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RELATIONSHIP BETWEEN DIFFERENCE SPECTRA AND METABOLISM

BARBITURATES, DRUG INTERACTION AND SPECIES DIFFERENCE

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Abstract—The characteristics of difference spectra induced by a series of barbiturates in a rat liver microsomal system are presented. This data is related to the structures, duration of pharmacological action and metabolic fate of the compounds.

The inhibition of the pharmacological action of tremorine and the potentiation of hexobarbital sleeping time in mice by concommitant administration of SKF525A or "Inderal"* (propranolol) is also correlated with the characteristics of the difference spectra of these compounds.

A partial correlation between difference spectra and species differences in metabolism is also demonstrated.

THE INDUCTION of a difference spectrum by the addition of substrates to liver microsomal suspensions is considered to be indicative of binding of the substrate to the terminal cytochrome (P-450) of the microsomal electron transport chain.[1–2] This binding is thought to be essential for the transfer of electrons from the substrate to the cytochrome.[3] Two types of difference spectra have been observed.† The Spectral Dissociation constant K_s is regarded as a measure of the affinity of a substrate for cytochrome P-450. The size of the difference spectrum (i.e. Δ O.D. Max/mμmole P-450) may be considered to be a measure of the capacity of the binding site (i.e. number of molecules of substrate bound/molecule of cytochrome)[4] or the strength of the substrate–cytochrome bond. Using rat liver microsomes studies with a series of thiazole acetic acids permitted their classification into three groups (1) none-inducers of spectral change (2) Type I compounds (3) Type II compounds. Metabolic studies[5–6] showed that the non-inducers were not metabolised by the liver. The factors determining whether a Type I or Type II difference spectrum was formed appeared to be more subtle but in the case of the thiazole acetic acids it seemed that the nature of the substituent at the position susceptible to metabolic attack was of crucial importance. In order to broaden the understanding of the interpretation of difference spectra a series of barbiturates have been examined in the same rat liver system.

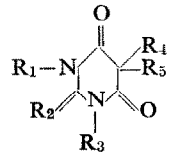
MATERIALS AND METHODS

Liver microsomal suspensions were prepared by the method of Kato and Gillette[7] from male outbred Alderley Park albino rats (200–250 g body wt.), male Alderley

* "Inderal" is a trademark, the property of Imperial Chemical Industries Ltd.

† Type I spectral change is characterised by a trough in the difference spectrum at 420 mμ and a peak at 385–390 mμ. Type II spectral change is characterised by a trough in the difference spectrum at about 385–390 mμ and a peak at about 420–425 mμ.

TABLE 1. DURATION OF ACTION, METABOLISM AND DIFFERENCE SPECTRA OF BARBITURATES IN RATS



Compound	Duration of action (Refs. 10, 11)	R_1	R_2	R_3	R_4	R_5	K_s $\times 10^{-3}$ M	ΔOD MAX ($\times 10^3$) /mg protein	Spectral type	Metabolism (Refs. 10, 11)
Barbital	Long	H	O	H	C_2H_5	C_2H_5	No difference spectrum			Minimal ω-oxidation and dealkylation of s.c.
Butabarbitone	Medium	H	O	H	C_2H_5	Sec. butyl	0·22	18	I	ω-oxidation of s.c.
Amylobarbitone	Medium	H	O	H	C_2H_5	3′ methyl butyl	0·04	25	I	(ω − 1) oxid. of s.c.
Pentabarbital	Medium	H	O	H	C_2H_5	1′ methyl butyl	0·075	19	I	ω oxid. of s.c.
Phenobarbital	Long	H	O	H	C_2H_5	phenyl	0·12	15	I	*p*-hydroxylation
5 ethyl-5-(1:2) cyclo-hexenyl) barbituric acid	Medium	H	O	H	C_2H_5	1:2 cyclo-hexenyl	0·65	11	I	Keto formation
5 methyl-5-(1:2) cyclo-hexenyl) barbituric acid	Medium	H	O	H	CH_3	1:2 cyclo-hexenyl	0·08	6	I	Keto formation
N-methylbarbital	Short	H	O	CH_3	C_2H_5	C_2H_5	No difference spectrum			*N*-demethylation
N,*N*′-dimethylbarbital	Short	CH_3	O	CH_3	C_2H_5	C_2H_5	No difference spectrum			*N*-demethylation
Hexobarbital	Ultra short	H	O	CH_3	CH_3	1:2 cyclo-hexenyl	0·09	25	I	ring hydroxylation keto formation, *N*-demethyl-ation, ring scission
Methyl-phenobarbitone	Short	H	O	CH_3	C_2H_5	phenyl	0·02	18	I	*p*-hydroxylation and *N*-demethylation
Thiobarbital	Ultra short	H	S	H	C_2H_5	C_2H_5	0·06	11	II	S-oxidation
Thiopentone, sodium	Ultra short	H	S	H	C_2H_5	1′ methyl butyl	0·04	18	I	S-oxidation and ω-oxidation of s.c.
Thiophenobarbitone	Ultra short	H	S	H	C_2H_5	phenyl	0·03	11	II	S-oxidation and *p*-hydroxylation
Thialbarbitone, sodium	Ultra short	H	S	H	allyl	2:3 cyclo-hexenyl	0·02	14	I	S-oxidation, keto formation and ring scission.

Park strain mice and a male Rhesus monkey, K_s and Δ OD. Max/mμmole P-450 values were determined as described by Schenkman *et al.*,[2] using microsomes resuspended in 0·1 M phosphate buffer pH 7·4. The cytochrome P-450 content of the microsomal suspension used was determined as described by Omura and Sato[8] and was found to be in the range 0·85–0·95 mμmoles/mg microsomal protein for rats. (Values for mouse and monkey were 0·57–0·75 and 1·55–1·72 mμmoles/mg protein respectively.) Protein determinations were carried out as described by Lowry *et al.*[9]

RESULTS

Difference spectra of barbiturates

The compounds selected for study were chosen to give a range of duration of action, chemical structures and variety of known metabolic products formed by enzymic attack on different parts of the molecule. The results are summarised in Table 1. No difference spectra were induced by barbital, *N*-methyl barbital and *N,N'*dimethyl barbital but if the size of one of the substituents on C_5 (R_5) of the barbital molecule was increased Type I difference spectra were induced. Increasing chain length of aliphatic substituents at C_5 resulted in a progressive decrease in K_s (e.g. K_s butabarbitone $= 0{\cdot}22 \times 10^{-3}$ M; K_s amylobarbitone $0{\cdot}04 \times 10^{-3}$ M). Substitution with phenyl or cyclohexenyl groups at C_5 also induced Type I difference spectra. Those oxybarbiturates which have a methyl group as the other substituent at C_5 (R_4) have lower K_s values than the corresponding ethyl substituted compounds [e.g. 5-ethyl-5-(1:2) cyclohexenyl) barbituric acid $K_s = 0{\cdot}65 \times 10^{-3}$ M; 5-methyl-5-(1:2 cyclohexenyl) barbituric acid $K_s = 0{\cdot}08 \times 10^{-3}$ M]. The replacement of oxygen by sulphur in the barbital molecule as the substituent at C_2 (R_2) resulted in the induction of a strong Type II difference spectrum. A similar Type II spectrum was induced by thiophenobarbitone (K_s $0{\cdot}03 \times 10^{-3}$ M) but Type I spectra were obtained from thiopentone and thialbarbitone.

Competitive inhibition studies. Delayed metabolism in rats and mice

(*a*) *Tremorine.* Additional evidence for the view that K_s values represent a measure of the relative affinity of substrates comes from inhibitor studies *in vivo*. It may be predicted that compounds with a high affinity (low K_s) for the liver drug metabolising enzymes will competitively inhibit *in vivo* the metabolism of compounds with a lower affinity (high K_s) if both compounds were able to achieve adequate penetration to the site of oxidation in the liver. A variety of compounds including desmethylimipramine, SKF 525A[12–13] and propranolol[14] delay the formation of pharmacologically active metabolites of tremorine in mice and rats and potentiate the activity of oxotremorine. Comparison of the K_s values of propranolol and some related compounds with their effectiveness as tremorine metabolic inhibitors in mice reveals that those compounds with K_s values at least two orders of magnitude lower than that of tremorine itself were most effective but compounds with K_s values of the same order were almost completely ineffective (Table 2).

(*b*) *Other drugs.* It has also been demonstrated in mice that the pharmacological effect of chloral hydrate,[15] chlorpromazine,[16] phenobarbital[16] and hexobarbital[16–18] are prolonged in the presence of propranolol (Table 2). Although this effect has been considered to be mediated through a synertistic effect at the C.N.S. site of action it is

TABLE 2. SPECTRAL CHARACTERISTICS IN MOUSE MICROSOMES AND INHIBITION OF TREMORINE ACTIVITY

Compound	Spectral type	Inhibition of tremorine pharmacological activity in mice (Ref. 14)	K_s (M)
Tremorine	I		$0{\cdot}4 \times 10^{-3}$
Oxotremorine			$\sim 1 \times 10^{-2}$
Propranolol	I	+ + +	$0{\cdot}1 \times 10^{-5}$
,, (+)	I	+ + +	$0{\cdot}6 \times 10^{-6}$
,, (−)	I	+ + +	$0{\cdot}1 \times 10^{-6}$
Pronethalol	I	+	$0{\cdot}8 \times 10^{-4}$
Practolol		±	$> 0{\cdot}25 \times 10^{-3}$
Chlorpromazine	I		$0{\cdot}34 \times 10^{-4}$
Hexobarbital	I		$0{\cdot}13 \times 10^{-3}$
Phenobarbital	I		$0{\cdot}2 \times 10^{-3}$
SKF525A	I		$0{\cdot}3 \times 10^{-7}$

possible that the potentiation observed may be partly due to inhibition by propranolol of liver oxidative metabolism to inactive derivatives.

An alternative complementary way of demonstrating drug interaction *in vivo* is to measure the blood levels of a compound with a high K_s in the presence and absence of another drug which would be expected to interfere with its metabolism by virtue of its lower K_s. Preliminary results with amidopyrine (Type I, $K_s = 0{\cdot}3 \times 10^{-3}$ M) and propranolol (Type I, $K_s = 0{\cdot}4 \times 10^{-6}$ M) in rats indicate that administration of propranolol 10 min before amidopyrine delays the formation of some metabolites of the latter by about 0·5–1·0 hr.

Species differences in the induction of difference spectra. The difference spectra induced by the addition of SKF525A, amidopyrine, propranolol and I.C.I. 54,450 (4-chloro-2-phenyl-thiazole-4-yl acetic acid) to liver microsomal suspensions from male rats, mice and a male rhesus monkey are given in Table 3. The spectra induced by aniline and amidopyrine were similar in all three species. The K_s of SKF525A in monkey

TABLE 3. CHARACTERISTICS OF DIFFERENCE SPECTRA INDUCED IN MICROSOMES OF DIFFERENT SPECIES

Compound	Species	Spectral type	K_s	ΔOD/mμmole P-450
SKF525A	Rat	I	$0{\cdot}6 \times 10^{-6}$ M	0·022
	Mouse	I	$0{\cdot}3 \times 10^{-7}$ M	0·010
	Monkey	I	$0{\cdot}45 \times 10^{-4}$ M	0·007
Aniline	Rat	II	$0{\cdot}67 \times 10^{-3}$ M	0·025
	Mouse	II	$0{\cdot}9 \times 10^{-3}$ M	0·030
	Monkey	II	$0{\cdot}5 \times 10^{-3}$ M	0·024
Amidopyrine	Rat	I	$0{\cdot}3 \times 10^{-3}$ M	0·013
	Mouse	I	$0{\cdot}2 \times 10^{-3}$ M	0·009
	Monkey	I	$0{\cdot}47 \times 10^{-3}$ M	0·005
Propranolol	Rat	I	$0{\cdot}4 \times 10^{-6}$ M	0·013
	Monkey	II (Poorly defined)	$0{\cdot}18 \times 10^{-2}$ M	0·033
I.C.I. 54,450	Rat	II	$0{\cdot}7 \times 10^{-3}$ M	0·005
	Monkey	II	$0{\cdot}5 \times 10^{-3}$ M	0·004

microsomes was as least two orders of magnitude greater than in the rat or mouse, I.C.I. 54,450 induced similar spectral changes in rat and monkey microsomes.

In the rat propranolol induced a Type I difference spectrum (K_s 0·4 $\times$ 10^{-6} M), but in monkey a poorly defined Type II difference spectrum was observed (K_s 0·18 $\times$ 10^{-2} M). Some other species differences between rat and mouse may be noted by comparison of Tables 1, 2 and 3.

DISCUSSION

Difference spectra and mode of metabolism

The relationship between difference spectra and metabolism is complex and it is doubtful if spectral data will ever be more than a guide to the metabolic fate of a compound *in vivo*. Other factors (e.g. absorption and excretion) also play an important part in determining the persistance and degree of metabolism of a compound. For example I.C.I. 54,450 induced similar spectral changes in rat and monkey microsomes but there are marked species differences in metabolism[5, 19] which may be due to inadequate penetration of the compound into monkey liver *in vivo*. On the other hand propranolol, which is metabolised differently by rat and monkey,[20] induced dissimilar microsomal changes in these two species. The relatively high K_s of SKF 525A in monkey microsomes suggests that in this species it is a poor metabolic inhibitor.

However, in consideration of a closely related group of compounds in one species (i.e. the barbiturates in the rat) it is possible to discern some correlation between mode of metabolism, chemical structure and difference spectra.

The correlation is clearer if one postulates that each substituent group has a characteristic Type I or Type II "directing" influence (e.g. sulphur at C_2 being "Type II directing" and cyclohexenyl at C_5 being "Type I directing"). The strength of this influence being dependent on the particular group but may be modified by its position in the molecule (e.g. 3'methyl butyl>sec. butyl$\gg$ethyl at C_5; sulphur > oxygen at C_2; l'methyl butyl at C_5 > sulphur at C_2 > phenyl at C_5). When two opposite directing groups are present in the same molecule the stronger group should determine which type of binding site (I or II) will be occupied and thus may dictate which part of the molecule will be preferentially subject to metabolic oxidation.

The major pathways of barbiturate metabolism are:[9]

(1) oxidation of the substituent at C_5
   (a) ω and (ω-1) oxidation of alkyl groups to the corresponding alcohol, carboxylic acid and keto derivatives.
   (b) *p*-hydroxylation of an aromatic ring.
   (c) formation of keto and hydroxy derivatives of cycloalkenyl groups.
(2) Replacement of sulphur by oxygen at C_2.
(3) *N*-dealkylation.
(4) Ring Scission (the enzyme responsible may not be located in the liver endoplasmic reticulum).

For all except the ultra short acting barbiturates, which are rapidly localised in fat, oxidative metabolic conversion prior to excretion and/or conjugation is of major importance in determining the duration of the pharmacological action *in vivo*.[4, 10] With the exception of the *N*-methyl barbiturates, which did not induce difference spectra but are metabolised, the progressive decrease in K_s from the long through the medium to the short acting compounds correlates fairly well with the decreasing dura-

tion of action. The short action of the *N*-methyl barbiturates is probably not due to rapid *N*-demethylation as this would produce barbital (a long acting compound). It has been shown that the microsomal *N*-demethylation of hexobarbital only occurs after hydroxylation and subsequent formation of the keto derivative of the alkenyl ring.[21]

Substitution of sulphur by oxygen at C_2 in barbital induces a Type II difference spectrum which correlates with metabolic attack at that position in the molecule. The influence of sulphur substitution is also strong enough to induce a Type II difference spectrum in the "Type I" phenobarbitone molecule, suggesting that sulphur replacement may proceed more readily and before *p*-hydroxylation of thiophenobarbitone. However, sulphur substitution in the other two compounds, both having large alkyl substituents at C_5 and low K_s values, failed to "convert" them to Type II compounds suggesting that the substituents at C_5 in these molecules are more susceptible to metabolic attack than the sulphur atom at C_2. The correlation although incomplete may be useful in stimulating further work on the relationship between difference spectra and the mechanisms of drug metabolism in the liver.

Substrate binding sites and metabolism of Type II compounds

Critical comparison of data derived from difference spectra and the relative rates of metabolism of various substrates shows that the observed differences in affinity for cytochrome P-450 alone are insufficient to account for all the variations in rate of metabolism observed.[22, 23] Consideration of the probable sequence of reactions involved in the oxidation of foreign compounds by the microsomal electron transport chain indicates that the activity of cytochrome P-450 reductase may be of critical importance, and therefore as substrates bind to oxidised P-450 the rate limiting step in the cycle is the reduction of the substrate–cytochrome P-450 complex.[23] It has been suggested that oxidised cytochrome P-450 may occur in two forms, the native cytochrome and the substrate–cytochrome complex.[24] The native cytochrome in its oxidised state has a spectrum with a major absorption peak at 420 mμ, and the peak seen at about 390 mμ on addition on Type I substrates is typical of the substrate-cytochrome complex. How then can the formation of difference spectra with a peak at 420 mμ by Type II compounds and their subsequent metabolism be explained? Schenkman *et al.*[2, 3] have ascribed this type of spectral change to ferrihaemochrome formation. This interpretation is supported by the similarity of the absolute absorption spectrum of oxidised P-450 and that induced by Type II compounds[4, 24, 25] and the displacement of carbon monoxide from reduced P-450 by Type II compounds whereas Type I compounds increased the size of the P-450-carbon monoxide difference spectrum.[2] Other evidence for competition between Type II compounds and oxygen for a single binding site comes from studies with P-450 reductase which was inhibited by Type II compounds but stimulated by Type I substrates.[22] Given that a Type II compound may bind to the site "normally" occupied by oxygen how can metabolic conversion occur? Possibly the substrate molecule could be "activated" in a manner analogous to that of the "activation" of oxygen which is postulated to occur immediately prior to attack on a Type I substrate molecule bound to P-450.[3] Under such conditions the "activated Type II substrate molecule" may then undergo substitution (e.g. replacement of sulphur by oxygen) and/or rearrangement (e.g. NIH Shift[26]) reactions. Several other possible schemes may be postulated as the sequence of events

leading to microsomal metabolism of Type II compounds is not yet so well defined as that involved in the metabolism of Type I compounds.[27] However, even though this mechanism is not understood it is possible to use difference spectra data as useful guides to the probable metabolism of novel compounds either by comparison with existing data or by assembling data on a few members of a series of related compounds. Another useful application is in drug interaction studies when determination of spectral dissociation constants would give a rapid indication of whether the observed antagonism or synergism was due to competition at the pharmacological site of action or at the site of detoxication or activation in the liver.

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