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N-DEMETHYLATION OF AMINOPYRINE *IN VIVO* AND IN THE ISOLATED HEPATOCYTE OF THE RAT

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Abstract—The *N*-demethylation of [dimethylamine-^{14}C]aminopyrine was monitored *in vivo* and in isolated hepatocytes of the rat by collection of $^{14}CO_2$. The isolated hepatocyte is capable of aminopyrine *N*-demethylation rates similar to those occurring *in vivo*. The kinetics of *N*-demethylation in the isolated cells indicate the presence of two separate reactions with K_m values of 32 and 710 μM. The kinetics of *N*-demethylation *in vivo*, however, indicates the presence of one reaction with an intermediate K_m value of 260 μM. It is thought that the two reactions observed in the hepatocytes also occur *in vivo* but that it is not possible to differentiate between them.

Broken cell preparations such as tissue homogenates and microsomal fractions have been used extensively for the study of drug metabolism in the liver. Although information can be obtained about the types of reactions occurring and the kinetic parameters, extrapolation from this type of data to reaction rates in the liver *in vivo* is often difficult. The problem is that conditions are not the same as in the intact cell and that the reaction rates are very much dependent on the conditions chosen by the investigator. For instance, the rate of *N*-demethylation of aminopyrine in microsomes is affected markedly by co-factor supply, a factor in the soluble fraction of rat liver, EDTA, and the semicarbazide used to trap the formaldehyde formed [1–3]. Since the development of a simple isolation procedure by Berry and Friend [4], the isolated liver cell has been available for *in vitro* studies of drug metabolism. Only recently has use been made of the hepatocyte for studies on drug metabolism [5–7]. The isolated hepatocyte, because it is an intact cell, should provide useful information on the role of the liver in the kinetics of drug metabolism *in vivo*. In this study, a comparison was made between *in vivo* and *in vitro* *N*-demethylation of aminopyrine To simplify the comparison, the same measurement technique was used both *in vivo* and *in vitro*. *N*-demethylation can be monitored *in vivo* by administering *N*-methyl-^{14}C-labeled aminopyrine [8, 9] and collecting the $^{14}CO_2$ from the respired air. As described here, the same procedure was adapted to the isolated hepatocyte preparation.

MATERIALS AND METHODS

Hepatocyte isolation

The method used is a modification of the procedure of Berry and Friend [4]. Male Wistar rats were anesthetized with sodium pentobarbital (60 mg/kg). The abdomen was opened and 2500 U.S.P. units of heparin were injected into the spleen to prevent blood coagulation in the liver during cannulation. The portal vein was cannulated and a phosphate Ringer solution minus calcium was perfused through to clear the liver of blood (flow rate, 30 ml/min and 37°). The composition of the Ringer solution was 150 mM Na^+, 5.9 mM K^+, 1.19 mM Mg^{2+}, 132 mM Cl^-, 15 mM HPO_4^{2-}, 1.19 mM SO_4^{2-} and pH 7.4. The Ringer solution was saturated with oxygen. The liver was excised from the rat while still maintaining perfusion and transferred to a recirculating perfusion apparatus. The apparatus maintained the perfusion solution at 37° and a saturated oxygen tension. The liver was perfused with 60 ml of the solution described above containing 0.05% collagenase (Worthington Biochemical Corp., Freehold, NJ, CLS II, Type II). After about 20 min the liver was swollen with blisters on the surface. The liver was carefully transferred to a 250-ml beaker containing 90 ml of the same medium with which the liver was perfused and to which was added 0.25% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, fraction V). The temperature of the medium was 21–24°. The liver was broken up with scissors and the mixture transferred to two 250-ml Erlenmeyer flasks. The flasks were gassed with 100% O_2 and shaken at 37° for 20 min. The contents of the flasks were filtered through nylon mesh [10] into a 1000-ml beaker. The beaker was placed in a cold bath of about 12° and the cells were allowed to sediment out by gravity for 15 min. The supernatant fluid was carefully decanted and the cells were resuspended in phosphate Ringer containing 1.5 mM Ca^{2+} and 0.25% albumin. The cell suspension was transferred to 250-ml Pyrex spinner flasks (Johns Scientific, Toronto) and stirred continuously at 37°. Oxygen was supplied above the cell suspension. Cell content was estimated by centrifuging a 4-ml aliquot at 1500 *g* for 5 min (swing bucket rotor) and weighing the sedimented cells.

Two modifications of previous methods were employed which increased the viability of the cells in the final preparation. One was to cool the liver to room temperature while dispensing it after the collagenase perfusion step. Another was to cool the

cells to 12° while sedimenting the cells under normal gravity. Cells are subjected to anoxia during both steps. Reducing the temperature extends the period the cells can be exposed to anoxia without damage. The sedimentation of cells by gravity eliminates cell damage that can occur during centrifugation [11]. It was also observed that perfusion of the liver without interruption of blood flow improved the viability of the final cell preparation. The use of hyaluronidase was found to be unnecessary. Since the cells were used shortly after harvesting, maintenance of asepsis was not critical. Nonetheless, all solutions were sterilized by filtering through 0.2 μm Nalgene filter units.

Measurement of $^{14}CO_2$ release by hepatocytes

Three ml of cells (12 mg/ml) was incubated with 0.1 μCi of [dimethylamine-^{14}C]aminopyrine (New England Nuclear, Boston, MA) and varying amounts of unlabeled aminopyrine in sealed 25-ml Erlenmeyer flasks at 37° in a New Brunswick shaking bath (60 cycles/min). Cells were suspended in phosphate Ringer and atmospheric air was used as the gas phase. The released CO_2 was collected in a center well containing 0.2 ml of 1.0 N NaOH plus a filter paper wick (1 cm × 2 cm). The reaction was stopped and the dissolved CO_2 driven off by the addition of 0.8 ml of 0.4 N H_2SO_4 to the outer chamber containing the cells. The flasks were left overnight at room temperature to ensure complete recovery of the CO_2. Efficiency of recovery was estimated by adding $NaH^{14}CO_3$ to a flask containing 3 ml of buffer and carrying through the same procedure as for the cell incubation. Recovery was 98–102 per cent.

Measurement of $^{14}CO_2$ release in vivo

Rats with an average weight of 300 g were injected intraperitoneally with [^{14}C]aminopyrine (1 μCi) and indicated amounts of cold aminopyrine. The rats were placed in tubular Plexiglas chambers [6 cm (inside diameter) × 22 cm]. The tail of the rat was allowed to project through a small hole in one end of the chamber. Air from each chamber was pulled through two gas washing bottles equipped with fritted glass air dispersion tubes (Act Glass, Vineland, NJ) at 160–200 ml/min. The first bottle contained 100 ml of 1 N NaOH. The second contained 50 ml of 1 N NaOH. Over 99 per cent of the $^{14}CO_2$ was collected in the first bottle. Aliquots of 0.7 ml were taken at 15-min intervals for liquid scintillation counting. One ml water plus 15 ml Aquasol (New England Nuclear) were added to the aliquot in the counting vial.

RESULTS

Isolated hepatocytes

The cells isolated by the procedure described here were in good condition by a number of criteria. Only 1–4 per cent of the cells were stained by Trypan blue. Usually less than 1 per cent had blisters on the cell surface. The oxygen consumption of the cells was 2.4 to 2.5 μmoles O_2/g/min, a rate comparable to that obtained in this laboratory with the perfused liver preparation. Addition of 5 mM succinate stimulated respiration by 20–25 per cent indicating good permeability characteristics for the cell membrane. If the hepatocyte membrane was made permeable by the addition of 0.001 % digitonin, cell respiration was suppressed to about 0.65 μmole/g/min and the addition of succinate stimulated respiration by 900–1000 per cent.

Metabolism of aminopyrine by isolated hepatocytes. The cells were incubated with *N*-methyl-^{14}C-labeled aminopyrine and 25 μM–2 mM concentrations of aminopyrine in sealed flasks. The release of $^{14}CO_2$ was linear for the first 30 min. The effect of aminopyrine concentration on *N*-demethylation was examined. The data are shown both as a velocity

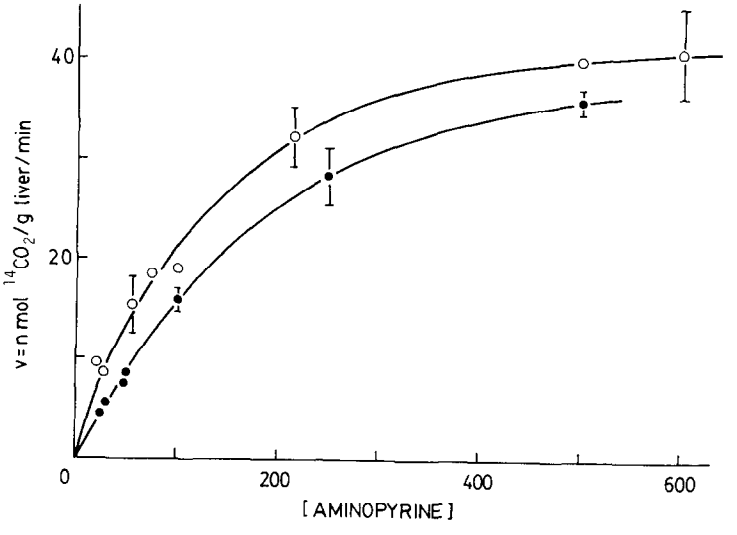

Fig. 1. Comparison of *N*-demethylation rates of aminopyrine in the rat *in vivo* and in isolated hepatocytes. Where no standard error is indicated, the values represent single observations. Other values are the mean of three or more observations. In isolated hepatocytes (open circles), the aminopyrine concentration is μmoles/liter of medium. Values at substrate concentration above 600 μM have been omitted. The *in vivo* aminopyrine dose is μmoles/kg body weight (closed circles).

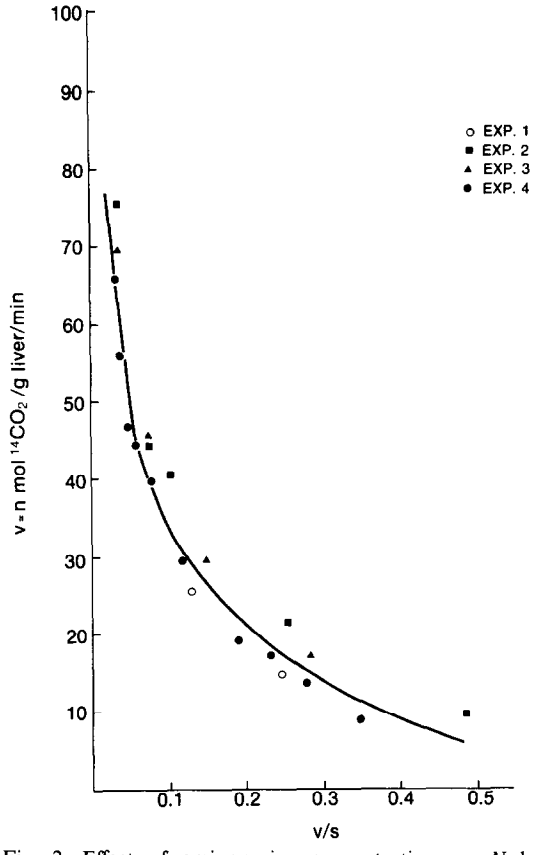

Fig. 2. Effect of aminopyrine concentration on *N*-demethylation of aminopyrine in isolated liver cells. Aminopyrine concentration varied from 25 to 2000 μM. Incubation time was 30 min. (For details, see Materials and Methods.)

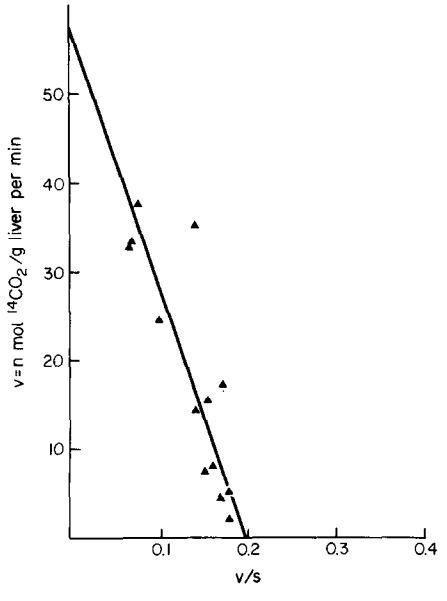

Fig. 3. Effect of dose of aminopyrine on *N*-demethylation rate of aminopyrine *in vivo*. Rates are expressed per g (wet weight) of liver. Liver weight was determined at the end of the measurement period. Aminopyrine dose varied from 25 to 500 μmoles/kg. (For details, see Materials and Methods.)

vs substrate concentration plot and as an Eadie–Hofstee kinetic plot (Figs. 1 and 2). The kinetic plot obtained was not linear as would be expected for a single reaction. On the assumption that the curve results from two *N*-demethylation reactions, the curve was subjected to graphic analysis by the method of Rosenthal [12]. The high affinity reaction would have a K_m of 32 μM and a V_{max} of 17 nmoles/g/min. The low affinity reaction would have a K_m of 710 μM and a V_{max} of 63 nmoles/g/min.

N-*Demethylation of aminopyrine* in vivo. Rats were injected intraperitoneally with *N*-methyl-labeled aminopyrine and the expired $^{14}CO_2$ was collected. The rate of $^{14}CO_2$ production was linear for the first 45 min. For calculating rates of $^{14}CO_2$ production the initial slope was used. The effect of the aminopyrine dose on *N*-demethylation was examined over a dose range of 25 500 μmoles/kg body weight. The rate of $^{14}CO_2$ production was calculated per g of liver. The liver weight, determined at the end of the collection period was 8.42 $\pm$ 0.6 g (S. D.) and the rat body weight was 303 $\pm$ 4.6 g. The *in vivo* rates of metabolism are compared with those of isolated hepatocytes in Fig. 1. The *in vivo* rate is somewhat lower than that observed for the isolated cells. The data are also presented as an Eadie–Hofstee plot (Fig. 3). Unlike the *in vitro* data, the *in vivo* data showed a linear curve. The V_{max} is estimated to be 58 nmoles/g of liver/min, and the K_m 260 μmoles aminopyrine/kg body weight.

DISCUSSION

In this study, aminopyrine was used for making *in vivo* and *in vitro* comparisons because it shows an apparent volume of distribution of 90 per cent of body weight [13] and, therefore, allows an estimate of drug concentration at the site of *N*-demethylation. The microsomal demethylation of aminopyrine results in the formation of formaldehyde which is further converted to CO_2 [14]. It is assumed that the demethylation step is rate-limiting, since the subsequent reactions are much faster [15]. Measurements of *N*-demethylation *in vivo* by $^{14}CO_2$ production underestimates the true reaction rate by about 20 per cent. After the injection of [^{14}C]formaldehyde, rats exhale about 82 per cent of the label as $^{14}CO_2$ [16].

The *N*-demethylation reaction in the isolated hepatocytes produced a non-linear Eadie–Hofstee plot, indicating the presence of more than one *N*-demethylation reaction. On the basis of graphic analysis [12] one reaction would have a K_m of 32 μM and a second reaction would have a K_m of 710 μM. Non-linear Lineweaver–Burk plots have been reported previously for aminopyrine *N*-demethylation in rat liver microsomes [17, 18].

Matsubara and Touchi [18] postulated that two reactions with K_m values of 55 and 677 μM were present. These estimates are in agreement with the hepatocyte data. It is unlikely that the nonlinear kinetic plots result from the second *N*-demethylation reaction occurring on the aminopyrine molecule. The Lineweaver–Burk plot for *N*-demethylation of 4-monomethylaminoantipyrine is also non-linear [17]. It is improbable that the non-linearity results from the conversion of formaldehyde to CO_2.

This step has a very high K_m and V_{max} [15]. It is also unlikely that uptake of aminopyrine by the hepatocytes is rate-limiting. The labeled aminopyrine in the cells had equilibrated with the surrounding medium in less than 5 min (unpublished observations).

A possibility is that the non-linearity is a reflection of the multiple forms of cytochrome P-450 which have been demonstrated in recent years (for examples Ref. 19–21).

The *in vivo* Eadie–Hofstee plot indicates a V_{max} of 58 nmoles of $^{14}CO_2$/g of liver/min and a K_m of 260 μmoles/kg (Fig. 3). The estimates are almost identical to those of Lauterburg and Bircher [8] for aminopyrine *N*-demethylation *in vivo* using the same technique. The *in vivo* estimates of these parameters are intermediate to those obtained with the isolated hepatocytes. It would appear that the *in vivo* data are the result of both reactions and that the range of *in vivo* concentrations of aminopyrine used is not sufficient to distinguish between the two components. The maximum dose of aminopyrine that the normal rats can tolerate is 500 μmoles/kg [8]. Concentrations higher than this are required to resolve the high K_m component.

The reason the low K_m component cannot be resolved at low aminopyrine doses *in vivo* is not clear. One possibility is that part of the aminopyrine is bound to extrahepatic sites, thus reducing availability at the liver. Aminopyrine does exhibit some tissue partitioning. The ratio of aminopyrine in the hepatocytes to the medium was 1.18 ± 0.03. Another is competition with other substrates for the *N*-demethylase. The effect would be to increase the apparent K_m.

An objective of the present study was to attempt to determine whether isolated hepatocytes are capable of *N*-demethylation rates approaching those in the intact liver *in vivo*. A number of factors complicate such a comparison. One complication is the presence of extra hepatic metabolism *in vivo*. Lauterburg and Bircher [8] compared *N*-demethylation of aminopyrine *in vivo* in normal rats and rats that had been subjected to $\frac{2}{3}$ hepatectomy. If their data are extrapolated to zero liver mass, 18 per cent of the total *N*-demethylation would still remain. The *in vivo* rates expressed per g of liver are about 18 per cent high. A second factor is that isolated hepatocytes are not completely representative of the whole liver. Connective tissue and most of the Kupffer cells are removed. The metabolism rate extrapolated to whole liver will, therefore, be high. The above two factors would tend to cancel one another out. Another factor is that the isolated hepatocyte and the hepatocyte *in vivo* are exposed to different environments. As discussed above, there appears to be a factor suppressing *N*-demethylation *in vivo* at low aminopyrine concentrations. The percentage difference in *in vivo* and *in vitro* rates is much greater at lower aminopyrine doses (Fig. 1). Despite these problems, as Fig. 1 shows, the *in vivo* and *in vitro* rates are remarkably close.

In conclusion, it would appear that the isolated hepatocyte is capable of aminopyrine *N*-demethylation rates similar to those occurring *in vivo*. The kinetics of *N*-demethylation in the isolated cells, however, indicate the presence of at least two reactions, whereas only one is evident from *in vivo* kinetics. It is thought that both reactions are present *in vivo* but that it is not possible to differentiate between them.

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