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The in-vitro metabolism of [^{14}C]pentobarbitone and [^{14}C]phenobarbitone by hamster liver microsomes

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The metabolism of [^{14}C]pentobarbitone and [^{14}C]phenobarbitone has been reinvestigated using an in-vitro hepatic microsomal system (Syrian hamsters, Aroclor 1254 induction). The incubation system was routinely supplemented with EDTA (1 mM) and a substrate concentration study revealed the metabolism of [^{14}C]pentobarbitone to be concentration-dependent, with the greatest overall metabolism (>50%) occurring at 0·054 μmol per 3·5 mL. With [^{14}C]phenobarbitone as substrate, overall metabolism was extremely low (3%) and independent of substrate concentration. Addition of further cofactors to the incubation mixture at 20 min intervals over an extended period resulted in almost complete metabolism of [^{14}C]pentobarbitone (100 min), 3′-hydroxypentobarbitone and 3′-oxopentobarbitone being identified as metabolites together with many minor, unidentified products. With [^{14}C]phenobarbitone as the substrate, cofactor addition up to 120 min resulted in 8% overall metabolism; *p*-hydroxyphenobarbitone was identified as a product of metabolism; other minor products were unidentified. The metabolism studies failed to produce a metabolite having the properties of the *N*-hydroxylated product of either [^{14}C]pentobarbitone or [^{14}C]phenobarbitone within the detection limits available (0·02% of 0·5 μmol per incubate).

Barbiturate drugs have been widely used medicinally as hypnotics and sedatives since early this century, although their use is now being curtailed due to unwanted side-effects of tolerance and their abuse. The reports of Tang et al (1975, 1977a, b) that barbiturates were metabolized in man in-vivo to the corresponding *N*-hydroxy metabolites, and that amobarbitone underwent *N*-hydroxylation in-vitro using dog liver 9000*g* and microsomal fractions (Reilly & Kadar 1977), aroused our interest and prompted us to re-examine the in-vitro metabolism of certain barbiturates. *N*-Oxidation of barbiturates gives rise to the formation of cyclic hydroxamic acids. Similarly, 2-acetylaminofluorene (AAF) and other amides are metabolized to hydroxamic acid products which are associated with increased toxicity and carcinogenicity compared with the parent amides (Cramer et al 1960; Miller et al 1964; Miller 1970). Therefore, from a toxicological viewpoint we felt it necessary to confirm whether a similar reaction occurs in the metabolism of barbiturates in-vitro.

Subsequent studies by Tang et al (1978, 1979) revealed that the previously supposed *N*-hydroxylation product of amobarbitone and phenobarbitone was the *N*-glucoside. Amobarbitone *N*-glucoside is formed in man in-vivo and in-vitro but not with other species (Tang & Carro-Ciampi 1980; Tang et al 1980). More recently, Carro-Ciampi et al (1985) have shown the formation of the *N*-glucoside of amobarbitone in the cat both in-vivo and in-vitro.

In the light of the above we now report our findings on the in-vitro metabolism of pentobarbitone and phenobarbitone using ^{14}C-labelled substrate to enable detection of minor metabolic products. A preliminary account of this work has been published (Seago & Gorrod 1985).

MATERIALS AND METHODS

Phenobarbitone (5-ethyl-5-phenyl barbituric acid) was purchased from May and Baker Ltd, Dagenham, UK, and pentobarbitone (5-ethyl-5-(1-methylbutyl) barbituric acid) was purchased from Sigma Chemical Company Ltd, Poole, UK. The reference compounds *p*-hydroxyphenobarbitone, *m*-hydroxyphenobarbitone and phenylethylmalondiamide were donated by Dr J. Caldwell, St Mary's Hospital Medical School, London, UK; 3′-hydroxypentobarbitone, 3′-oxopentobarbitone and the terminal oxidation product 5-ethyl-5-(1′-methyl-3′-carboxypropyl) barbituric acid were gifts from Dr A. C. Moffat, Home Office Research Establishment, Aldermaston, UK; *N*-hydroxyphenobarbitone and *N*-hydroxypentobarbitone were prepared from the alkyl substituted malonyl chloride and

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benzyloxyurea using the method of Safir et al (1953) as described by Seago (1982), the *N*-hydroxy product was produced by hydrogenation of the *N*-benzyloxybarbiturate in the presence of platinum oxide; [2-^{14}C]phenobarbitone (50 μCi mL^{-1}; 23 mCi mmol^{-1}) was purchased from Amersham International plc, UK, and [2-^{14}C]pentobarbitone sodium (50 μCi/0·5 mL, 37 mCi mmol^{-1}) was purchased from Laboratory Impex Ltd, Twickenham, UK. Lumagel liquid scintillant was obtained from LKB Instruments, Selsdon, UK. X-Ray film, DC-80 developer and FX-40 X-ray liquid fixer were from Kodak Ltd, Swallowdale, UK. All other chemicals and biochemicals were used as commercially available.

Chromatography

Thin-layer chromatography (TLC) was carried out on silica gel 60F_{254} (0·2 mm, thick 20 × 20 cm) precoated plastic plates purchased from E. Merck, Darmstadt, Germany. Chromatograms were routinely developed in benzene–acetic acid–methanol (88:10:2) (System A). *n*-Octanol, saturated with water (System B), was used as the second system in two-dimensional chromatography to separate *N*-hydroxypentobarbitone from 3′-oxopentobarbitone.

Animals

Male Syrian hamsters, 80–100 g, were obtained from Olac, Oxon, UK and fed on diet FFGM (E. Dixon and Sons Ltd, Ware, UK). The animals were deprived of food on the night before tissue preparation but had free access to water.

In-vitro metabolism

Washed hepatic microsomes were prepared using the method of Gorrod et al (1975) with isotonic Tris/KCl buffer pH 7·4 at 0–4 °C. Protein was assayed according to Lowry et al as adapted by Miller (1959) using bovine serum albumin (Grade V) as standard. Microsomal cytochrome P450 content was assayed by the method of Omura & Sato (1964). Substrate (0·054–5·0 μmol; 2·25 μCi) was incubated in stoppered 25 mL Ehrlenmeyer flasks containing hepatic washed microsomes (1·0 mL equivalent to 0·5 g liver) and a cofactor solution of nicotinamide adenine dinucleotide phosphate (NADP; 2 μmol), glucose-6-phosphate (G-6-P; 10 μmol), glucose-6-phosphate dehydrogenase (G-6-P-D; 1 unit) and $MgCl_2$ (50% v/v solution, 20 μmol) in 2·5 mL phosphate buffer (0·01 M; pH 7·4). Cofactors and substrate were incubated at 37 °C for 5 min before the addition of the washed microsomes; incubations were routinely for 60 min. In experiments where additional cofactors were used at 20 min intervals they were added in a final volume of 50 μL buffer.

Determination of metabolite

Metabolic activity was terminated by placing the flasks on ice and addition of the extraction solvent. Unreacted substrate and metabolites were extracted with ethyl acetate (3 × 4 mL) after addition of sodium chloride (1 g). The combined organic extract was reduced to dryness under nitrogen at 45 °C, the residue taken up in ethanol (25 μL), and an aliquot (10 μL) applied to a TLC plate which was run against authentic standards. ^{14}C-Activity was detected by exposing the chromatogram to X-ray film for 5 days, which on development revealed the radioactivity as dark areas which were traced on to the original chromatogram, then the silica was removed, eluted with methanol and subjected to liquid scintillation counting. The ^{14}C-activity in each area was calculated as a percentage of total activity. Scintillation counting with methanol and silica in the presence of a known amount of ^{14}C-activity confirmed that with the quantities routinely involved in these experiments no quenching effect was observed.

Determination of material in aqueous residue

An accurately measured amount (0·5 mL) of the aqueous phase was transferred to a low-potassium glass vial and to this was added 20 mL of scintillant (Scintran Cocktail T, BDH) for ^{14}C activity determination.

Determination of material in tissue pellet

The tissue pellet, with as little aqueous phase as possible, was transferred to a low-potassium glass vial and digested with Soluene (Packard, 2·0 mL), at 40 °C. The resulting solution was bleached with hydrogen peroxide solution (1·0 mL; 100 volume); excess hydrogen peroxide was removed with ascorbic acid solution (15%, 0·5 mL) before the addition of Lumagel scintillant (8 mL) for radioactivity determination.

RESULTS

To ensure complete extraction of ^{14}C-activity into the organic phase, aliquots of the aqueous residue and tissue pellet were taken and the ^{14}C-content determined. For both substrates less than 1% ^{14}C-activity was detected in either the aqueous phase or tissue residue samples, and for subsequent experiments the extraction into the organic phase was considered to be complete.

Table 1. The effect of substrate concentration in the in-vitro metabolism of phenobarbitone by Syrian hamster liver microsomes.

R_F*	Substrate concentration (μ mol/3·5 mL) 0·097	0·18	0·5	1·0	5·0
0·03	0·25	0·46†			
		0·22	0·23	0·11	ND‡
0·08	0·73	0·63			
		0·50	0·56	0·63	0·36
0·15	0·21	0·18			
		0·18	0·20	0·29	0·17
0·26	0·42	0·32			
		0·43	0·48	0·55	0·21
0·30	1·24	0·97			
		0·84	1·05	0·76	0·29
0·36	96·91	97·30			
		97·61	97·14	97·43	98·88
0·52	0·23	0·09			
		0·06	0·08	0·06	ND
0·55	ND	0·07			
		0·07	0·16	0·16	0·10

* Radioactive compounds detected using solvent system A expressed as a percentage of the radioactivity present.
† The results are shown from two experiments carried out on separate occasions with different tissue preparations.
‡ ND = not detected.

Table 2. The effect of substrate concentration on the in-vitro metabolism of pentobarbitone by Syrian hamster liver microsomes.

R_F*	Substrate concentration (μ mol/3·5 mL) 0·054	0·10	0·50	1·0	5·0
0·02	0·96	0·88†			
		0·37	0·31	0·23	0·34
0·08	49·23	38·31			
		21·05	16·37	14·16	3·97
0·14	0·85	0·71			
		0·42	0·41	0·28	0·13
0·16	0·89	0·70			
		0·25	0·22	0·23	0·11
0·24	—	—			
		0·43	0·51	0·44	0·27
0·27	0·67	1·15			
		0·50	0·35	0·72	0·43
0·40	47·40	58·25			
		76·98	81·85	83·94	94·75

* Radioactive compounds detected using solvent system A expressed as a percentage of the radioactivity present.
† The results are shown for two experiments carried out on separate occasions with different tissue preparations.

Using a microsomal incubation system supplemented with EDTA (1 mM), the effect of varying substrate concentration from 0·054 μmol (pentobarbitone) or 0·097 μmol (phenobarbitone) to 5·0 μmol/3·5 ml was investigated. For [^{14}C] phenobarbitone the extent of microsomal metabolism was very low (~3%) and independent of substrate concentration (Table 1). With [^{14}C]pentobarbitone, decreasing the substrate concentration resulted in an increase in total metabolism (Table 2). At 0·054 μmol, less than 50% of the substrate remained (60 min at 37 °C), forming a major metabolite of R_F 0·08 (System A; co-chromatographing with authentic 3′-hydroxypentobarbitone, R_F = 0·09). Although this very low substrate concentration gave the highest metabolic conversion, 0·5 μmol was routinely used in the current studies in order that larger quantities of metabolites might be available for identification.

To increase further the overall microsomal metabolism of [^{14}C]pentobarbitone and [^{14}C]phenobarbitone, male Syrian hamsters were routinely pretreated with Aroclor 1254, a general inducer of cytochrome P450 (Alvares et al 1973). Cofactors were added to the incubation mixture at 20 min intervals up to 100 min ([^{14}C]pentobarbitone) or 120 min ([^{14}C]phenobarbitone), with samples taken at intermediate time points. The results obtained for [^{14}C]pentobarbitone are shown in Table 3. Incubations for periods longer than 20 min, with no supplementation, resulted in no further increase in metabolism. If, at 20 min, and 20 min intervals thereafter, cofactors were added to the incubation mixture, metabolism continued so that by 100 min less than 1% of the substrate remained (Table 3). The amount of the major metabolite (R_F 0·09 3′-hydroxypentobarbitone) increased initially, but after 40 min this product decreased concomitant with an increase in a metabolite at R_F 0·19 (R_F of authentic 3′-oxopentobarbitone and *N*-hydroxypentobarbitone). At 100 min this product represented 9·84% of the total radioactivity extracted; there was also present a third metabolite (2·56%) with R_F 0·15 (5-ethyl-5-(1′-methyl-3′-carboxy-propyl)barbituric acid). To elucidate further the nature of the [^{14}C]pentobarbitone metabolites, the 100 min incubation extract was subjected to two-way chromatography (System A then B). By comparison of the R_F values of metabolites with authentic material run in the same system, the major metabolite was shown to be 3′-hydroxypentobarbitone and the second metabolite 3′-oxopentabarbitone. No *N*-hydroxypentobarbitone could be detected using this method (limit of detection: 0·2% of 0·5 μmol per incubate).

Incubation of [^{14}C]phenobarbitone with the supplemented microsomal system still only resulted in 8% metabolism over 120 min (Table 4). After TLC separation (System A) some ^{14}C-activity (1·60%)

Table 3. Effect of adding further cofactors on the in-vitro metabolism of pentobarbitone, (0·5 μmol/3·5 mL), using Aroclor-induced Syrian hamster liver microsomes.

	Incubation time (min)								
R_F*	20	40	60	80	100	40†	60‡	80§	100¶
0·02	0·72	0·73	1·04	0·69	0·99	1·66	2·18	2·92	2·71
0·09	64·09	59·18	63·30	58·41	60·51	84·54	87·66	85·34	82·78
0·15	0·80	0·73	0·92	0·67	0·77	1·63	2·01	2·21	2·56
0·19	1·00	0·94	0·92	0·84	0·94	3·01	5·31	7·29	9·84
0·23	0·29	0·28	0·36	0·26	0·48	0·64	0·69	0·65	0·83
0·28	0·09	1·13	0·86	1·05	1·44	0·78	0·61	0·75	0·75
0·42	32·21	37·01	32·60	38·08	34·86	7·73	1·53	0·81	0·53

* Radioactive compounds detected using solvent system A, expressed as a percentage of the radioactivity present.
† Cofactors added at 0, 20 and terminated at 40 min.
‡ Cofactors added at 0, 20, 40 and terminated at 60 min.
§ Cofactors added at 0, 20, 40, 60 and terminated at 80 min.
¶ Cofactors added at 0, 20, 40, 60, 80 and terminated at 100 min.

Table 4. The effect of adding further cofactors on the in-vitro metabolism of phenobarbitone (0·5 μmol/3·5 mL), using Aroclor-induced Syrian hamster liver microsomes.

	Incubation time (min)		
R_F*	80†	100‡	120§
0·01	0·56	0·82	1·60
0·09	2·46	3·07	3·15
0·23	0·67	0·88	0·94
0·27	2·79	0·91	2·18
0·32	93·53	94·32	92·14

* Radioactive compounds detected using solvent system A, expressed as a percentage of the radioactivity present.
† Cofactors added at 0, 20, 40, 60 and terminated at 80 min.
‡ Cofactors added at 0, 20, 40, 60, 80 and terminated at 100 min.
§ Cofactors added at 0, 20, 40, 60, 80, 100 and terminated at 120 min.

remained on the origin, indicating the formation of highly polar material. Radiolabel was associated with products of R_F 0·09 (identical to *m*-hydroxyphenobarbitone and phenylethylmalondiamide, 3·15%) and at R_F 0·27 (*p*-hydroxyphenobarbitone, 2·20%). There was also material containing ^{14}C-activity at R_F 0·23. No *N*-hydroxyphenobarbitone (R_F 0·12) could be detected (limit of detection 0·02% of 0·5 μmol per incubate).

DISCUSSION

Jacobson et al (1973) have previously shown that addition of EDTA (1 mM) to rat hepatic microsomal preparations decreases lipid peroxidation and the breakdown of cytochrome P450 resulting in an increase in overall metabolism of pentobarbitone. Using [^{14}C]pentobarbitone we have confirmed this increase in microsomal metabolism in the presence of EDTA (1 mM) (Seago 1982); thus EDTA has been routinely incorporated for these studies.

The present results reveal the metabolism of [^{14}C]pentobarbitone to be concentration-dependent in accord with previously published studies on substrate inhibition of microsomal metabolism. Daly et al (1967) showed that amphetamine (5 μmol), on incubation with rat liver microsomes yielded only 0·002% *p*-hydroxyamphetamine. Reduction of the substrate concentration to 0·2 μmol (Jonsson 1974) resulted in 3% of *p*-hydroxyamphetamine being formed; in the same study, 1 μmol amphetamine caused complete inhibition of microsomal metabolism. In this respect it is of interest that both barbiturates and amphetamines which so clearly demonstrate excess substrate inhibition are also known to strongly bind with cytochrome P450. Thus, Franklin (1974) showed that amphetamine produced a type II spectra, whereas it has long been known that barbiturates form type I spectra (Remmer et al 1966; Schenkman et al 1967). It would be of interest to establish whether the rates of metabolism of other compounds which bind strongly to cytochrome P450 and yet are substrates for the monooxygenase system, are similarly affected. Jacobson et al (1973) showed microsomal metabolism of pentobarbitone to occur within the concentration range 0·5-0·75 μmol, and for our studies 0·5 μmol/3·5 mL was selected as the substrate concentration routinely used.

The addition of cofactors at timed intervals as first reported by Jacobson et al (1973) has also been investigated as a means of increasing metabolism. For [^{14}C]pentobarbitone, this resulted in complete metabolism of substrate (0·5 μmol), with 3′-hydroxy-

pentobarbitone and 3′-oxopentobarbitone being identified as metabolites from their TLC characteristics, compared with authentic material. The terminal acid, 5-ethyl-5-(1′-methyl-3′-carboxypropyl)barbituric acid may also be formed as a minor metabolite as a compound was detected at R_F 0·15 which was similar in value to the authentic acid (0·13). However, the conversion of alcohol to acid would require the participation of aldehyde oxidase, an enzyme predominantly present in cell cytoplasm; positive identification of this minor metabolite at R_F 0·15 was not possible. After two-dimensional TLC of the 100 min incubation extract, as many as 16 radioactive areas were located on the autoradiogram, only three of which have been identified. Thus, areas of radioactivity located after single dimension TLC may be composed of one or more compounds, or the component(s) may be unstable during the process of two-dimensional TLC. None of the compounds detected ran at an R_F similar to synthetic *N*-hydroxypentobarbitone. Our findings for the metabolism of [^{14}C]pentobarbitone in-vitro correlate well with previously published reports. 3′-Hydroxypentobarbitone has been shown to be the major metabolite of pentobarbitone in-vitro (Cooper & Brodie 1957; Kuntzman et al 1966). The (−)-isomer of 3′-hydroxypentobarbitone is further metabolized by 100 000*g* soluble fraction supplemented with NAD and NADPH to a product thought to be the ketone (Kuntzman et al 1967). These workers also established that the metabolism of pentobarbitone was linear for only a short time (Kuntzman et al 1967), all metabolism having ceased by 60 min incubation (Cooper & Brodie 1957). The terminal acid has not previously been reported as an in-vitro metabolite but as a urinary metabolite in man (Baldeo et al 1980), horses (Nicholson 1968) and rats (Titus & Weiss 1955). If formed in our system, further oxidation of the terminal acid could lead to shortening of the side-chain resulting in the formation of several other metabolites, the ultimate stage being the complete removal of one C_5 substituent, as detected in the metabolism of barbitone (5,5-diethyl barbituric acid) in-vivo (Goldschmidt & Wehr 1957; Ebert et al 1964). In addition, ω-hydroxylation of the ethyl side-chain has been shown to be a route of metabolism for phenobarbitone (Harvey et al 1972) and barbitone (Goldschmidt & Wehr 1957) in-vivo. Metabolic ring scission has been postulated for barbiturates (Williams 1959). In agreement with this [^{14}C] pentobarbitone has been shown to give small amounts of labelled urea as a metabolite (Titus & Weiss 1955) in-vivo. Al Sharifi et al (1983) detected small amounts of 4′-hydroxypentobarbitone as a human urinary metabolite.

Much lower overall metabolism of [^{14}C]phenobarbitone (compared with pentobarbitone) has been found in our studies. The substrate concentration study revealed metabolism to be concentration independent and to proceed at a very low rate. However, by supplementing the in-vitro system with cofactors and incubating for a prolonged period (120 min), 8% metabolism occurred. This is in contrast to studies of Peters et al (1973) and Crayford & Hutson (1980) who failed to detect any metabolism of phenobarbitone in-vitro. Interestingly, *p*-hydroxylation of phenobarbitone has been observed in-vitro using immobilized microsomal enzymes trapped in polyvinylpyrrolidone (PVP) (Denti et al 1976). In this case one can imagine that the PVP acted as a barrier, effectively reducing the substrate concentration at the enzyme active centre. The metabolites detected in this in-vitro study have previously been reported as metabolic products of phenobarbitone in-vivo (Seago 1982).

The results obtained from these studies clearly show [^{14}C]pentobarbitone and [^{14}C]phenobarbitone to be metabolized by the hepatic microsomal system to a variety of products. The level of formation of these metabolites is, in general, very low and dependent on substrate concentration. Further positive identification of the metabolites for [^{14}C]phenobarbitone and [^{14}C]pentobarbitone by removal of areas from TLC, elution and analysis by mass spectrometry was not possible due to the very low concentrations involved.

The lack of *N*-hydroxylation of either substrate would appear to confirm the findings of Tang et al (1978, 1979), since the *N*-hydroxy metabolite was later shown to be the *N*-glucoside and is of interest as hydroxamic acids often have diverse pharmacological and toxicological properties (Buu-Hoi et al 1970; Miller 1970) including mutagenicity. In separate experiments synthetic *N*-hydroxypentobarbitone and *N*-hydroxyphenobarbitone were examined for mutagenicity against *Salmonella typhimurium* TA 98 and TA 100 using the method of McCann et al (1975) but omitting the microsomal activating system (10–1000 μg plate). These compounds were negative at the concentrations used against either organism (Seago 1982). However as hydroxamic acids generally require a second activating step (Miller 1970), the mutagenic activity of these compounds may not have been able to be expressed in our system. Similarly incubates of phenobarbitone with aroclor-induced hamster liver microsomes subsequently

assayed against *S. typhimurium* failed to show the metabolic generation of mutagenic material (Seago, unpublished results).

We therefore conclude that phenobarbitone and pentobarbitone are not converted to *N*-hydroxy derivatives in-vitro and that this type of cyclic hydroxamic acid is not a primary mutagen.

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