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THE RELATIONSHIP BETWEEN BINDING TO CYTOCHROME P-450 AND METABOLISM OF *n*-ALKYL CARBAMATES IN ISOLATED RAT HEPATOCYTES

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Abstract—The binding constants of an homologous series of *n*-alkyl (C_2–C_{10}) carbamates ($CH_3(CH_2)_nOCNH_2$) to the cytochrome P-450 of suspensions of isolated, viable rat hepatocytes have been measured. All the carbamates except ethyl and propyl carbamate produced type I difference spectra and their binding affinities ($1/K_s$) were found to be directly dependent upon their lipophilicity. These binding affinities were similar to those determined in rat liver microsomes. Maximum development of the binding spectrum in hepatocytes was always within one second of the addition of each carbamate, indicating that for these carbamates membrane permeability was not rate limiting for access to, and metabolism by, cytochrome P-450 and that much of the cells' cytochrome P-450 was unoccupied by endogenous substrates. The major metabolites of C_4–C_8 carbamates were unconjugated ω-1 oxidation products. Below hexyl carbamate only the ω-1 hydroxylated metabolite was observed but for the more lipophilic carbamates the keto metabolite was also a major product. The same products were found in blood after i.p. dosing of rats with hexyl carbamate. A direct relationship was observed between the affinity constant of the carbamate for cytochrome P-450 and the total rate of oxidative metabolism in the ω-1 position. Hydrolysis of the carbamate group was a minor metabolic pathway in contrast to the *in vivo* situation.

Binding of xenobiotics to hepatic microsomal cytochrome P-450 typically produces a type I spectral change which is thought to be due to the formation of a complex between the substrate and the apoprotein of cytochrome P-450 [1]. For a structurally related series of compounds the affinity of type I binding is usually directly related to lipophilicity of the substrate [2]. In hepatic microsomes, in the absence of added exogenous substrates, it has been shown that at temperatures of 20–36° between 28 and 35% of the cytochrome P-450 is available for low to high spin transition [3–5].

Type I binding spectra have also been demonstrated in suspensions of freshly isolated rat hepatocytes [6–8]. Von Bahr *et al.* [7] have shown that the binding of hexobarbitone to cytochrome P-450 in isolated hepatocytes does not require energy, hexobarbitone reaching the binding site by simple diffusion. The rate of binding was dependent upon temperature and the concentration of hexobarbitone. Maximum binding occurred within one second of the addition of hexobarbitone. Using three other barbiturates with a very narrow range of oil/water partition coefficient (1.84–7.62) they also showed the rate of appearance of the (type 1) spectrum was proportional to the partition coefficient, however they could find no correlation between partition coefficient and binding constant, K_s.

Type I spectral changes in liver microsomes appear to correlate with Michaelis constants for the oxidation of a number of substrates [1–9]. However, the correlation of K_s with metabolism rate has been reported for the barbiturates and various *O* and *N* alkylated compounds [10–12] to be very poor. Such studies have generally been performed using compounds covering a rather narrow range of partition coefficients (usually less than ten-fold) and none have involved an homologous series. The relationship between K_s and K_m does not appear to have been examined in intact cells or *in vivo*.

The homologous aliphatic carbamate series C_2–C_8 has provided a useful model for examining the influence of lipophilicity on drug absorption [13, 14] distribution and metabolism [15] and binding [16] because it covers over a thousand-fold range of partition coefficients and is free of ionizable group and major hydrogen bonding effects. In the present paper this series of compounds is used to examine the relationship between binding to cytochrome P-450 and metabolism in isolated adult rat hepatocytes.

MATERIALS AND METHODS

Chemicals. Methyl (Koch-Light Laboratories Ltd., Bucks, U.K.), ethyl (BDH Ltd., Poole, U.K.), *n*-propyl (Kodak Ltd., Liverpool, U.K.) and *n*-butyl (Eastman Ltd., Rochester, NY) carbamates were obtained commercially. The remaining carbamates

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including the radio-labelled carbamates were synthesized in the Chemistry Division, C.D.E. Porton Down. All were shown to be pure by gas–liquid chromatography (GLC), thin layer chromatography (TLC) and melting point determinations [12].

Partition coefficients. These were determined in 0.1 M phosphate buffer pH 7.4 *n*-octan-1-ol at 37°.

Cell preparations. Male Wistar albino rats weighing about 80 g and allowed food and water *ad lib.* were pretreated with sodium phenobarbitone, 80 mg/kg daily for 3 days, by i.p. injection. Hepatocytes were isolated by incubation of liver slices with 0.05% w/v collagenase and 0.1% w/v hyaluronidase [17]. The cells were washed and resuspended in Dulbecco's phosphate buffered saline, PBSA, without phenol red but supplemented with 100 mg% D-glucose (i.e., 5.56 mM).

Binding spectra. To obtain binding spectra *n*-alkyl carbamates dissolved in *N,N*-dimethylformamide were added to one of a matched pair of 1 cm^2 cuvettes each of which contained 2.5 ml of a suspension of hepatocytes 2×10^6 cells per ml (final concn) in PBSA incorporating 2.5% w/v gelatin powder (BDH Ltd., Poole, U.K.) and 100 mg % glucose. Carbamate solutions were made up in *N,N*-dimethyl formamide such that the addition of 1 μl to a solution of 2.5 ml cell suspension produced a concentration equal to that of the dissociation constant, K_s, of each carbamate as determined by Al-Gailany *et al.* [2] for the interaction of these carbamates with cytochrome P-450 in rat liver microsomes. Spectral measurements were made at 37° in a Varian Cary 219 Spectrophotometer. Following the addition of the carbamate solution; cell suspensions were mixed in the cuvettes using a piston-type rapid mixing device and the absorption difference measured at 390 nm to determine the time taken for its maximum development. The spectrum was then scanned between 370 and 450 nm. The suspension was mixed upon each addition of carbamate solution and before each scan both to ensure adequate oxygenation of the suspension and to produce an even distribution of cells in the suspension. No noticeable sedimentation of cells occurred during the scans.

Spectral changes were measured from the peak to trough of the type I spectra, i.e. $\Delta A_{390\text{–}420nm}$, and a Lineweaver–Burk plot made of substrate concentration, [*S*], versus spectral change, from which the dissociation constant, K_s, and maximum spectral change, ΔA_{max}, were determined:

$$\frac{1}{\Delta A} = \frac{1}{[S]} \cdot \frac{K_s}{\Delta A_{max}} + \frac{1}{\Delta A_{max}}$$

To examine the effect of anaerobicity, suspensions were bubbled with O_2-free N_2 for 2 min then sealed under Parafilm. Cell viability was checked using trypan blue [17] at the end of each study. In each case cell viability was above 80–85%.

Metabolism studies. In order to study carbamate metabolism, hepatocytes (2×10^6 per ml, final concentration) contained in a conical flask (10 ml) were suspended in one ml of Liebovitz L15 medium + 10% foetal calf serum (FCS). Radiolabelled carbamate (0.5 mM and 1.5 μCi/μmole) was added and the preparation incubated in a shaking water bath at 37°. The reaction was stopped at various times by cooling on ice. The medium was subjected to TLC after separating the hepatocytes by centrifugation but without further purification.

In some experiments in which the radiolabelled carbon dioxide produced by the preparation was measured, incubations were carried out as above but in a sealed Warburg flask. The central well contained 10% KOH to trap $^{14}CO_2$ and hepatocytes were destroyed at set intervals by injection of 1 ml methanol.

Where cell viability was checked at the end of each experiment prior to methanol addition typically it did not drop below 85–90%. Because it was found very difficult to obtain sufficient amounts of the major metabolites for isolation and identification using hepatocyte suspensions, *n*-hexyl carbamate was administered *in vivo* and metabolites isolated from plasma. Comparisons were then made with *in vitro* produced metabolites. The *in vivo* metabolites of *n*-hexyl [^{14}C]carbamate were identified by dosing adult male Wistar albino Porton strain rats i.p. with this compound dissolved in polyethylene glycol 300 at a dose of 0.7 mmole/mg. After 3 hr the rats were exsanguinated from the abdominal aorta under diethyl ether anaesthesia. The blood was immediately heparinized and centrifuged and the plasma fraction separated and shaken with four changes of 2 vol. of diethyl ether. The pooled ether extracts were dried with anhydrous sodium sulphate and subjected to rotary evaporation at 20° and 260 mm Hg. The residue was applied to a TLC plate and run in chloroform: butan-1-ol (9:1 v/v). Radioactive spots were identified by radiochromatogram scanning, scraped off and re-run in ethyl acetate:methanol (7:3 v/v). Spots were then eluted with diethyl ether, dried under a stream of N_2 and stored dessicated under nitrogen at −20°. Chemical ionization mass spectra were recorded on a V.G. Micromass 7070F mass spectrometer using isobutane as the ionizing gas; inlet temperature 50–60°; source temperature 200°; source pressure 10^{-4} Torr; ionization voltage 70 eV; acceleration voltage 4000V.

RESULTS

In common with the findings in rat liver microsomes, ethyl and propyl carbamate did not give a binding spectrum in hepatocytes. All the other carbamates gave a typical type 1 spectrum (Fig. 1) and showed a linear relationship between the reciprocals of carbamate concentration and absorption difference between 390 and 420 nm (Fig. 2). The maximum binding spectrum always developed within 1 sec of the addition of each carbamate (the time taken to add the carbamate, mix and measure the absorption spectrum). The affinity of binding ($1/K_s$) was linearly related to partition coefficient from *n*-butyl to *n*-octyl carbamate but *n*-decyl carbamate had a lower than expected affinity coefficient (Table 1).

Studies on the nature of the metabolites appearing in the blood after the i.p. administration of hexyl carbamate showed that the predominant metabolites were not conjugated. TLC demonstrated the presence of two major metabolites.

The fragmentation patterns of the mass spectra of

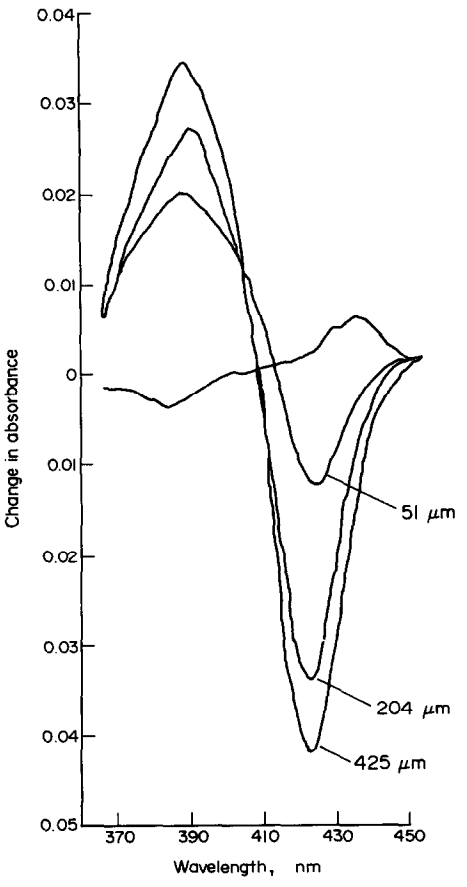

Fig. 1. Type 1 difference spectrum obtained on addition of various concentrations of *n*-heptyl carbamate to a suspension of rat hepatocytes.

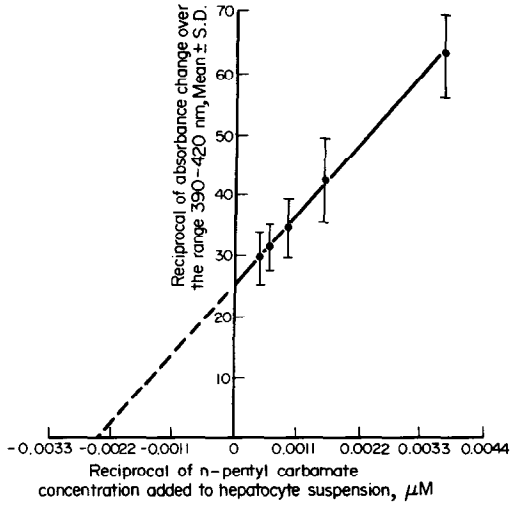

Fig. 2. A Lineweaver–Burk plot to determine the dissociation constant and maximum spectral change for the interaction of *n*-pentyl carbamate with cytochrome P-450 in isolated rat hepatocytes.

the isolated metabolites were consistent with the two major metabolites being the (ω-1) hydroxylated product and its further oxidation product, the ketone. Thus the following mass peaks were observed.

Suspected (ω-1) hydroxy metabolite
m/e 162: (molecular ion)
m/e 144: (indicating loss of water from M^+)
m/e 101: indicating breaking ester link to give $CH_3CH{\cdot}OH(CH_2)_4^+$
m/e 43: CH_3CO^+.

Suspected (ω-1) ketone metabolite
m/e 160: (molecular ion)
m/e 99: indicating breaking of ester link to product $CH_3CO{\cdot}(CH_2)_4^+$
m/e 43: (indicating CH_3CO^+).

Close similiarity between TLC patterns in various solvent systems indicated that the *in vivo* and *in vitro* metabolites of hexyl carbamate were the same. A number of very minor metabolites were apparent

Table 1. Type 1 binding spectra of various aliphatic carbamates to rat hepatocytes

Carbamate	Partition coefficient	Microsomal K_s (μM)	Hepatocyte K_s* as a fraction of microsomal $\frac{\Delta A_{max390-420\ nm}}{K_s}$	Absolute hepatocyte K_s (μM)	$\Delta A_{max390-420\ nm}$	*n*
Ethyl	0.7	ND	ND	ND	ND	4
n-Propyl	2.3	ND	ND	ND	ND	4
n-Butyl	7	1900	0.80 ± 0.94	1520 ± 1786	0.032 ± 0.008	4
n-Pentyl	23	930	0.47 ± 0.15	437 ± 140	0.041 ± 0.008	3
n-Hexyl	70	500	0.44 ± 0.26	220 ± 130	0.041 ± 0.012	4
n-Heptyl	250	170	0.26 ± 0.13	44.2 ± 22.1	0.054 ± 0.028	4
n-Octyl	700	71	0.25 ± 0.14	17.8 ± 9.9	0.052 ± 0.007	4
n-Decyl	7000	35	0.71 ± 0.25	24.8 ± 8.8	0.046 ± 0.010	3

Hepatocytes were isolated from phenobarbitone pretreated rats.
Microsomal data from Ref. 2.
Parition coefficients between octanol and phosphate buffer pH 7.4 taken from Ref. 13.
* Calculated from the ratio, hepatocyte K_s: microsomal K_s.
ND, not detectable.

on the thin layer chromatograms of the more lipophilic carbamates in addition to the two major (ω-1) oxidative metabolites. These metabolites have yet to be identified. It was apparent from the TLC profiles of the major metabolites of the other carbamates that ω-1 hydroxylation was the predominant fate of carbamates above C4.

In the two cases in which detectable levels of a ketone metabolite appeared (i.e., *n*-hexyl and *n*-octyl carbamates) the amounts of this metabolite in proportion to those of the hydroxy metabolite increased with increasing lipophilicity of the parent compound. However for a particular homologue this proportion remained constant with time.

Small amounts of radiolabelled carbon dioxide were produced with time from each carbamate through the hydrolysis of the [^{14}C]carbamate group (date not shown). The more lipophilic carbamates tended to show the greater hydrolysis, the maximum conversion being approximately 5% over 4 hr.

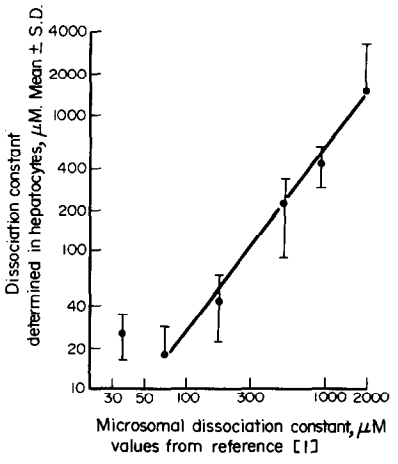

Fig. 3. The relation between the dissociation constants of *n*-alkyl carbamates interacting with cytochrome P-450 in hepatic microsomes and in isolated hepatocytes. The equation of the line is: $\ln y = \ln y_0 + m.\ln x$, where $y_0 = 0.050\,\mu M$; $m = 1.35$; correlation coefficient = 0.9970. The outlier, *n*-decyl carbamate, has been omitted from the above regression analysis.

DISCUSSION

Measurement of cytochrome P-450 binding spectra in hepatocytes provides a very useful direct indication of the uptake of xenobiotics by hepatocytes and permits an assessment of the *in vivo* relevance of the results of microsomal cytochrome P-450 binding spectra investigation. The binding constants determined in the rat hepatocytes were within an order of magnitude (i.e. 0.1–1.0) of those found in rat liver microsomes (Table 1). The higher than expected K_s for *n*-decyl carbamate may be due to its being bound by membrane lipids thereby limiting its accessibility to cytochrome P-450. (It should be noted that the maximum aqueous solubility of *n*-decyl carbamate is 10.5 μM, calculated from the linear regression correlation between the number of carbon atoms in the *n*-alkyl side chain and the logarithm of the aqueous solubility.) In all cases the spectra were completely developed within one second of the addition of substrate. It can be concluded from this that in hepatocytes, membrane permeability does not limit the rate of access of these compounds to cytochrome P-450. The formation of a type 1 binding spectrum upon the addition of the carbamates and the close relationship between K_s values determined in hepatocytes and those obtained in liver microsomes from the same species (Fig. 3) demonstrates that a significant proportion of the cellular cytochrome P-450 is free of endogenous substrate, implying either that a particular form of cytochrome P-450 exists predominantly for exogenous substrates or that in the hepatocyte preparation used endogenous substrates, e.g. cholesterol, steroids, fatty acids, were sufficiently well bound to limit their access to cytochrome P-450.

Hydrophobic bonding appeared to be the main driving force for P-450 binding (Table 2). In contrast to previous findings [6–8] with less lipophilic substrates, the type 1 binding spectrum formed by the carbamates were persistent for many minutes under either aerobic or anaerobic conditions after the addition of 100 μM *n*-octyl carbamate, despite the fact that metabolism of octyl carbamate was linear with time for up to 1 hr. The maximum amounts of substrate metabolised in one hour was less than 33% of the initial substrate concentration. This indicates that the extent of cytochrome P-450 saturation under the experimental conditions used here is not rate limiting for metabolism. Only the carbamates which formed type 1 binding spectra with cytochrome P-450 formed ω-1 oxidation products. The presence of a stable type 1 binding spectrum indicates that the rate limiting step in the oxidation of the carbamates

Table 2. Concentration of metabolites of *n*-alkyl carbamates in the cell suspension medium after incubation with rat hepatocytes for one hour*

Carbamate	Metabolite concentration (μM) Ketone	Hydroxy	Total	Ratio [ketone]:[hydroxy]
Ethyl	0	0	0	—
n-Propyl	0	17.5 ± 6.3	17.5 ± 6.3	—
n-Butyl	0	41.3 ± 13.8	41.3 ± 13.8	—
n-Pentyl	0	47.4 ± 15.9	47.4 ± 15.9	—
n-Hexyl	28.1 ± 10.4	66.3 ± 23.7	94.4 ± 30.6	0.42 ± 0.19
n-Octyl	63.5 ± 26.4	62.6 ± 19.0	126.1 ± 45.8	1.01 ± 0.48

* Results are expressed as means ± S.D. of four experiments.

is at a stage beyond the binding of substrate to cytochrome P-450. Previous work has demonstrated that the concentration of NADPH in hepatocytes is not normally rate limiting for metabolism [18, 19]. It is probable therefore that the rate limiting step for oxidation of these substrates by hepatocytes was at the level of the reductases [20].

The predominant metabolic fate of all of the carbamates which formed type 1 binding spectra with cytochrome P-450 was ω-1 oxidation. Trace amounts of other metabolites of the aliphatic chain were detected but little conjugation was observed despite the fact that hepatocytes prepared in an identical manner are active in glucuronidation, sulphation and glutathione conjugation [18, 19, 21, 22]. ω-1 Oxidation products are also found as major metabolites of the aliphatic carbamates *in vivo* [15]; however, in contrast to the *in vivo* situation [15] in hepatocytes only low levels of $^{14}CO_2$ were formed. This may imply that unlike ω-1 oxidation of carbamates, hydrolysis is almost entirely an extrahepatic metabolic reaction.

The more lipophilic carbamates formed an ω-1 ketone as well as an ω-1 hydroxylated product (Table 2). The proportion of ketone to hydroxylated metabolite was constant with time in each case, thus it is likely that the ketone is formed from the hydroxylated metabolite by a non-cytochrome P-450 dependant reaction and that the initial ω-1 hydroxylation is the rate limiting step for each carbamate. Whether the form of cytochrome P-450 involved in the oxidation of carbamates is the same as that concerned with ω-1 oxidation of laurate and other fatty acids remains to be established.

The total rate of oxidative metabolism increased proportionally with both increasing substrate lipophilicity and with cytochrome P-450 binding constant, but the rate of metabolism did not appear to be related with $\Delta A_{max390-420nm}$. Failure of previous workers to show a clear relationship between K_s and rate of metabolism [10], may be attributable, at least in part, to the fact that the compounds they studied were either not a homologous series or covered too limited a range of K_s values or contained basic groups or partial sterically hindered sites of metabolic attack.

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