.2	29.5 ± 2.1†	29.2 ± 4.7‡	13.5 ± 2.17†	378 ± 50†
% Difference	+ 4	− 2	− 9	− 32	− 26	− 40	− 47

* Values are expressed as the mean ± S. E. M.
† $P < 0.01$, as compared to control values by the Mann–Whitney U-test.
‡ $P < 0.05$, as compared to control values by the Mann–Whitney U-test.

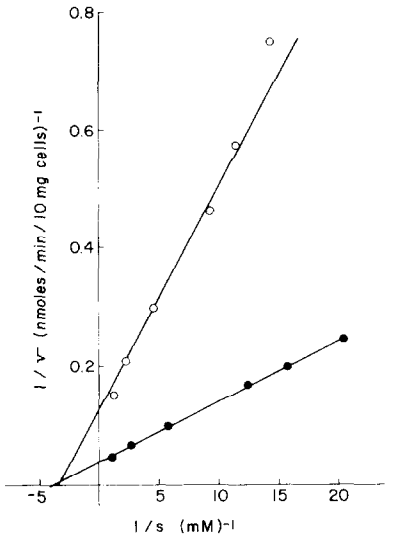

Fig. 3. Lineweaver Burk plot of the metabolism of [^{3}H]-hexobarbital in isolated hepatocytes from control (n = 6, closed circles) and cirrhotic (n = 6, open circles) rat livers.

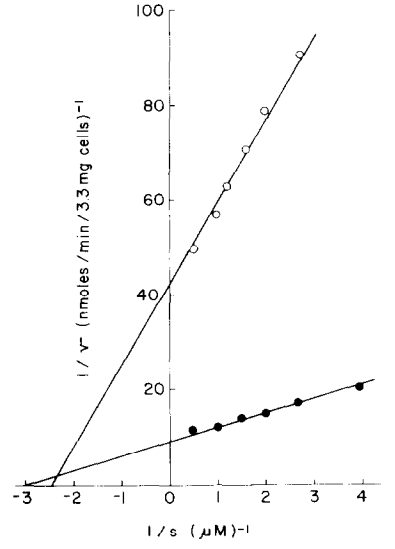

Fig. 4. Lineweaver–Burk plot of the metabolism of [^{3}H]-propranolol in isolated hepatocytes from control (n = 5, closed circles) and cirrhotic (n = 6, open circles) rat livers.

DISCUSSION

A major objective of this study was to evaluate whether this model of chronic liver disease in the rat simulates cirrhosis in man, particularly with reference to histopathology and its influence on drug metabolism. We have confirmed, first, that the structural changes within the liver closely resemble human cirrhosis [3] and, second, that the *in vitro* metabolism of aminopyrine, hexobarbital and propranolol was impaired in both the 9000 g supernatant fraction and isolated hepatocyte preparations. The elimination of each of the three substrates evaluated is also impaired in patients with cirrhosis [15–17]. On this basis, we feel that the present model is a suitable one, and may be used to further our understanding of the mechanisms involved in impaired drug metabolism in cirrhosis.

A potential problem in using the present method for producing cirrhosis is the use of the enzyme-inducing drug, phenobarbital, in order to potentiate the toxicity of carbon tetrachloride. This has required the use of appropriate phenobarbital-treated controls. The [^{14}C]aminopyrine breath tests *in vivo* confirmed that drug elimination was impaired at the end of 10 weeks of administration of phenobarbital and carbon tetrachloride (Fig. 1). On stopping treatment, the initial half-life fell to a stable plateau by day 7 (Fig. 1), suggesting that drug metabolism studies performed after this time would be representative of hepatic function in the chronic disease state rather than a consequence of acute hepatotoxicity from carbon tetrachloride. Histology also confirmed the absence of acute inflammatory changes by day 7 after stopping the administration of CCl_4. Impaired *in vivo* metabolism of aminopyrine in cirrhotic animals, as indicated by a prolonged half-life of $^{14}CO_2$ pulmonary excretion, provided a stimulus to evaluate this model more extensively *in vitro*.

Impaired drug metabolism was confirmed and extended in studies using a 9000 g supernatant fraction from liver homogenates. The metabolism of three drugs was significantly reduced in cirrhotic livers, the change with propranolol and hexobarbital being similar to each other but greater than that with aminopyrine (Table 1). These changes were associated with a reduction in the concentration of cytochrome P450. In contrast, DNA and protein concentrations

Table 2. Comparison of Michaelis–Menten constants and intrinsic clearance of aminopyrine, hexobarbital and propranolol in isolated hepatocytes from control and cirrhotic rats*

	n	K_m (μmoles)	V_{max} (nmoles·min·g cells^{-1})	Intrinsic clearance (ml·min·g cells^{-1})
Aminopyrine				
Control	4	449 ± 26	14.1 ± 1.6	0.031 ± 0.002
Cirrhosis	4	451 ± 92	10.4 ± 2.8	0.023 ± 0.005
Hexobarbital				
Control	6	198 ± 27	2170 ± 320	10.8 ± 0.3
Cirrhosis	6	512 ± 164	1059 ± 265†	2.8 ± 0.5‡
Propranolol				
Control	5	0.27 ± 0.04	31.1 ± 4.3	118 ± 11
Cirrhosis	6	0.58 ± 0.20	8.1 ± 1.7‡	30 ± 12‡

* Values are expressed as the mean ± S.E.M.
† $P < 0.05$, as compared to control values by the Mann Whitney U-test.
‡ $P < 0.01$, as compared to control values by the Mann Whitney U-test.

remained at control levels. A problem of interpretation of 9000 *g* supernatant metabolism is that cells other than hepatocytes (particularly from fibrous tissue) might contribute to protein and DNA and dilute drug-metabolizing enzyme activity. In order to evaluate whether the observed reduction in metabolism was an experimental artifact, intact hepatocytes were isolated from rat livers. With this preparation, optimal conditions for defining Michaelis-Menten kinetics were obtained for each of the three test substrates.

In control rats, the V_{max} values of the three substrates were qualitatively similar to results in 9000 *g* supernatant fractions, with propranolol and aminopyrine having similar values and hexobarbital having a far greater value (Table 2). However, the greater affinity of drug-metabolizing enzymes for propranolol resulted in its having the lowest apparent K_m. The Michaelis constants obtained *in vitro* can be used to estimate and predict hepatic extraction ratios for drugs when they are present in substrate concentrations that are associated with first-order elimination [14]. Applying this principle to the K_m and V_{max} measured in isolated hepatocytes, an approximate assessment of whole organ intrinsic clearance can be obtained as V_{max}/K_m (Table 2). These estimates confirm that aminopyrine has a low intrinsic clearance and propranolol a high intrinsic clearance, with hexobarbital being intermediate.

This rank order for intrinsic clearance is in contrast to the rank order in V_{max} and is illustrative of the relative contributions of K_m and V_{max} to *in vivo* drug elimination. In cirrhotic rats, there is evidence of a reduction of V_{max} without a change in the apparent K_m (Figs. 2–4). Thus, the amount of drug-metabolizing enzyme per g of hepatocytes was reduced, while the affinity of enzyme for each substrate remained unchanged. It is unclear whether a reduced V_{max} and a normal DNA content of the liver are due to each hepatocyte being deficient in enzyme or result from a portion of the cells having defective enzyme activity diluting the activity of the remaining cells with a normal enzyme complement. In both the 9000 *g* supernatant and hepatocyte experiments, the reduction in V_{max} with propranolol was similar to that of hexobarbital with both being greater than that of aminopyrine. This might suggest that cirrhosis produced the greatest reduction in intrinsic clearance of those drugs with the largest initial values, as suggested previously in man [2]. Knowledge of the anatomical location of the enzymes responsible for metabolism of various substrates and their relation to the site of the toxic lesion would contribute to an understanding of the relationship between microscopic anatomy of the disease process and altered drug metabolism. In relating *in vitro* results to expected results *in vivo*, changes in liver blood flow and the presence of intra- or extrahepatic shunts should also be considered as they may alter the pharmacokinetic drug disposition of high clearance drugs to a greater extent than low clearance drugs. Their importance is presently being studied.

In conclusion, the administration of phenobarbital and carbon tetrachloride for 10 weeks to rats produced a model of liver disease which was similar histologically to cirrhosis in man. In this model, hepatic drug metabolism of the three substrates tested was impaired due to a reduction of the V_{max} for each drug. This model should be appropriate to further understanding of the mechanisms of abnormal drug disposition in liver disease.

REFERENCES

1. G. R. Wilkinson and S. Schenker, *Biochem. Pharmac.* **25**, 2675 (1976).
2. R. A. Branch and D. G. Shand, *Clin. Pharmacokinet* **1**, 264 (1976).
3. E. K. McLean, A. E. M. McLean and P. M. Sutton, *Br. J. exp. Path.* **50**, 502 (1969).
4. W. J. Marshall and A. E. M. McLean, *Br. J. exp. Path.* **50**, 578 (1969).
5. D. G. Shand and J. A. Oates, *Biochem. Pharmac.* **20**, 1720 (1971).
6. R. R. Holcomb, N. Gerber and M. T. Bush, *J. Pharmac. exp. Ther.* **188**, 14 (1974).
7. T. Nash, *Biochem. J.* **55**, 416 (1953).
8. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. K. Burton, *Biochem. J.* **62**, 315 (1956).
11. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
12. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
13. I. H. Siegel, *Enzyme Kinetics*, p. 54. John Wiley, New York (1975).
14. A. Rane, G. R. Wilkinson and D. G. Shand, *J. Pharmac. exp. Ther.* **200**, 420 (1977).
15. G. W. Hepner and E. S. Vesell, *Ann. intern. Med.* **83**, 632 (1975).
16. D. D. Briemer, *Clin. Pharmacokinet.* **2**, 93 (1977).
17. R. A. Branch, J. James and A. E. Read, *Br. J. clin. Pharmac.* **3**, 242 (1976).