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APPARENT MICHAELIS CONSTANTS FOR THE METABOLISM OF [UREYL-^{14}C]TOLBUTAMIDE BY HUMAN LIVER MICROSOMAL PREPARATIONS

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Abstract—A method is described for the measurement of the amount of 1-butyl-3-*p*-hydroxymethylphenylsulphonyl-[^{14}C]-urea (hydroxy-[ureyl-^{14}C]tolbutamide) formed *in vitro* from [ureyl-^{14}C]tolbutamide. Very low rates of metabolism can be assayed accurately. K_m and V_{max} have been determined for the metabolism of [ureyl-^{14}C]-tolbutamide by microsomal preparations from human liver obtained (a) postmortem; (b) as operation biopsy material; (c) from tissue frozen for 1 week at −20°. Frozen microsomal pellets have also been investigated. No significant differences have been found between the Michaelis constants for the four types of preparation, although the values reported are generally lower than those in the literature for the metabolism of other drugs by human liver preparations. Pentobarbitone inhibits the rate of metabolism *in vitro* of [ureyl-^{14}C]tolbutamide in a manner neither fully competitive nor fully non-competitive.

IN A PREVIOUS paper,[1] we described attempts we had made to measure the rates of metabolism of aniline, codeine and hexobarbitone by microsomal preparations from human livers obtained post-mortem. The sensitivity of the methods used was low. In this paper, the use of radioactive tolbutamide is shown to be a way of obtaining information about the kinetics of drug-metabolism even when rates of metabolism are very low. This is particularly important when the quantity of tissue available is small—for example, with human liver biopsy material. Kuntzman, Ikeda, Jacobson and Conney[2] have described the use of [2-^{14}C]pentobarbitone for kinetic determinations with rat liver microsomal preparations. Daly[3] has described the use of [4-^{3}H]acetanilide for kinetic determinations with rat liver microsomal preparations.

The employment of radioactive drugs eliminates the problem of correcting for high amounts of endogenous material in tissue preparations. For example the colorimetric assay of formaldehyde—commonly used to follow the rates of metabolism of ethylmorphine, codeine, aminopyrine and several other drugs—is subject to a large error when the rates of metabolism are low. In addition, the error which may result from measuring the rates of disappearance of substrate—for example in following low rates of metabolism of hexobarbitone—is eliminated.

MATERIALS AND METHODS

Materials

All common reagents were of AnalaR grade. Glucose-6-phosphate (disodium salt) and $NADP^+$ were purchased from Boehringer Corporation, London, W5. Glucose-6-phosphate dehydrogenase was purchased from Sigma (London) Limited, Lettice

Street, London NW3. [Ureyl-^{14}C]tolbutamide was a gift from Farbwerke Hoechst A/G., Frankfurt/Main, Germany.

It was found to be necessary to remove from the [ureyl-^{14}C]tolbutamide a small proportion of radioactive impurity with properties similar to those of the hydroxylated metabolite. This was done by suspending the radioactive tolbutamide in water and extracting the bulk of it with successive portions of heptane. The impurity was almost completely left in the aqueous layer. Slow evaporation of the heptane layers to dryness resulted in the recovery of the [ureyl-^{14}C]tolbutamide as fine white needles. Thin layer chromatography on commercial silica gel plates (E. Merck, G.m.b.H., Darmstadt, Germany) showed only one radioactive and u.v.-light absorbing spot; the solvent[4] was chloroform–methanol 95:5 v/v, and the R_f value found was 0·71. A solution of the tolbutamide was made up in the stoichiometric volume of 0·1 M NaOH so that after dilution with water, 0·2 ml contained 1·0 μmole (3·75 μc).

Human liver samples obtained from cases of sudden death were packed in ice as soon as possible after death (2½–4 hr in all cases), and taken to the laboratory for processing. Some were frozen at −20° for 2 weeks before thawing for use. Human liver operation biopsy samples were placed immediately after excision in ice-cold buffered (pH 7·4) 1·15% KCl which was 0·005 M with respect to potassium phosphate. They were taken to the laboratory for processing 6–14 min later.

METHODS

Preparation of microsomal fractions. Biopsy samples were weighed (0·4–1·4 g) and homogenized in the ice-cold buffered KCl (4 ml/g tissue) using a PTFE/glass Potter–Elvehjem homogenizer. Homogenates were centrifuged at 10,000 *g* for 20 min at 4°. The supernatants were decanted leaving behind a fluffy layer as well as the mitochondrial and cell-debris pellet, and the supernatants were centrifuged at 105,000 *g* for 60 min. The resulting red gelatinous pellets were resuspended in the ice-cold buffered KCl so 1 ml contained 80–140 mg wet liver equivalent. Liver samples obtained post-mortem were treated as described by Darby *et al.*[1] except that some samples of tissue were homogenized using an Ultra–Turrax homogenizer (Janke u. Kunkel A/G., Stauffen i. Br., Germany; Scientific Instrument Centre, Ltd., Leeke Street, London WC1), so that 25 g of tissue were dispersed in 100 ml of buffered KCl. The pellets formed by the final high-speed centrifugation of the homogenates were always resuspended so that 1 ml of buffered KCl contained 800 mg wet liver equivalent. Mitchard[5] has carried out a comparative study of homogenizations performed using the Potter homogenizer, the Ultra-Turrax machine, or the MSE top-drive macerator.

Incubation mixtures contained, in a final volume of 2·5 ml, KH_2PO_4 (150 μmole); MgSO4 (10 μmole); NADPH-generating mixture ($NADP^+$ 0·5 μmole; glucose-6-phosphate 8 μmole; glucose-6-phosphate dehydrogenase 1·8 units); microsomal suspension 0·5 ml: and [ureyl-^{14}C]tolbutamide (1·0 μmole, or 0·375–1·0 μmole in the kinetic experiments), added last. Incubations were carried out in 50 ml centrifuge tubes at 37° with shaking at 150 oscillations per min. Appropriate recovery flasks were run simultaneously, the [ureyl-^{14}C]tolbutamide being added at the end of the incubation period.

Measurement of 1-*butyl*-3-(*p-hydroxymethylphenyl*)*sulphonyl*-[^{14}C]-*urea* (*hydroxy* [*ureyl*-^{14}C]*tolbutamide*). The method of Tagg *et al.*[6] has been extensively modified by us. Enzyme activity was stopped by the addition of 2 ml of ice-cold 1·0 M sodium

acetate solution pH 5·0 to the 50 ml centrifuge tubes. Ten ml of petroleum spirit (boiling range 40–60°) 1·5% v/v with respect to 3-methylbutan-1-ol were added next, and the mixtures were extracted by vortex-mixing for 15 sec. The tubes were centrifuged at 2000 rev/min for 10 min and the top layers discarded. The extractions with petroleum spirit were repeated three times, the top layers being discarded every time. Finally, two extractions were made with 10 ml of ethyl acetate, the two top layers being pooled after centrifugation. The pooled ethyl acetate extracts were taken to dryness at 50° under a stream of nitrogen, and the residues dissolved in 5·0 ml of ethyl acetate. Radioactivity in aliquots of the ethyl acetate solutions was measured using a liquid scintillation counter (Packard 3320 model) following the addition of 10 ml of Bray's dioxan-based solution.[7] No quench corrections were necessary. The counting rates obtained with recovery flasks were subtracted from those obtained as a result of incubation for the appropriate time, before calculation of molar yields. The radioactivity extracted by ethyl acetate from the recovery flasks routinely represented 0·15 per cent or less of the radioactivity added to the flasks. Not less than 95 per cent of hydroxylated metabolite was routinely recovered in the ethyl acetate extracts. This was shown in the following way: an incubation similar to the one described above but on a large scale was carried out using a rat-liver microsomal preparation of high drug-metabolizing activity. To the resulting ethyl acetate extract were added 30 mg of authentic non-radioactive hydroxytolbutamide (gift of Hoechst Pharmaceuticals Ltd., Brentford, Middlesex, England). The extract was evaporated to a small volume and the whole streaked on to a 20 × 20 cm thin-layer chromatography plate (E. Merck, G.m.b.H., Darmstadt, Germany). After developing[4] with chloroform–methanol (95:5, v/v) the areas (R_f 0·34) corresponding to the metabolite were scraped off and eluted with ethyl acetate. The ethyl acetate was evaporated leaving radioactive metabolite. After recrystallization to constant specific activity, a known weight was carried through a typical incubation and extraction procedure.

Microsomal protein was estimated according to the method of Lowry *et al.*,[8] with bovine serum albumin as standard.

Experimental results

Figure 1 demonstrates that the rate of hydroxylation of [ureyl-^{14}C]tolbutamide by hepatic microsomal preparations obtained from a human biopsy sample (female) is approximately linear for only the first 15 min. All incubations were carried out for this length of time, which had also been found to be the optimum for microsomal preparations from rat liver.

The rate of hydroxylation is proportional to the amount of microsomal protein present, as Fig. 2 shows for a microsomal preparation from a second human liver biopsy preparation (female).

While an incubation with a fixed amount of [ureyl-^{14}C]tolbutamide for a fixed time allows a measurement of the rate of metabolite formation per unit weight of tissue or tissue-protein, there is no way of knowing if the linearities demonstrated in Figs. 1 and 2 will hold in every experiment. One of the advantages of carrying out Michaelis–Menten kinetic experiments, even though only simply constructed, is that departure from the linear relationships of Figs. 1 and 2 will be apparent from the departure from linearity of Lineweaver–Burk plots. The apparent Michaelis parameters K_m and V_{max}[9] obtained may be used quite reliably for comparative purposes,

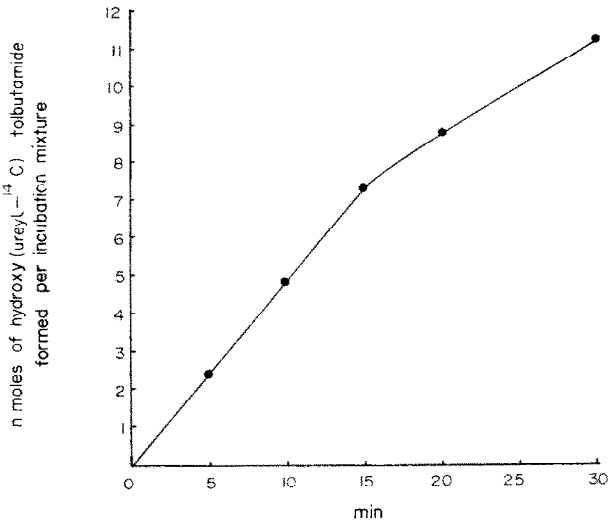

FIG. 1. Amount of hydroxy [ureyl-^{14}C]tolbutamide formed in different periods of time from 1 μmole of [ureyl-^{14}C]tolbutamide by aliquots of a microsomal preparation from a biopsy sample of human liver (female). The incubation mixture, 2·5 ml pH 7·4, contained $MgSO_4$, potassium phosphate, NADPH-generating mixture, [ureyl-^{14}C]tolbutamide and microsomal suspension equivalent to 72 mg liver (1·43 mg protein; 0·5 ml).

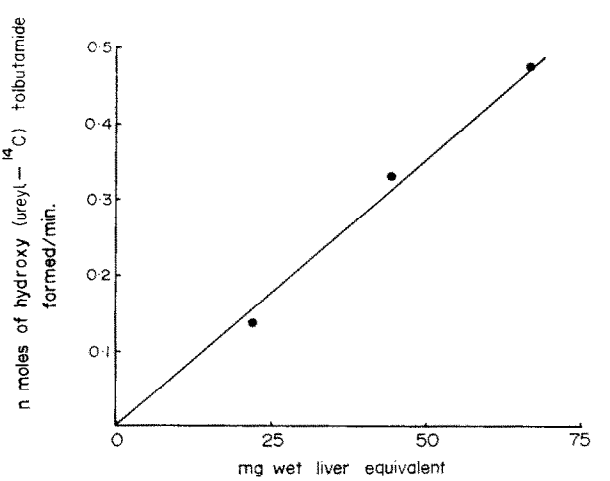

FIG. 2. Rate of formation of hydroxy [ureyl-^{14}C]tolbutamide from 1 μmole of [ureyl-^{14}C]tolbutamide by different quantities of a microsomal suspension prepared from a biopsy sample of human liver (female). The incubation mixture pH 7·4 contained $MgSO_4$, potassium phosphate, NADPH-generating mixture, [ureyl-^{14}C]tolbutamide and microsomal suspension (44·4 mg wet liver equivalent/ml; 1·14 mg protein/ml).

subject only to uncertainty about the correct units in which to express rates of metabolism. The yield of microsomal membrane fragments from a given weight of tissue varies according to the homogenization and centrifugation procedure used and according to the treatment of the tissue beforehand. Reliable estimates of microsomal protein content cannot be obtained from samples of liver obtained post-mortem. Values ranging from 9 to 17 mg protein/g wet liver have been found by us. Biopsy samples are subject to error in the extent to which tissue taken from the outer edge of a lobe of liver includes capsular tissue, connective tissue and fatty material. Values of 14·8–23·6 mg protein/g wet liver have been found by us.

With the rates of metabolism expressed in terms of nmoles of tolbutamide metabolized/min/mg microsomal protein, Fig. 3 illustrates typical Lineweaver–Burk graphical presentations of the relationship between [ureyl-^{14}C]tolbutamide concentration and the rate of the metabolism of the drug *in vitro* by different human hepatic microsomal preparations obtained from a (male) biopsy sample, from a male post-mortem, from a tissue sample frozen at −20° for 1 week post-mortem (male), and from microsomal pellets frozen for 2 weeks at −20° after preparation from tissue obtained post-mortem (male). Straight lines were fitted to the experimentally obtained points by a standard "least squares" regression calculation. Riggs[10] has criticized the use of the least squares method for this purpose on the grounds that the reciprocals of the lowest concentrations of substrates in a wide range are subject to a very large error. The range of substrate concentrations used here is very narrow, in fact, and the fit of the straight lines by the method of least squares is valid.

Table 1 summarizes the values for the Michaelis parameters obtained with human liver samples, and includes for comparison, some figures obtained using microsomes made from rat livers processed immediately after killing by the procedure of Darby.[11] The rats were of the Wistar strain (Scientific Products Farm, Ash, Kent, England), and weighed 200–400 g.

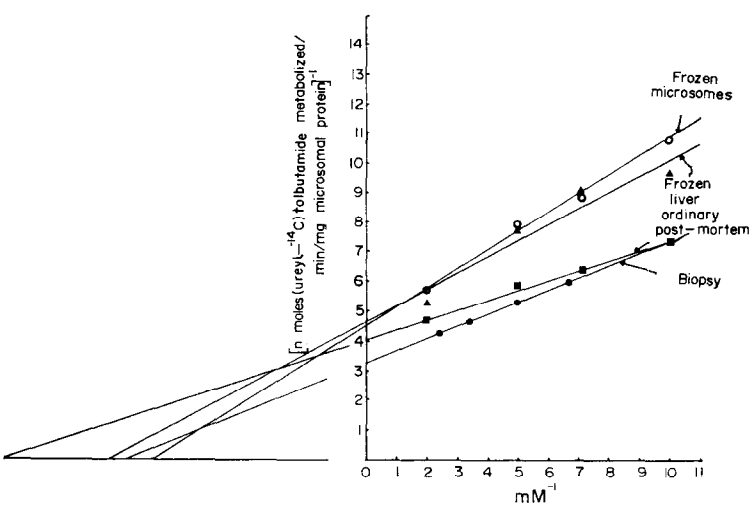

FIG. 3. Lineweaver–Burk plots of the reciprocals of the [ureyl-^{14}C]tolbutamide concentrations vs. the corresponding reciprocals of the rates of formation of hydroxy [ureyl-^{14}C]tolbutamide by different male human liver microsomal preparations. ● biopsy ($P < 0{\cdot}001$). ■ ordinary post-mortem ($P < 0{\cdot}01$). ▲ frozen liver ($P < 0{\cdot}05$). ○ frozen microsome pellet ($P < 0{\cdot}01$).

TABLE 1. APPARENT MICHAELIS PARAMETERS FOR THE METABOLISM *in vitro* OF [UREYL-^{14}C]TOLBUTAMIDE BY MICROSOMAL PREPARATIONS FROM HUMAN LIVER AND RAT LIVER

Source of tissue	Sex	K_m (mM)	V_{max} (nmoles of hydroxy-[ureyl-^{14}C]-tolbutamide formed/min/mg microsomal protein)
Human biopsy	Male	0·13	0·31
	Male	0·09	0·27
	Female	0·03	0·29
	*Male	0·13	0·40
Human post-mortem			
Fresh	Male	0·12 ± 0·02 $n = 5$	0·21 ± 0·03 $n = 5$
Frozen liver	Male	0·19 ± 0·03 $n = 3$	0·20 ± 0·03 $n = 3$
Frozen microsomes	Male	0·19 ± 0·03 $n = 3$	0·26 ± 0·03 $n = 3$
Rat	Male	0·50 ± 0·06 $n = 8$	1·15 ± 0·16 $n = 8$
	Female	0·53 ± 0·10 $n = 5$	0·53 ± 0·08 $n = 5$

* This patient had been treated for some time with phenobarbitone.
n = number of different tissue samples tested. Results, where appropriate, are expressed as means ± S.E.M.
K_m and V_{max} were not statistically significantly different when the respective values found with microsomal preparations from livers obtained fresh, postmortem, were compared with those found with frozen liver samples, or with frozen microsomal pellets. Comparison with the values found with microsomal preparations from rat liver (male or female) invariably showed significant or highly significant differences.

Figure 4 demonstrates that pentobarbitone at a concentration of 1·0 mM inhibits the conversion by human liver microsomal preparations *in vitro* of [ureyl-^{14}C]tolbutamide to its hydroxylated metabolite. The pattern, which could be duplicated, is consistent with that commonly described as 'mixed inhibition'—neither fully competitive nor fully non-competitive.[9]

We have not been able to substantiate our earlier report[1] that cytochrome P-450 decays rapidly in suspensions of microsomal pellets made from human liver samples obtained post-mortem. However, we could not positively detect any cytochrome P-450 in the microsomal preparations described here as originating from human livers obtained post-mortem. Pelkonen *et al.*[12] were unable to detect cytochrome P-450 in human foetal liver microsomal preparations obtained post-mortem. Microsomal preparations originating from biopsy samples contained cytochrome P-450 which we found did not decay as a result of standing the microsomal suspension at 0° for 1½ hr. The concentrations (39–55 nmoles/100 mg microsomal protein) were approximately twice those reported by Alvares *et al.*[13] and Ackerman,[14] but approximately half those reported by Thorgeirsson and Davies.[15] Activities of NADPH-cytochrome *c* reductase have been found by us to be much the same whether microsomal prepara-

tions were derived from biopsy samples or from liver samples obtained post-mortem. The activities ranged from 96 to 179 nmoles of cytochrome *c* reduced/min/mg microsomal protein, and were in good agreement with the values reported by Ackermann.[14]

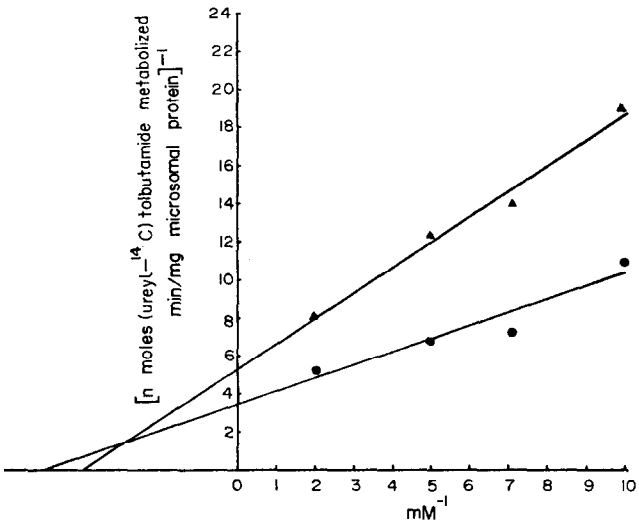

FIG. 4. Lineweaver–Burk plots of the reciprocals of the [ureyl-^{14}C]tolbutamide concentrations vs. the corresponding reciprocals of the rates of formation of hydroxy [ureyl-^{14}C]tolbutamide for a microsomal preparation from an ordinary liver sample obtained from a male human post-mortem, in the presence and absence of 1×10^{-3} M pentobarbitone sodium (Nembutal). ● no inhibitor ($P < 0{\cdot}05$). ▲ 1×10^{-3} M pentobarbitone ($P < 0{\cdot}01$).

DISCUSSION

The values for the apparent Michaelis constants for the rate of metabolism of [ureyl-^{14}C]tolbutamide by microsomal preparations from human liver (Table 1) are similar whether the liver sample was biopsy material or obtained fresh post-mortem, or frozen after obtaining post-mortem. This suggests that all the microsomal pellets isolated represented comparable types of fragments of the endoplasmic reticulum of the cells even if the protein contents varied considerably according to the method of homogenization and centrifugation and the treatment of the tissue beforehand. Freezing microsomal pellets did not significantly alter the apparent Michaelis constants for the metabolism of [ureyl-^{14}C]tolbutamide, and this may be of use in estimating the capacity of the livers of patients undergoing drug therapy to metabolize drugs at different times in the period of treatment. Levin *et al.*[16] have reported that microsomal pellets made from rat liver and frozen at −25° for 20 days retain all their capacity to metabolize pentobarbitone.

Comparison of the values in Table 1 with those reported for the metabolism of chlorpromazine[12] (human foetal liver samples; K_m 0·077 mM; V_{max} 0·29 μmoles of chlorpromazine metabolized per g tissue per hr), and ethylmorphine[15] (K_m 0·8–4·0 mM; V_{max} 2·0–8·8 nmoles/min/mg microsomal protein), codeine[14] (male human liver biopsy samples: K_m 15 mM; V_{max} 5·7 μmoles/min/g protein), and aminophenazone[14] (male human liver biopsy samples: K_m 6·1 mM; V_{max} 3·7 μmoles/min/g microsomal protein) shows that K_m and V_{max} for tolbutamide are, in general, much lower. Alvares *et al.*[13]

have reported K_m of 0·02 mM for the hydroxylation of 3,4-benzpyrene by 9000 *g* supernatant made from human liver.

The inhibition by pentobarbitone of tolbutamide metabolism illustrated in Fig. 4 is closely paralleled by that described by Ackermann,[14] who used SKF-525A to inhibit the metabolism of aminophenazone *in vitro* by human liver microsomal preparations. Such inhibition patterns may be a useful guide in choosing suitable combinations of drugs in therapy. Only small samples of tissue are needed if radioactive drugs are tested *in vitro* with microsomal preparations.

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