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KINETIC STUDIES ON THE METABOLISM OF ETHYLMORPHINE BY ISOLATED HEPATOCYTES FROM ADULT RATS*

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Abstract—Hepatocytes of adult rats were isolated by infusion of a hyaluronidase collagenase mixture. High yields of cells excluding trypan blue were obtained. These cells, in Hank's buffer containing rat serum and 0.1% glucose, *N*-demethylate [^{3}H-CH$_3$-N]ethylmorphine. The formaldehyde initially formed is further metabolized to tritiated water. Fifteen per cent of the original metabolic activity was observed after 21 hr at 37° in 5% CO_2–air, and cumulative metabolism is linear for up to 90 min under these conditions. The K_m for the *N*-demethylation of [^{3}H-CH$_3$-N]ethylmorphine is 50 μM, 20 per cent of the value observed for this reaction by microsomal preparations. An active transport of the substrate into the cell is postulated to account for this difference.

The study of drug metabolism in the past has generally been restricted to the use of whole animals, perfused organs or tissue homogenates. Although these studies have provided valuable information concerning the biochemical, physiological and pharmacological mechanisms regulating drug metabolism, they often have significant limitations. Whole animals or isolated organ studies are often subject to uncontrollable and frequently unidentified factors which may have significant effects on the results. On the other hand, while more amenable to manipulations, tissue homogenates suffer from the fact that they do not retain much of the organization of the native cells, which may play a prominent role in the regulation of metabolism in the intact animal.

Workers in many areas of biochemistry have sought models which retain cellular organization yet are readily manipulated. In the main they have developed techniques for preparing isolated, metabolically active cells. Yet, in spite of the success over the years in culturing a wide range of mammalian cells, only recently have techniques been reported for the preparation of hepatocytes which could serve as a potential model in the study of drug metabolism.

Nebert and Gelboin [1] first reported the use of the cultured explants or dispersions of fetal rodent livers for drug metabolism studies. Although studies with this model led to a marked increase in our knowledge concerning the inductive process, it does not appear to be a good model for the nonreplicating adult liver. Further, it is difficult to obtain large quantities of material for many biochemical studies. Finally, these cells, and the original tissue from which they are derived, seem to metabolize a relatively narrow range of substrates [1]. These limitations place significant restrictions on the areas of drug metabolism which can be studied with this system.

Mechanical [2,3], chemical [4] and enzymatic [5] methods have been utilized to prepare relatively pure suspensions of hepatocytes from adult animals. Of these methods, the perfusion of the liver with a mixture of collagenase and hyaluronidase gives cells which appeared to be the best model for metabolic studies [5]. We [6], Cantrell and Bresnick [7], and Henderson and Dewaide [8] examined early versions of these enzyme techniques for the preparation of cells for drug studies. None proved to be very successful for broader investigations.

Recently Seglen [9,10] has reported a modification of these methods in which the liver is continuously perfused with the enzyme mixture at 37° using a perfusion pump. Further, she added Ca^{2+} ion to the perfusion medium, which is known to be an obligate cofactor for clostridinal collagenase [11] and which Howard *et al.* [12] have found will help to maintain the integrity of the mitochondria. This method gives a yield of cells of 50 per cent or more. Further, a much higher fraction of these cells excluded trypan blue, suggesting that they are metabolically active.

We have examined this method for its application to drug studies by ascertaining the ability of these cells to metabolize [^{3}H-CH$_3$-N]ethylmorphine and at the same time sought to establish the ideal incubation conditions. Our studies indicate that these cells very actively metabolize this substrate in the presence of glucose as the sole source of energy and in the absence of added pyridine nucleotides or their precursors. Further, kinetic studies suggest that there is an active transport of ethylmorphine from the medium into the cell. Concurrent with these studies, Moldeus *et al.* [13] have examined the activity of this preparation for another substrate, alprenolol, and found excellent activity.

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MATERIALS AND METHODS

[^{3}H-CH_3-N]ethylmorphine was prepared as previously described by the method of Abdel-Monem and Portoghese [14], in which norethylmorphine phenylcarbamate was reduced by $LiAl^3H_4$. HEPES buffer was obtained from Sigma Chemical Co. (St. Louis, Mo.); the hyaluronidase and collagenase from Worthington Biochemical Corp. (Freehold, N.J.); and the trypan blue (direct blue 14) from Matheson Scientific (Elk Grove Village, Ill.).

All animals used in these experiments were fed male C-D rats (200–250 g) obtained from Charles River (North Willmington, Mass.).

Isolated hepatocytes were prepared essentially by the method of Seglen [9]. The animals were anesthetized with ether, the abdomen was opened by a midline incision, and the portal vein was cannulated with a 21 gauge butterfly needle. The liver was then infused *in situ* with oxygenated 0.5 mM EGTA buffer* at 20 ml/min while the liver was being excised and placed in the perfusion apparatus. The perfusion rate was then increased to 40 ml/min for 5 min, after which 50 mM $CaCl_2$† (4.5 ml) was added, followed by calcium-free HEPES buffer (6 ml) containing collagenase (44 mg) and hyaluronidase (25 mg). The perfusion was continued for 10 min, at which time the liver was placed in a polypropylene beaker, the capsule ruptured and the suspension filtered through 110 mesh nylon. The filtrate was centrifuged at 100 *g* for 2 min at room temperature. The supernatant was discarded and the pellet was resuspended in Ca^{2+}-free HEPES buffer‡ containing 20% rat serum and 0.1% glucose. The cells were resedimented and washed twice. They were finally suspended in Hanks buffer§ with 20% rat serum to the desired concentration and maintained at room termperature with very gentle agitation under a constant flow of air with 5% CO_2.

The cells were counted with a hemocytometer. A 0.05% trypan blue solution in Ca^{2+}-free buffer was used to determine the integrity of the plasma membrane. For the purposes of this study, those cells excluding the dye were defined as viable, although we realize that this may or may not be related to their metabolic acitivity.

Metabolic activity was further assessed by determination of oxygen uptake. In these studies Hanks buffer was equilibrated with 5% CO_2 in air at 37°. The buffer was then placed in a jacket incubation vessel fitted with a YSI 5331 oxygen electrode (Yellow Springs Instrument, Yellow Springs, Ohio). The reaction was initiated by the addition of the cell suspension. The concentration of oxygen was derived from standard tables [15].

Three ml of the cell suspension was placed in unstoppered serum vials and the substrate added. The vials were incubated in air for 10–90 min at 37° as indicated. The reaction was stopped by adding 2 ml of 8.9% $ZnSO_4 \cdot 7H_2O$. In early experiments, an aliquot (1 ml) of this mixture was diluted with H_2O (6 ml) and a portion run through a column of XAD-2 polystyrene resin (Mallinckrodt Incorp., St. Louis, Mo.), prepared as described previously [16]. Aliquots (1 ml) of the untreated and resin-treated solutions were suspended in Aquasol (New England Nuclear Corp., Boston, Mass.) and counted in a Beckman LS 100 liquid scintillation counter. We have previously shown that since ethylmorphine is quantitatively absorbed by the resin [16], the radioactivity of the effluent is due solely to ^3HCHO and ^3H_2O. In later experiments, the incubation system was lyophylized, the water trapped in a U-tube at −77°, and the ^3H_2O activity determined as above. In the latter technique, the water phase is trapped in a U-tube which is cooled in a dry ice ethanol bath. Since the formaldehyde remains with the solids, it can be separated from the ethylmorphine by forming the dimedon derivative, precipitating the derivative, and counting the precipitate [16].

Glucose was determined by the phosphomolybdic acid method of Lauber and Mattice [17]. Hepatic microsomes were prepared after homogenization of the liver in KCl–Tris (150 mM, 50 mM, pH 7.4) and differential centrifugation in a Sorval RC-2B and Beckman L2-65 centrifuge [18]. Microsomal protein was determined by the method of Sutherland *et al.* [19]. Microsomal ethylmorphine *N*-demethylase was determined by incubating KCl–Tris–Mg (150 mM, 20 mM, 5 mM, pH 7.4) (3 ml) with NADP (0.33 mM), glucose 6-phosphate (25 mM), and glucose 6-phosphate dehydrogenase (0.67 units/ml) at 37° for 10 min with 0.1 to 2 mM ethylmorphine [18]. The reaction was terminated by the addition of $ZnSO_4$ (1 ml) followed by BaOH (sat.) (1.5 ml) and NaB_2O_7 (sat.) (0.5 ml) and the suspension centrifuged. The formaldehyde was determined in the supernatant by the method of Nash [20].

RESULTS

We obtained an average of $3.2 \pm 0.4 \times 10^8$ cells from a liver, and 77 per cent of these cells excluded trypan blue. These values agree with the report of Seglen [10]. Further examination of the cell preparation at 400× magnification revealed little contaminating debris. A recent report by Ingebretsen and Wagle [21] suggested that satisfactory preparations could be obtained in the absence of hyaluronidase. Unfortunately, in our hands, deletion of this enzyme led to clumped cells with poor activity.

Examining the products of the *N*-demethylation of [^{3}H]ethylmorphine by the cells, we could not detect any HCHO colorimetrically, although significant amounts of metabolism were observed by the radioassay (0.91 nmole $HCHO/10^6$ cells). This suggested that all the HCHO formed during the metabolism of the ethylmorphine was itself further metabolized to H_2O, presumably by the mitochondrial aldehyde dehydrogenase. Further, we found that all the enzymatically released activity could be lyophylized, while in pre-

*EGTA buffer: 8.00 g NaCl, 0.35 g KCl, 0.16 g KH_2PO_4, 0.16 g $MgSO_4 \cdot 7H_2O$, 2.38 g HEPES, and 0.19 g EGTA in 1 liter H_2O.

† Ca^{2+} buffer: 4.90 NaCl, 0.55 mg KCl, 0.13 g $MgCl_2 \cdot 6H_2O$, 2.38 g HEPES, and 7.35 g $CaCl_2 \cdot 2H_2O$ in 1 liter H_2O.

‡ Ca^{2+}-free buffer: 8.18 g NaCl, 0.35 g KCl, 0.16 g KH_2PO_4, 0.16 g $MgSO_4 \cdot 7H_2O$, and 2.38 g HEPES in 1 liter H_2O.

§ Hanks buffer: $NaHCO_3$, 2.2 g/l.; NaCl, 6.8 g/l; glucose, 1 g/l.; KCl, 0.4 g/l.; $CaCl_2$, 0.2 g/l.; $NaH_2PO_4 \cdot H_2O$, 0.14 g/l.; and $MgSO_4$, 0.1 g/l.

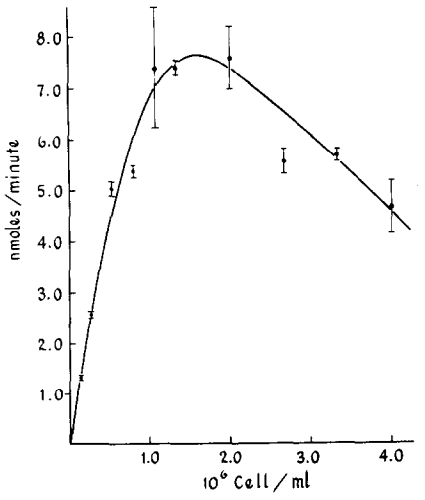

Fig. 1. Effect of hepatocyte concentration on *N*-demethylation of [^{3}H]ethylmorphine. Hepatocytes of increasing concentrations were incubated with 2 mM [^{3}H]ethylmorphine for 30 min as described in the text. Values are the average of triplicate incubations ±S.E.M.

vious studies (Thompson and Holtzman, unpublished data) we found that the formaldehyde remained behind with the solids. Finally, when carrier formaldehyde was added and precipitated with dimedon, a procedure which removes all HCHO from solution [16], all the activity remained in solution. Because of the close agreement between the resin and lyophilization methods, the two were used interchangeably in subsequent experiments.

In view of the significant activity of this preparation, we next investigated the behavior of the system with regard to a number of parameters. The first was the effect of cell concentration on metabolism (Fig. 1). Clearly this preparation is quite linear to about 5 × 10^5 cells/ml. The loss of linearity after this concentration of cells is not due to a decrease in the glucose concentration or to a drop in the pH of the suspension medium since both are quite stable. Nor was there a significant decrease in the oxygen content over the 10 min of the incubation. During subsequent incubations, the cell concentration was routinely maintained at 2 to 3 × 10^5 cells/ml.

We next examined the linearity of the activity with time and found that the reaction was quite linear to 90 min (Fig. 2). In view of the linearity with time and cell concentrations, we were able to compare the initial activity of cell preparations and the isolated microsomes. In later studies, the activity of these cell preparations was about 3 nmoles/min-10^6 cells which is about equal to the V_{max} usually observed for the isolated microsomes run under optimal conditions. This comparison is based on our usual yield of 20 mg of microsomal protein/g of liver, which is about half the microsomes present in the intact liver. Hence there would be about 40 mg of microsomal protein/g of liver. Further, it has been estimated that there are about 2 × 10^8 cells/g so that 10^6 cells would equal about 0.2 mg of microsomal protein and the metabolism would be about 15 nmoles/mg of microsomal protein-min[22].

About the time we had completed this portion of the studies, it was reported that CO_2 appeared to be an important nutrient for cells, above and beyond its buffering capacity [23]. Up to this time we had been using the HEPES buffer of Seglen [10], which contained neither CO_2 nor Ca^{2+} ion. Hence, we decided to investigate the importance of these two components on cellular metabolism (Fig. 3). Clearly there is improved metabolism when CO_2 is included in the medium. All studies reported below were, therefore, performed in Hanks bicarbonate buffer with Ca^{2+} included.

In all of these studies, serum was added to give a concentration of about 20%. The reason for this requirement is unclear but has been reported for a wide variety of culture systems [24]. Yet it seems to be only beneficial in Hanks bicarbonate buffer (Fig. 3). In view of these results, all subsequent studies were performed with Hanks bicarbonate buffer and 20% rat serum.

We next examined the viability of the hepatocytes as determined by three indices of function. The first was the ability of the cells to exclude trypan blue.

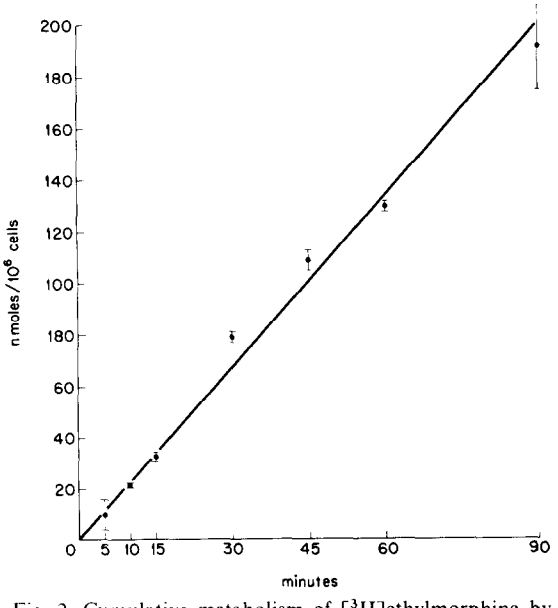

Fig. 2. Cumulative metabolism of [^{3}H]ethylmorphine by isolated hepatocytes. Isolated hepatocytes (3 × 10^5 viable cells/ml) were incubated with 2 mM [^{3}H]ethylmorphine in medium containing 20% rat serum and 0.1% glucose as described in the text. The cumulative metabolism was measured at the times indicated.

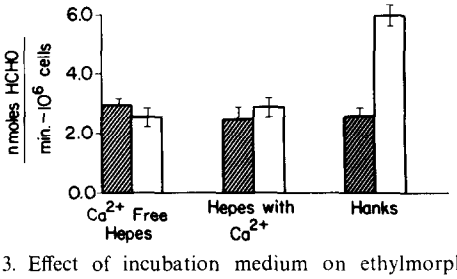

Fig. 3. Effect of incubation medium on ethylmorphine *N*-demethylation by isolated hepatocytes. The effect of 20% rat serum (open boxes) was assessed in Ca^{2+}-free HEPES, HEPES with 5 mM Ca^{2+}, and in Hanks buffer. Cells were incubated for 10 min at 37° in air (HEPES) or 5% CO_2–air (Hanks).

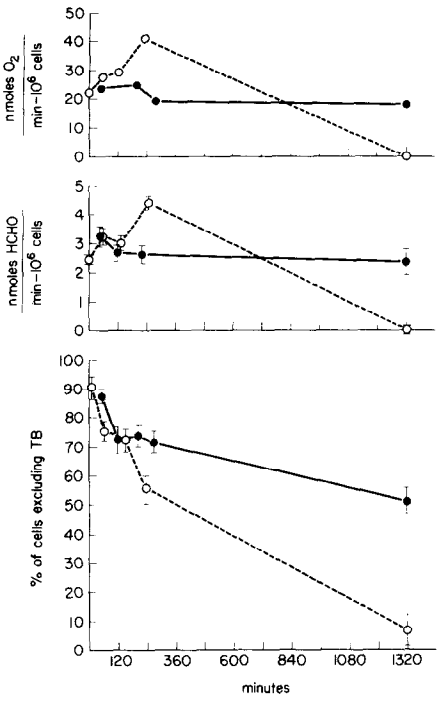

Fig. 4. Effect of preincubation on oxygen uptake (top), formaldehyde formation (middle) and trypan blue exclusion (bottom) by isolated hepatocytes in the absence (O– –O) and presence (●—●) of penicillin G (100 μg/ml) and streptomycin (100 μg/ml). Cells were withdrawn at the specified times and counted. Further, the oxygen uptake and formaldehyde formation were determined at 37° in Hanks buffer under 5% CO_2 air.

which indicates whether the plasma membrane is intact; second, the ability of the cell to respire; and finally, the rate of ethylmorphine *N*-demethylation (Fig. 4). Clearly on the basis of these indices, this preparation showed good but decreasing metabolism for at least several hr. Further, the three indices appear to parallel each other during the course of the preincubation. This is not unreasonable since it is clear that the hepatocyte with a ruptured plasma membrane will not be able to retain critical cofactors, as the pyridine nucleotides. Yet, these studies were not performed in a totally aseptic environment, so that it may well be that bacterial contamination compromised the cells. We than added penicillin (100 μg/ml) and streptomycin (100 μg/ml) and followed these same indices (Fig. 4). At the end of 24 hr the concentration of cells viable was 15% of the zero time, but their specific activity was constant. Interestingly, at 24 hr the cells which still excluded trypan blue tended to be clumped, suggesting that aggregation could prove to be a problem in further studies.

The question also arises as to whether specific hormones, such as insulin, cortisone and thyroxine, may affect the stability of the drug-metabolizing systems, much as these and other hormones have been shown to affect casein synthesis in mammary gland explants [25–27]. Of these, only thyroxine appears to have a significant effect (Fig. 5). This is not surprising, since this hormone has been shown to have profound effects on drug metabolism in intact subjects [28]. But the failure of insulin to be active is surprising, since a number of workers have shown that it has significant effects on isolated hepatocytes [29–33]. All these agents, however, are metabolized by the liver so that the actual concentrations are not known. Hence, the cells may have reduced hormone concentrations to a point where they have no effect. This question will require further evaluation. In subsequent studies, hormones were not included.

In a final study, we examined the substrate-dependent kinetics of the ethylmorphine *N*-demethylase (Fig. 6). The K_m for this metabolism (50 μM) is significantly lower than the K_m found for the isolated microsomes (250 μM) determined in a parallel study in Hanks bicarbonate. This difference could be due to a number of causes in such a complex system. For example, oxygen depletion or suboptimal pyridine nucleotide concentrations could reduce the K_m by reducing the turnover number of the enzyme. Alternatively,

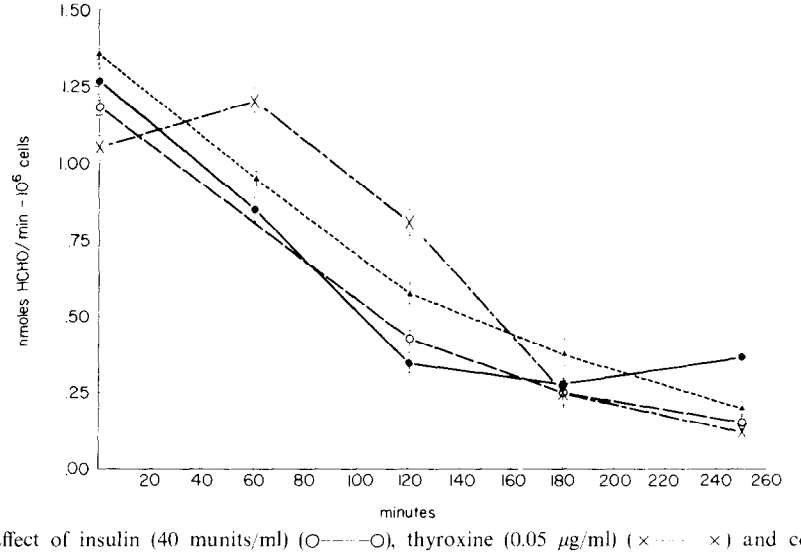

Fig. 5. Effect of insulin (40 munits/ml) (O– –O), thyroxine (0.05 μg/ml) (× – –×) and cortisone (30 μg/ml) (▲– –▲) on the control (●—●) ethylmorphine *N*-demethylase of hepatocytes from male rats. Cells were incubated for 10 min in Hanks buffer in 5% CO_2 air.

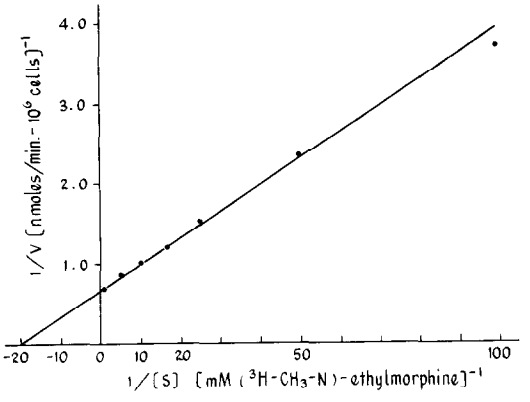

Fig. 6. K_m for ethylmorphine *N*-demethylase of isolated hepatocytes. [^{3}H]ethylmorphine (0.01 to 2.0 mM) was incubated with 1.3×10^5 viable cells/ml in 20% rat serum and 0.1% glucose for 10 min in 5% CO_2–air at 37°. Metabolism was measured as described in the text.

the cells may transport drug into the cytoplasm to increase the drug concentration bathing the enzymes. This point has been investigated in only a preliminary fashion.

One possible explanation for the differences in K_m values of *N*-demethylation observed for hepatocytes and microsomes could be due to the removal of product from the reaction site of the membrane by glucuronidation. Hence, if the product acted as a competitive inhibitor of *N*-demethylation, then its removal by glucuronidation should decrease the apparent K_m. To rule this out, varying concentrations of ethylmorphine were incubated with microsomes and an NADPH-generating system in the presence and absence of 2 mM UDPGA. The K_m for the demethylation of ethylmorphine was unaffected by the addition of UDPGA, while the V_{max} was slightly increased. Under similar conditions Von Bahr and Bestilsson [34] found that half of the 2-HO-desmethylimipramine formed from desmethylimipramine is conjugated with UDPGA, suggesting that the lack of effect is not due to a failure of our system to form the glucuronide. Thus, if glucuronide formation takes place in the cells, there appears to be no effect on the K_m.

Interestingly, Moldeus *et al.* [13] found no such difference in K_m values for alprenolol. This agent is a highly lipid-soluble drug which exhibits first pass metabolism, much like its congener propranolol [35], where a large fraction of the drug is removed from the portal system before ever entering the systemic circulation. This phenomenon has never been reported for ethylmorphine. It is unclear how this difference between the kinetics of ethylmorphine and alprenolol metabolism could be related to the presence or absence of first pass metabolism.

DISCUSSION

These studies clearly indicate that the preparation of hepatocytes by the method of Seglen gives an abundant yield of metabolically active cells capable of rapidly metabolizing ethylmorphine. In agreement with Bissell *et al.* [36], this high activity can be observed on a chemically defined medium in the total absence of added cofactors, but in the presence of rat serum. This preparation should permit a thorough evaluation of the many factors which may control drug metabolism in a system where hormonal and other homeostatic mechanisms can be carefully controlled.

A second significant result of these studies is that they suggest that the hepatocyte is capable of actively transporting drugs into its cytosol. We feel, on the basis of present evidence, that this is the most likely explanation for the marked discrepancy between the K_m for *N*-demethylation by the intact hepatocyte and the microsomes. This result is not entirely unexpected since the usual therapeutic concentrations for many drugs, such as barbiturates, hydantoins and opiates, rarely exceed 100 μM in the serum. Yet, the microsomal hydroxylations are only at 30 per cent of V_{max} at these concentrations. This value is quite low, especially for an enzyme system with a low turnover number of no more than 20 [37]. These considerations would suggest that the evolutionary process would lead to mechanisms to enhance the metabolism of potentially toxic xenobiotics. The transport process may represent such a mechanism.

It is of interest that Moldeus *et al.* [13] have recently reported metabolic studies with this hepatocyte preparation. They found that the K_m values for the metabolism of alprenolol by the hepatocyte and the isolated microsomes are both about 9 μM, suggesting that there is no active transport of this drug into the cell. This is a very lipophilic agent with a high affinity for microsomes. Taking their results along with our own would suggest that the more water-soluble compounds with a low affinity for the microsomes may be transported into the cell, while the more lipophilic agents with high affinities for the microsomes may rapidly enter by simple diffusion through the plasma membrane. Preliminary results in our laboratory would directly suggest that the water-soluble compounds are transported.

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