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KINETIC STUDIES ON THE DEETHYLATION OF ETHOXYBENZAMIDE

A COMPARATIVE STUDY WITH ISOLATED HEPATOCYTES AND LIVER MICROSOMES OF RAT*

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Abstract—Kinetic parameters (K_m and V_{max}) of ethoxybenzamide deethylation in isolated rat hepatocytes and liver microsomes were compared. Adjustment of cofactors in microsomal deethylation, such as NADPH and Mg^{2+}, to give optimum conditions, and appropriate correction of the apparent kinetic parameters for nonspecific binding and microsomal yield resulted in good agreement among the kinetic parameters of isolated hepatocytes [$V_{max} = 0.0863\ \mu mole \cdot min^{-1} \cdot (g\ liver)^{-1}$ and $K_m = 0.459$ mM] and microsomes [$V_{max} = 0.124\ \mu moles \cdot min^{-1} \cdot (g\ liver)^{-1}$ and $K_m = 0.378$ mM].

A long-term goal in the study of drug metabolism is the quantitative prediction of *in vivo* drug metabolism from enzymatic parameters (V_{max}, the maximum velocity of reaction, and K_m, the Michaelis constant) obtained *in vitro*. Recently, based on comparisons of drug metabolism in perfused liver preparations with metabolism in subcellular liver fractions and in isolated hepatocytes, Billings *et al.* [1] suggested that drug metabolism in isolated hepatocytes correlates better with *in vivo* drug metabolism than does metabolism in 9000 *g* supernatant fractions or microsomes. Drug metabolism studies with isolated hepatocytes from small animals are easily performed, but the preparation of isolated hepatocytes from large animals or humans presents some difficulties. In large animals and humans, *in vitro* drug metabolism can only be investigated by using subcellular liver fractions as enzyme sources. In the present study, the authors used ethoxybenzamide (EB), which is deethylated primarily in the liver by the microsomal monooxygenase system to form salicylamide (SAM) [2], as a model drug to investigate whether liver microsomes can be used as a source of enzymes whose kinetic parameters correlate well with those obtained with isolated hepatocytes. This required close examination of the experimental conditions for the liver microsomes in relation to those used for isolated hepatocytes, as well as of other factors which affect the estimation of kinetic parameters, such as nonspecific substrate binding and microsomal losses incurred during differential centrifugation.

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MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: EB from the Takeda Chemical Co. (Tokyo, Japan); SAM from the Tokyo Kasei Chemical Co. (Tokyo, Japan); collagenase type I and carbonyl cyanide phenylhydrazone (CCP) from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); β-glucuronidase/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1) and NADPH from the Boehringer Co. (Mannheim, F.R.G.); and sodium succinate, trypan blue, and general reagents from the Wako Pure Chemical Co. (Osaka, Japan).

Animals. All animals used in these experiments were male Wistar rats (230–270 g) obtained from the Shihashi Co. (Tokyo, Japan), and fed *ad lib.*

Preparation and incubation of isolated hepatocytes. Isolated hepatocytes were prepared essentially by the method of Baur *et al.* [3], except that prolonged pre-perfusion (35 ml/min, 15 min) with Ca^{2+}-free perfusion buffer was performed to ensure complete removal of the intercellular adhesion factor, Ca^{2+}. This was followed by collagenase perfusion (4 mM Ca^{2+}, 0.05% collagenase) instead of hyaluronidase and collagenase perfusion (0.5 mM Ca^{2+}, 0.05% collagenase and 0.1% hyaluronidase), according to Seglen [4].

The compositions of the main buffers were as follows: (a) perfusion buffer: 121 mM NaCl, 6 mM KCl, 0.6 mM $MgSO_4$, 12 mM $NaHCO_3$, 0.74 mM KH_2PO_4, and 5 mM glucose at pH 7.1–7.2; (b) wash and incubation buffer: 131 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$, 3 mM phosphate buffer, and 10 mM Tris–HCl, at pH 7.4.

After preincubation for 5 min, the reaction was started by adding an equal volume of EB solution to the cell suspension (the final volume of the reaction mixture was 2 ml); no cofactor was added unless

otherwise stated. The incubations were performed at 37° for a specified period (usually 5 min) with shaking (90 oscillations/min) in 10 ml round-bottom vials under air. In some cases, the vials were flushed with 95% O_2–5% CO_2. After incubation, the reaction was terminated by adding 0.5 ml of 15% trichloroacetic acid/ml of incubation mixture.

Cell viability. We obtained an average of $2.6 \pm 0.4 \times 10^8$ cells from one liver, and more than 95 per cent of these cells excluded trypan blue (0.3 per cent). The endogenous cellular respiration was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The respiratory control ratio and the respiratory stimulation ratio were measured as the ratios of respiration with and without 2 μM CCP and with and without 1 mM sodium succinate, respectively. The value of endogenous cellular respiration was about 14 nmoles $\cdot$ $min^{-1} \cdot 10^6$ $cells^{-1}$, and the respiratory control and stimulation ratios were 1.6 and 1.1, respectively, in agreement with the report of Baur *et al.* [3]. All experiments with isolated hepatocytes were performed within 2 hr after cell preparation.

Damaged isolated hepatocytes and sonicated cell homogenates. In some experiments, damaged isolated hepatocytes and cell homogenates were used as enzyme sources. Homogenates from isolated hepatocytes were prepared by treatment of the suspended cells in an ultrasonic disintegrator (Branson 185 cell disrupter) for 3 min, and damaged isolated hepatocytes, defined as those stained 100 per cent by trypan blue (0.3%), were prepared by high-speed mixing in an automatic mixer for more than 5 min.

Preparation and incubation of liver microsomes. Isolation of microsomes was performed as described previously [2]. Incubations with microsomes were carried out as follows: the incubation mixture contained various concentrations of EB, 0.5 ml of microsomal suspension, 0.6 mM NADPH, and 6 mM $MgCl_2$ in a final volume of 1.7 ml of 0.05 M Tris–HCl buffer (pH 7.4). All incubations were carried out at 37° for a specified period (usually 5 min) with shaking (90 oscillations/min) under air.

Enzyme activity assay. EB is deethylated *in vivo* to SAM, and SAM undergoes subsequent metabolic conversion to glucuronide and sulfate. In incubations with hepatocytes, damaged hepatocytes or cell homogenates, the total SAM (free SAM + SAM glucuronide + SAM sulfate) was determined after hydrolysis with β-glucuronidase/arylsulfatase. On the other hand, free SAM in the incubation mixture was determined without enzyme hydrolysis, and conjugates of SAM were estimated by subtracting the free SAM from the total SAM. SAM formed in all experimental systems was extracted with 1,2-dichloroethane and assayed fluorometrically as reported elsewhere [2]. Complete enzyme hydrolysis of SAM conjugates was confirmed by the following procedures. To obtain an adequate amount of SAM conjugates, a higher cell concentration (2×10^7 cells/ml) was used in this experiment. After incubation for 1 hr at 37°, the reaction was stopped by adding 15% trichloroacetic acid, a 1-ml aliquot was pipetted off, and the pH was adjusted to 5.5. The samples were then incubated after adding 20 μl of β-glucuronidase/arylsulfatase (activity of β-glucuronidase: approximately 5.2 units/ml at 38° with phenolphthalein monoglucuronide as a substrate; activity of arysulfatase: 2.6 units/ml at 38° with phenolphthalein disulfate as a substrate) at 24-hr intervals. Incubation for 48 hr (40 μl of enzyme in total) was required for complete hydrolysis of SAM conjugates; no additional hydrolysis of the conjugates was detected on further incubation. Control experiments showed that no nonenzymatic deethylation of EB to SAM occurred under these experimental conditions.

Determination of cytochrome P-450 and protein. Cytochrome P-450 was measured according to Omura and Sato [5] with a Hitachi 356 dual wavelength double-beam spectrophotometer. Protein contents of the isolated microsomes were determined by the method of Lowry *et al.* [6], with bovine albumin as a standard. The content of cytochrome P-450 per mg of microsomal protein was 0.66 ± 0.052 nmole (mean ± S.D.).

Nonspecific binding to microsomes and isolated hepatocytes. Nonspecific binding to microsomes was reported elsewhere; no saturation of binding was observed, and the unbound fraction was virtually constant at about 0.65 [2]. The nonspecific binding to isolated hepatocytes was determined using the hepatocyte reaction mixture described earlier. After adding EB solution to the hepatocyte suspension, the mixture was centrifuged at room temperature and the supernatant fraction was pipetted off immediately. All procedures were completed within 1 min, so that the decrease of EB by metabolism in the hepatocytes could be neglected, and the concentration of EB in the supernatant fraction was determined. The levels of EB concentration, i.e. 0.25, 0.5, 1.5 and 3.0 mM, cover the actual concentrations studied. No saturation of binding to the isolated hepatocytes was observed, and the unbound fraction was virtually constant at about 0.9.

RESULTS

Conditions for the assay of EB deethylation in isolated hepatocytes. To test whether the oxygen in air is sufficient to support cytochrome P-450-linked drug monooxygenation, the time courses of EB deethylation in 95% O_2–5% CO_2 and in air were compared (Fig. 1). No difference was found between them at either high (3 mM) or low (0.25 mM) EB concentration. The EB deethylation proceeded almost linearly with time for 15 min in all cases, as shown in Fig. 1. Figure 2 shows the effect of cell concentration on EB deethylation; the relation is linear up to about 2.0×10^6 cells/ml of incubate.

The effect of exogenous NADPH on EB deethylation was investigated with isolated intact hepatocytes, damaged hepatocytes, and sonicated cell homogenates (Fig. 3). Exogenous NADPH had little effect on intact hepatocytes but increased enzyme activity in both damaged hepatocytes and cell homogenates. The concentration of exogenous NADPH required for maximal EB deethylation activity was about 0.4 mM in damaged hepatocytes. About 1.0 mM NADPH was required for maximal enzyme activity in cell homogenates; the activity in cell homogenates was lower than in the other two systems.

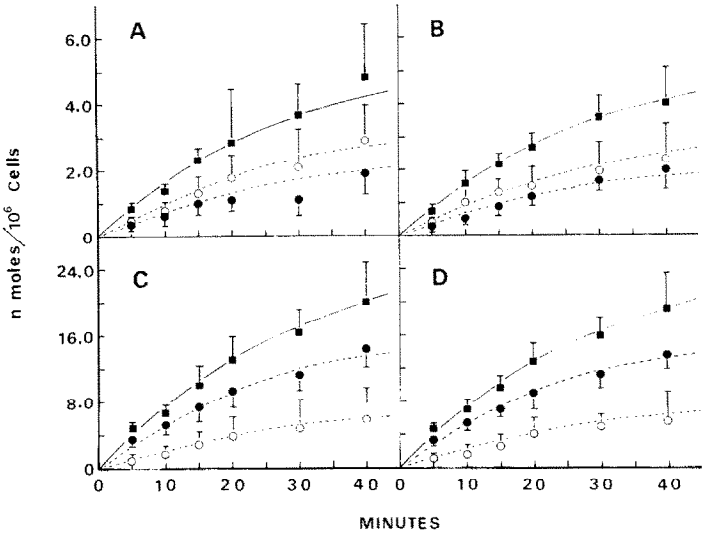

Fig. 1. Time course of EB deethylation and subsequent conjugation of SAM in isolated hepatocytes. Incubations were performed at 37° using 2×10^6 cells/ml. The concentration of EB was 0.25 mM (A, B) or 3 mM (C, D). Mixtures in B and D were incubated in 95% O_2 and 5% CO_2 whereas A and C were incubated in air. Key: total metabolites (■); conjugates of SAM (○); and free SAM (●). Each point is the mean ± S.D. of four experiments.

Possible explanations for this will be discussed later.

In view of these results, the following experimental conditions were adequate for the kinetic studies with isolated hepatocytes: (a) cell concentration, 1.5–2.0×10^6 cells/ml of incubate; (b) incubation time, 5 min; (c) in air; and (d) no cofactor added.

Conditions for the assay of EB deethylation in microsomes. Isolated microsomes catalyzed EB deethylation linearly for up to 10 min, and the rate was also linear with respect to microsomal protein concentration up to about 3 mg/ml of incubate (Fig. 4).

The effect of exogenous NADPH on EB deethylation was saturated to give constant velocities of deethylation above 0.5 mM NADPH at 0.3 and 3.5 mM EB, respectively.

The effect of exogenous Mg^{2+} on EB deethylation is shown in Fig. 5. EB deethylation increased to a maximum at 6 mM Mg^{2+} or less, followed by a decrease of enzyme activity at higher concentrations of exogenous Mg^{2+}. The kinetic studies on EB deethylation were carried out in the presence and absence of Mg^{2+} (Fig. 6). It is clear that Mg^{2+} affected K_m but not V_{max}.

In view of these results, the following experimental conditions were adequate for the kinetic studies with microsomes: (a) microsomal protein concentration, 2.5–3.0 mg/ml of incubate; (b) incubation time, 5 min; and (c) cofactors, NADPH (0.6 mM) and Mg^{2+}, (6 mM).

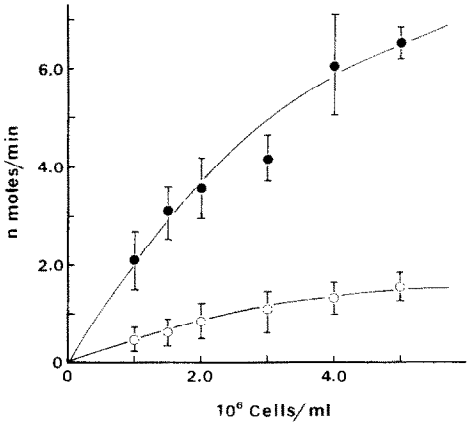

Fig. 2. Deethylation velocities of EB in isolated hepatocyte suspension at various cell concentrations. The velocities are expressed as nmoles of total SAM formed/min. The reactions were performed at 37° for 5 min in a 2-ml cell suspension. Key: 0.25 mM (○), and 3 mM (●) EB. Each point is the mean ± S.D. of four experiments.

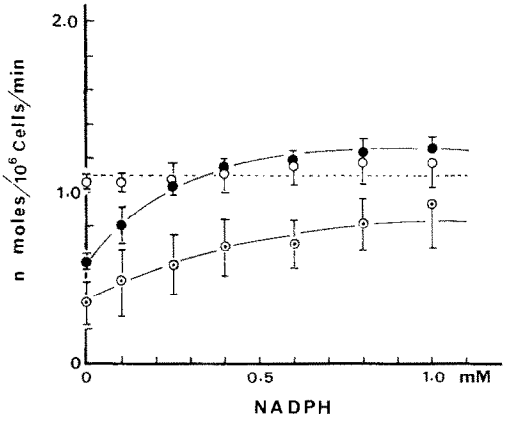

Fig. 3. Effect of exogenous NADPH on EB deethylation velocity in isolated hepatocytes (○), damaged hepatocytes (●), and sonicated cell homogenates (⊙). The velocities are expressed as nmoles of total SAM formed·10^6 cells^{-1}·min^{-1}. The reactions were performed at 37° for 5 min in a 2-ml cell suspension containing 2 mM EB and 2×10^6 cells/ml. Each point is the mean ± S.D. of four experiments.

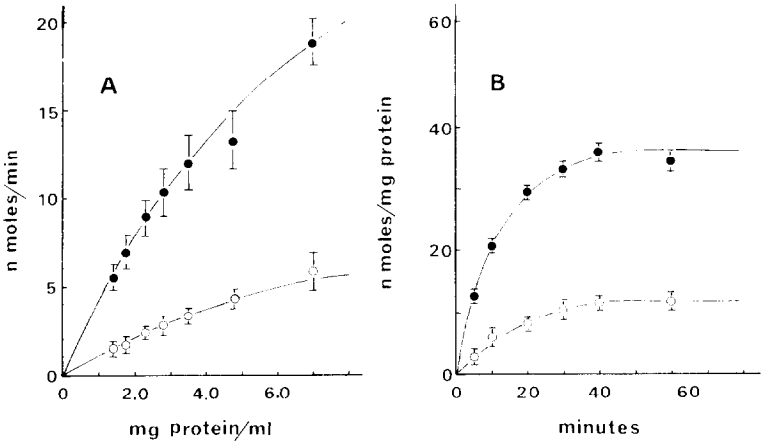

Fig. 4. Panel A: Deethylation of EB in hepatic microsomes at various microsomal protein concentrations. The enzyme activity is expressed as nmoles SAM formed/min. The reaction was performed at 37° for 5 min in 1.7 ml of the reaction mixture that contained 6 mM $MgCl_2$ and 0.6 mM NADPH. Panel B: Time course of EB deethylation in hepatic microsomes. The time course is expressed as nmoles SAM formed/mg microsomal protein. The reaction was performed at 37° in 1.7 ml of the reaction mixture that contained 6 mM $MgCl_2$, 0.6 mM NADPH, and 3 mg microsomal protein/ml. For A and B, the concentration of EB was 3.53 mM (●) and 0.294 mM (○). Each point is the mean ± S.D. of four experiments.

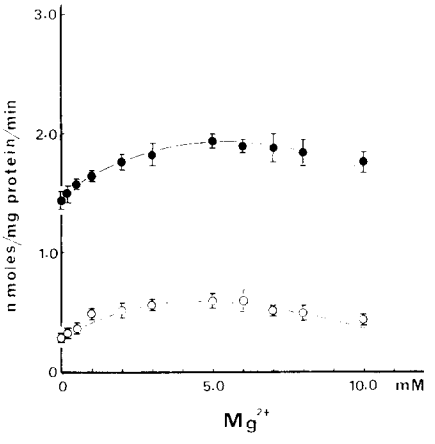

Fig. 5. Effect of exogenous Mg^{2+} on EB deethylation in hepatic microsomes. Incubations were performed at 37° for 5 min, and the mixtures contained 0.6 mM NADPH and 3 mg microsomal protein/ml. The concentration of EB was 3.53 mM (●) or 0.294 mM (○). Each point is the mean ± S.D. of four experiments.

Kinetic parameters of EB deethylation with microsomes and isolated hepatocytes. Various concentrations of EB were incubated with microsomes and isolated hepatocytes, and the amount of SAM formed was determined. K_m and V_{max} in the Michaelis–Menten equation were determined by an iterative nonlinear least squares method using a computer [2]. The apparent K_m values obtained from microsomes* and isolated hepatocytes were corrected for nonspecific binding to microsomes and isolated hepatocytes by factors of 0.65 [2] and 0.9, respectively. The V_{max} values were obtained in units of nmoles of SAM formed per min per 167 mg wet liver* and per 10^6 cells for microsomes and hepatocytes, respectively. In order to compare the V_{max} values of the two systems, both values were converted to μmoles of SAM formed per min per g liver, and are listed in Table 1.

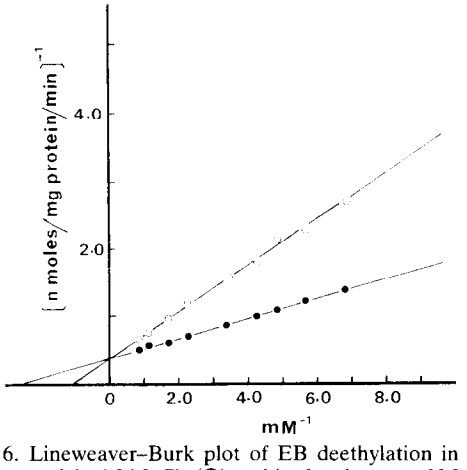

Fig. 6. Lineweaver–Burk plot of EB deethylation in the presence of 6 mM $MgCl_2$ (●) and in the absence of $MgCl_2$ (○). This shows the results of one experiment typical of three.

* The values of K_m and V_{max} in microsomes were taken from Table 4 of a preceding paper [2], where the V_{max} value is for 1.7 ml of the microsomal suspension that was obtained from 167 mg liver. As 32.5 mg microsomal protein was obtained from 1 g liver, 1.7 ml of the microsomal suspension contained 5.4 mg microsomal protein. Since the microsomal concentration of 3.2 mg/ml (= 5.4 mg/1.7 ml) almost passes the criterion for the assay condition of EB deethylation in microsomes (see text), the preceding values were taken for the comparison with the values in hepatocytes.

Table 1. Kinetic parameters of ethoxybenzamide deethylation in rat microsomes and isolated hepatocytes*

	V_{max} [μmoles·min^{-1}·g liver^{-1}]	K_m (mM)	N
Microsomes†	0.124 ± 0.003	0.378 ± 0.017	6
Hepatocytes‡	0.0863 ± 0.0163	0.459 ± 0.045	4

* Data are expressed as means ± S.D. and N denotes the number of experiments.

† Values are taken from Table 4 of a preceding paper [2]. The K_m value is corrected for nonspecific binding of EB to microsomes and is the same as the reported value. V_{max} is corrected for per gramm liver as follows. The microsomal protein obtained was 32.5 mg from 1 g of liver at that time, and the content of P-450 was 0.66 nmole P-450/mg microsomal protein as reported in the present Materials and Methods section. The content of cytochrome P-450 was estimated as 33.8 nmoles/g liver from the reported values [7–11]. Then the yield of the microsomes from liver was calculated as $0.66 \times 32.5/33.8 = 0.635$. Since the previous V_{max} value [2] was for the microsomal suspension that was obtained from 167 mg liver, the V_{max} value for 1 g of liver was calculated as $0.0131 \times 0.167^{-1} \times 0.635^{-1} = 0.124$ [μmoles·min^{-1}·(g liver)$^{-1}$].

‡ V_{max} value obtained for 10^6 cells is corrected per g liver, using the average content of hepatocytes (1.25×10^8 cells/g liver) [12, 12]. The K_m value is corrected for nonspecific binding of EB to the hepatocytes by multiplying by a factor of 0.9 (reported in the text).

DISCUSSION

Figure 1 indicates that the oxygen supply from the air or that available in the cells was sufficient for cytochrome P-450-linked drug monooxygenation. It is interesting that the ratios of conjugates to total SAM were high at low EB concentration (Fig. 1 A and B), but low at high EB concentration [Fig. 1 (C and D)]. This is not unreasonable, since the saturation of SAM conjugation *in vivo* has been reported by Levy and Matsuzawa [14].

It is noteworthy that the concentration of endogenous NADPH in rat liver has been reported to be 0.3–0.5 mM [15–17] and that the concentration of exogenous NADPH required for the maximal EB deethylation activity in damaged hepatocytes and microsomes is the same. Lehninger [18] showed that pyridine nucleotides cannot permeate into mitochondria, and Orrenius *et al.* [19] used pyridine nucleotide exclusion as a criterion to determine the viability of isolated hepatocytes. The small effect of exogenous NADPH on EB deethylation by isolated intact hepatocytes confirms that the isolated hepatocytes prepared in our laboratory possess intact plasma membranes. By comparing the maximal EB deethylation activity in damaged hepatocytes with that in intact hepatocytes, it is clear that NADPH generation from endogenous substrates is sufficient to support optimal deethylation in the intact hepatocytes. This is in agreement with the results of Moldéus *et al.* [20]. The enzyme activity in cell homogenates was significantly lower than in intact and damaged hepatocytes at every concentration of NADPH (Fig. 3). This may be the result of the homogenation procedure destroying the enzyme partially, or unmasking or activating inhibitors (in mitochondria or nuclei [21]) or pyrophosphatase (which destroys NADPH [22, 23]).

Peters and Fouts [24] studied the effect of Mg^{2+} on hepatic microsomal drug metabolism, classifying the substrates into three groups: (1) no effect on, or a decrease in, enzyme activity with increased concentration of Mg^{2+}; (2) increased enzyme activity with increasing Mg^{2+} concentration; and (3) peak enzyme activity at 6 mM Mg^{2+} or less. The Mg^{2+} concentration at the peak rate of EB metabolism is very close to that for aniline and benzphetamine metabolism [24], which belong to the third group. But Mg^{2+} did not change V_{max}, and decreased K_m, for EB (Fig. 6). These results contrast with those observed with aniline (increase in V_{max}, but no change in K_m) and benzphetamine (increase in both V_{max} and K_m) [25]. The reason for these differences is not clear. The endogenous Mg^{2+} concentration in rat liver has been reported to be 8 mM [26]. It is interesting that the optimal Mg^{2+} concentration for maximal EB deethylation activity with microsomes (about 6 mM), is in the range of the physiological Mg^{2+} concentration.

Adjustment of the cofactors NADPH and Mg^{2+} in microsomal deethylation to give optimum conditions, which are close to physiological conditions, and appropriate correction of the apparent kinetic parameters for nonspecific binding and microsomal yield resulted in rather good agreement between the kinetic parameters of microsomes and isolated hepatocytes. A lower V_{max} value, however, was observed in the isolated hepatocytes. For this reason, the estimate of the cell content in a gram of liver may be recalculated. Moldéus *et al.* [20] reported that the cellular concentration of microsomal protein is 0.33 mg/10^6 cells. Using this value and the value of 51 mg of microsomal protein/g liver (32.5 mg/0.635 = 51.2; see legend for Table 1), the number of cells per gram liver was calculated to be 1.55×10^8 cells. Then, the V_{max} value in hepatocytes per g liver can be recalculated as 0.107 [μmoles·min^{-1}·g liver)$^{-1}$], which is closer to the V_{max} value in microsomes.

Since Moldéus *et al.* [20] demonstrated that isolated hepatocytes are very useful for drug metabolism studies, some attempts have been made to

compare drug metabolism in subcellular liver fractions with that in hepatocytes. The apparent Michaelis constants for alprenolol in hepatic microsomes and in 9000 *g* supernatant fractions are 25 and 17 μM respectively [27], which are in reasonable accordance with that found in isolated hepatocytes—10 μM [28]. The apparent Michaelis constant for ethylmorphine, however, is significantly higher in microsomes (250 μM) than in isolated hepatocytes (50 μM) [29]. Furthermore, the apparent Michaelis constant for antipyrine in microsomes, 22.0 mM [30], is different from that in isolated hepatocytes, 1.4 mM [31]. The discrepancy between the kinetic parameters in microsomes and those in hepatocytes may be due, in part, to the experimental conditions, which were not optimal, and to other factors that affect the estimation of kinetic parameters.

Recently, in addition to drug metabolism linked to cytochrome P-450, an excellent correlation between drug glucuronidation in native microsomes and in isolated hepatocytes was reported by Andersson *et al.* [32].

The results of the present study on deethylation, as well as the investigation of Moldéus *et al.* described above, indicate that drug metabolism in microsomes may correlate well quantitatively with that in isolated hepatocytes, at least for some drugs, if optimal experimental conditions can be established, and if other factors which affect the estimation of kinetic parameters are considered.

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