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REVIEW

The concept of regioselectivity in drug metabolism

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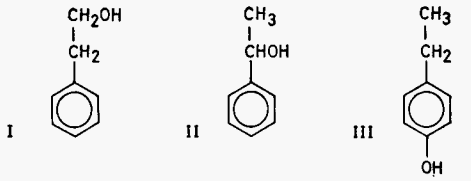
1. INTRODUCTION

Classically, metabolites generated by the biotransformation of a single parent drug differ in their molecular formulae. On the other hand, a number of metabolic routes may result in the production of isomeric metabolites, that is metabolites having the same molecular formula, but which differ in structure such that they are structural isomers.

Structural isomers are conveniently classified into constitutional isomers, that is those differing in the sequential arrangement of atoms regardless of direction in space, and stereoisomers (Mislow, 1966). The importance of stereochemical factors in drug metabolism is an accepted fact and has been considered at length in a recent review (Jenner & Testa, 1973). To the best of our knowledge, no in depth review has been devoted to drug metabolism in terms of constitutional isomerism. It is the scope of the present work to consider one class of constitutional isomers of great significance in drug metabolism, that is regioisomers.

In the broadest sense, regioisomers (usually called positional isomers) can be defined as molecules displaying not only the same molecular formula, but also possessing identical functional groups. By definition, and to discriminate between stereoisomers and regioisomers, one at least of these identical functional groups must be adjacent to different atoms. This definition in fact is limited by the definition of functional groups themselves. Consider for instance 2-phenylethanol (I), 1-phenylethanol (II) and *para*-hydroxyethylbenzene (III); these three compounds are regioisomers if abstraction is made of the differences between alcoholic and phenolic -OH. A more difficult case is presented by 2-aminoethanol (IV) and ethylhydroxylamine (V): biochemists considering these molecules as products of metabolic hydroxylation might classify them in a manner differing from that of structural chemists.



HO-CH_2-CH_2-NH_2 IV

CH_3-CH_2-NHOH V

When discussing stereochemical factors in drug metabolism, a discrimination is made between substrate stereoselectivity, that is differences in the biotransformation of stereoisomers, and product stereoselectivity which is the selective generation of stereoisomers from a single substrate (Jenner & Testa, 1973). By analogy, the concepts of substrate regioselectivity and of product regioselectivity can be discriminated. At the present time, substrate regioselectivity, that is the comparative metabolism of separate regioisomers, has been paid only limited and non-systematic attention. The present review will thus be restricted to the classification and discussion of cases relevant to product regioselectivity.

The object of a discussion on regioselectivity in drug metabolism is to enable some kind of differentiation of the metabolites arising by the same or different mechanisms at apparently equivalent sites on the substrate. It will become apparent that such distinctions may be of a predictive value when considering the metabolism of novel drug molecules. More important perhaps is the new insight allowed by the concept of regioselectivity into basic mechanisms of drug metabolism.

2. HYDROXYLATION OF ALKYL GROUPS

2.1. Alkanes

Alkanes are excellent model compounds for the study of hydroxylating enzymes and the special attention given to n-alkanes has shown the involvement of several distinct enzyme systems in their metabolism.

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The hydroxylation of n-pentane (Ullrich, Ruf & Mimoun, 1972) and n-heptane (Frommer, Ullrich & others, 1972) by rat liver microsomes is selective for the (ω-1)-position (Table 1). A careful study of the effect of inducers and inhibitors showed at least three monooxygenases to be active in the hydroxylation of n-heptane, each of these enzymes displaying its own regioselectivities (Frommer & others, 1972). The question of whether each enzyme is in fact regiospecific, that is, it displays an apparent complete selectivity, remains unanswered; results with n-heptane however suggest that the (ω-1)-hydroxylating system contributes significantly to ω-hydroxylation.

Table 1. *Relative regioisomeric hydroxylation of alkanes by rat liver microsomes.* Ullrich & others, 1972; Frommer & others, 1972).

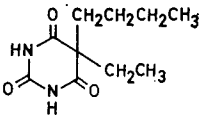
	Position of hydroxylation			
Substrate	ω	ω-1	ω-2	ω-3
n-Pentane	<0·5	84	16	—
n-Heptane	9·5	73·8	11·1	5·6

The oxidation of n-decane by mouse liver microsomes on the other hand occurs with a marked selectivity at the ω-position, the major products being n-decanol, decanoic acid and decamethyleneglycol. The enzyme system involved in the hydroxylation of n-decane appears to differ in enzymic properties from the systems hydroxylating lower and higher homologues (Ichihara, Kusunose & Kusunose, 1969). However, preferred ω-oxidation of long-chain hydrocarbons is also indicated by the metabolism of n-hexadecane to cetyl alcohol and palmitic acid using mouse liver microsomes (Kusunose, Ichihara & Kusunose, 1969).

Thus, the regioselectivity observed in the hydroxylation of n-alkanes appears to be determined primarily by the enzymes involved; the properties of the substrate, (e.g. molecular size) will have only an indirect influence in 'selecting' some enzymes. This general rule does not however hold for branched alkanes, where steric and perhaps electronic factors would be expected to play an important if little known role in directing the site of oxidative attack.

2.2. Alkyl groups adjacent to highly substituted carbon atoms

Since the metabolism of 5-alkyl-substituted barbituric acids is well documented, these compounds may serve as valuable models in the present context. For example, butobarbitone (VI) yields 3′-hydroxybutobarbitone as a major human metabolite arising from (ω-1)-hydroxylation of the n-butyl substituent. Isolation of the 3′-keto metabolite further stressed the importance of initial 3′-hydroxylation (Grove, Toseland & others, 1974). Extensive 3′-hydroxylation has also been reported for the 1′-methylbutyl substituent of secobarbitone (Waddell, 1965) although recent evidence conclusively demonstrated 4′-hydroxylation (ω-hydroxylation) also to be a significant route of secobarbitone metabolism in man (Gilbert, Hetherington & others, 1975).



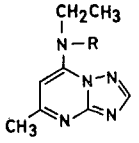
VI

A recent paper (Gilbert, Powell & Templeton, 1975) concludes that (ω-1)-hydroxylation is preferred for side-chains of 4 or more carbons in length, while 3-carbon chains are hydroxylated preferentially at the ω-positions with the penultimate position appearing somewhat resistant to oxidative attack. Hydroxylation at the (ω-2)-position is also documented for some 6-carbon chains.

2.3. N- and O-alkyl groups

As will be shown by the examples to follow, heteroatoms considerably influence the regioselectivity of alkyl group hydroxylation. Metabolism by *N*- and *O*-demethylation is often encountered and occurs with great ease, as shown by the fact that the products of such reactions usually represent major metabolites. The initial and rate-limiting step of these reactions is known to be hydroxylation of the α-carbon. The great facilitation of α-*C*-hydroxylation by the presence of an heteroatom is further indicated by the usually fast removal of *N*-ethyl groups by *N*-dealkylation. Recent examples however show that hydroxylation of *N*-ethyl groups may not always be completely regioselective for the α-position. Thus, trapidil (VII), besides being transformed into the secondary and primary amine metabolites found in the urine of man and animals, also yields a 2′-hydroxylated metabolite (VIII) resulting from β-hydroxylation that has been demonstrated in the urine of rats and dogs dosed with the drug (Borchert, Bornschein & others, 1974).

In the biotransformation of the hypoglycaemic agent chlorpropamide, the hydroxylation of the *N*-propyl substituent shows marked species variation. In man, total n-propyl hydroxylation accounts for



VII: R = $-CH_2CH_3$

VIII: R = $-CH_2CH_2OH$

ca 50 % of the urinary metabolites, with β-hydroxylation predominating (*ca* 40 %), α-hydroxylation (*N*-dealkylation) being minor (7–8 %) and γ-hydroxylation minute (2–3 %) (Thomas & Judy, 1972). In the rat and dog, total n-propyl hydroxylation represents *ca* 70 and 15 %, respectively, of urinary metabolites, with *N*-dealkylation being the major route (*ca* 50 and 10 %, respectively), β- and γ-hydroxylation being equally small in one case and minute in the other (Thomas & Judy, 1972; Taylor, 1974).

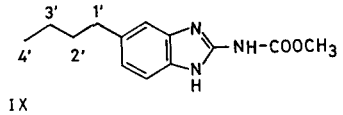
These examples stress the fact that if the presence of an heteroatom can exert a profound influence on the regioselectivity of *C*-hydroxylation, enzymic factors are of the utmost importance and can result in wide variation in the nature of the products.

Similar conclusions can be drawn from metabolic studies of n-propyl *p*-nitrophenyl ether. Using rat and guinea-pig hepatic microsomes (Mitoma, Dehn & Tanabe, 1971), β-hydroxylation (also designated 2′- or (ω-1)-hydroxylation) was shown to be the major route of metabolism. Pretreatment of animals with phenobarbitone and 3-methylcholanthrene greatly increased *O*-dealkylation (α-hydroxylation) in the rat but not in the guinea-pig. These and other results in this study again indicate that separate enzymes may be involved in the hydroxylation at the different sites; however, as pointed out by the authors, definite proof of the number of enzymes involved must await their purification (Mitoma & others, 1971).

2.4. Alkyl groups adjacent to aromatic rings

In a similar manner to heteroatoms, an aromatic ring has a marked influence on the chemical and biochemical reactivity of vicinal C-H bonds. However, the few examples to be found in the literature are rather confusing, because they do not point to any general rule governing the biological hydroxylation of aryl-adjacent n-alkyl groups.

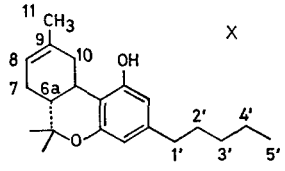
A major route of parbendazole (IX) metabolism in sheep and cattle is ultimate (4′-) oxidation of the n-butyl side-chain; 1′- and 2′-hydroxylation were



IX

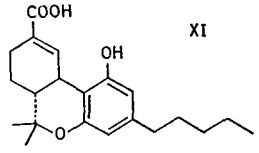
also shown to occur to a marked extent, leaving the (ω-1)-position as the only unattacked site (Dunn, Gallagher & others, 1973).

Another relevant example is seen in the metabolism of (—)-Δ^8-THC (tetrahydrocannabinol) (X, dibenzopyran nomenclature) and (—)-Δ^9-THC. Oxidative metabolism of the n-pentyl side-chain of Δ^8-THC gives rise to the 1′-hydroxy and 3′-hydroxy



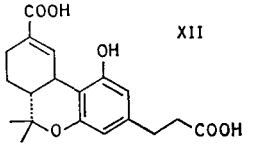
X

derivatives as major *in vitro* metabolites in dog liver preparations (Maynard, Gurny & others, 1971). On the other hand, metabolism of Δ^9-THC by the same route has recently been shown to produce the 3′-hydroxy and 4′-hydroxy derivatives using dog lung and liver; these derivatives were generated as major metabolites from the lung, but as minor metabolites from the liver (Widman, Nordqvist & others, 1975). In the rabbit two major urinary metabolites of Δ^9-THC were the 1′-hydroxy and 2′-hydroxy derivatives of Δ^9-THC-11-oic acid (XI) (Burstein, Rosenfeld & Wittstruck, 1972). These findings are important in the light of the reported pharmacological activity of 2′-, 3′- and 5′-hydroxy-Δ^8THC, and of the lack of activity of 1′-hydroxy-Δ^8-THC (Widman & others, 1975).



XI

The above findings exemplify the metabolic hydroxylation of four out of the five carbon atoms building the alkyl side-chain. Oxidation of the 5′-position is also likely when considering the acidic metabolite XII isolated from the urine of rabbits dosed with Δ^9-THC (Nordqvist, Agurell & others, 1974).



XII

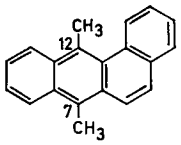
The metabolism of ethylbenzene in contrast occurs by high regio- and stereoselective hydroxylation at the 1′-position (e.g. McMahon & Sullivan, 1966),

in accordance with the known chemical reactivity of the benzylic position. The examples reported in this section indicate that this regioselectivity is markedly decreased, or even lost, by lengthening the alkyl side-chain adjacent to the aromatic ring. Opposing factors seem to be involved that require further investigation before being fully understood.

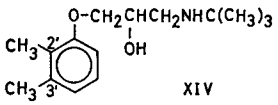
2.5. Oxidation of sterically non-equivalent methyl groups

Enantiotopic and diastereotopic groups are to be found in molecules yielding enantiomeric and diastereoisomeric products, respectively, upon selective metabolism. The term 'regiotopic' therefore seems appropriate to groups in substrates generating regioisomeric metabolites. According to the structure of the substrate a number of distinct cases involving oxidation of sterically non-equivalent methyl groups can be envisaged.

The two methyl groups of 7,12-dimethylbenz[a]-anthracene (XIII, DMBA) are non-equivalent, and the 7- and 12-hydroxylated metabolites will be regioisomers. (Such would not be the case for the corresponding dimethylanthracene, a molecule of



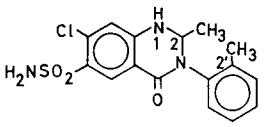
XIII



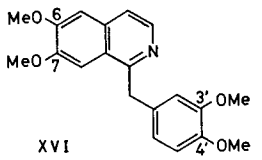
XIV

higher symmetry.) Recently, high pressure liquid chromatography (h.p.l.c.) has enabled quantitative analysis of the *in vitro* metabolites of DMBA (Yang & Dower, 1975). The results show 7-methyl- and 12-methyl-hydroxylation to be significant routes of metabolism of practically equal importance in control and 3-methylcholanthrene-induced rat liver microsomes. Assuming a single enzyme to be involved in the hydroxylation reaction, it is apparent that there is no molecular factor in DMBA that allows enzymic discrimination of the two methyl groups. In contrast, a clear regioselectivity is apparent in the *in vivo* metabolism of 1-*t*-butylamino-3-(2,3-dimethyl-phenoxy)-2-propanol (XIV), a β-adrenoceptor blocker. In the rat, rabbit and monkey, the pharmacologically active 3'-methyl-hydroxylated metabolite was produced in amounts far in excess (10 to 30 times) of the inactive 2'-methyl-hydroxylated metabolite (Honma & Kambekawa, 1975). Intuitively, steric hindrance factors appear responsible for this pharmacologically useful regioselectivity.

Metolazone (XV) is cited as an example of a compound having two methyl groups adjacent to dissimilar moieties, and hence expected to markedly differ in their biochemical reactivity. Metabolic hydroxylation of both methyl groups has been shown to occur, but no conclusion can be drawn about regioselectivity (Cohen, Hartman & others, 1973). Metabolic studies of compounds comparable to XV might allow an assessment of the role of electronic



XV



XVI

factors on regioselectivity, while compounds such as XIV should be useful in the study of predominant steric factors.

In a compound such as caffeine, hydroxylation of the three *N*-methyl groups is not fundamentally different from the above examples, although being brought about by what is considered to be a different metabolic pathway. The three mono-*N*-demethylated metabolites have been quantified in the urine of rats dosed with caffeine (Khanna, Rao & Cornish, 1972). Despite the complexity of the metabolic pattern (Rao, Khanna & Cornish, 1973), it is apparent that *N*-3 is the major site of *N*-demethylation (generating paraxanthine, pKa 8·5) while the minor site is *N*-7 (generating theophylline, pKa 8·7) and *N*-1 is the site of intermediate importance (generating theobromine, pKa 9·9). The pKa values of the products, as indices of the electronic densities at the nitrogen atoms, do not correlate with the observed importance of *N*-demethylation; this lack of correlation indicates that a variety of factors must be implicated in the regioselectivity of the process.

Recent fundamental studies of the metabolism of papaverine (XVI) have provided further evidence of regioselectivity in the metabolism of non-equivalent methyl groups (Belpaire & Bogaert, 1975; Belpaire, Bogaert & others, 1975). Thus, while no 3'-*O*-demethylation was found to occur, marked species

Table 2. *Regioselective O-demethylation of papaverine (XVI) in various species* (from Belpaire & Bogaert, 1975).

Species	Mean % of dose in 6 h bile	Metabolites in the bile*			
		A	B	C	D
Rat	84	42	35	10	3
Guinea-pig	50	53	29	11	2
Rabbit	44	53	7	33	2
Dog	57	68	4	14	7
Cat	50	17	4	62	5

* As % of total radioactivity chromatographed.
A: 4'-demethyl-papaverine. B: 7-demethyl-papaverine. C: 6-demethyl-papaverine. D: 4'6,-didemethyl-papaverine.

variation was detected in the biliary excretion of other *O*-demethylated metabolites (Table 2). Thus, 4'-*O*-demethylation is a major route in all species except the cat, 7-*O*-demethylation is an important route in the rat and guinea-pig only, while 6-*O*-demethylation is significant in the rabbit and the major route in the cat. *O*-Demethylation considered globally was enhanced in the rat by phenobarbitone pretreatment and inhibited by SKF-525A, while it remained unaffected by 3-methylcholanthrene pretreatment. Unfortunately, however, the individual reactions of *O*-demethylation were not considered separately in the latter experiments, and no conclusion can be attempted with regard to the multiplicity of enzymes involved.

3. HYDROXYLATION OF CYCLOALKYL GROUPS

3.1. Cyclohexyl groups

The simple molecule cyclohexylamine is of interest not only from the toxicological significance it has gained as a metabolite of cyclamate, but also with regard to the structural aspects of its metabolic hydroxylation. The stereochemical profile of cyclohexylamine *C*-hydroxylation has been discussed (Renwick & Williams, 1972; Jenner & Testa, 1973); it involves diastereoisomeric, enantiomeric and conformational aspects. Positional isomerism however is also involved in cyclohexylamine hydroxylation (Table 3). Marked species variation is apparent, with the 1-, 3- and 4- positions being the major sites of oxidative attack (Renwick & Williams, 1972).

An interesting comparison can be made between cyclohexylamine and a medicinal derivative, namely bromhexine (XVII). In the urine of rabbits dosed with this drug, no metabolites were found resulting

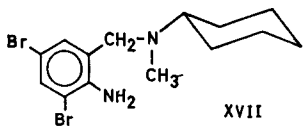
Table 3. *Relative oxidative attacks in the* in vivo *metabolism of cyclohexylamine as % of dose** (Renwick & Williams, 1972).

Position	Species			
	Rabbit	Rat	Guinea-pig	Man
N	0·2	0	0	0
C-1 (deamination)	14	0·05	3	1·6
C-2	0	0	0	0
C-3	11·9	2·3	1·4	0
C-4	0·6	2·2	0·4	0

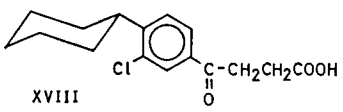
* Excreted in 24 h urine.

from *C*-1 and *C*-2 oxidation of the cyclohexyl moiety; *C*-4-hydroxylation (axial only) was the major route (35 % of urinary metabolites), with *C*-3-hydroxylation (axial and equatorial) also being a significant route (27 %) (Schraven, Koss & others, 1967). These results differ markedly from those obtained in the same species with cyclohexylamine (see above), thus indicating that the properties of the substrate may be even more important than the enzymic factors in determining the regioselectivity of cyclohexyl hydroxylation. This hypothesis is further substantiated by the known metabolism of several substituted cyclohexylamines in various species, where the 3- and 4-position of the alicycle are consistently major sites of hydroxylation (e.g. Testa & Jenner, 1976).

Comparable regioselectivities also appear in the literature for cyclohexyl rings adjacent to an aromatic ring, as exemplified by bucloxic acid (XVIII) (Gros, Davi & others, 1974). Here again, hydroxylation of the saturated ring occurs selectively at the 3- and 4-positions in all species studied including man.



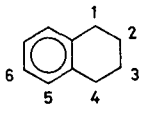
XVII



XVIII

3.2. Unsaturated cycloalkyl groups

Cycloalkyl rings fused to aryl rings have many similarities with the examples discussed above (see 2.4). A simple model compound is tetralin (XIX). Its metabolism in the rabbit has been shown to occur by

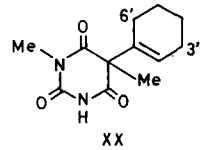


XIX

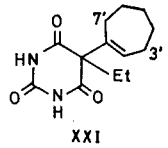
regioselective oxidation (Elliott & Hanam, 1968). Four isomeric monohydroxylated derivatives exist, namely 1-, 2-, 5- and 6-hydroxytetralin. No phenolic metabolites (5- and 6-hydroxy derivatives) were excreted, showing the oxidation to be specific for the alicyclic ring. Hydroxylation was found to be regioselective for the benzylic position (*C*-1, respectively *C*-4) since it accounted for 60% of the administered dose. The 2-position, however, is also a site of oxidative attack, accounting for 25 % of the dose. The formation of the latter metabolite is excluded by a free-radical attack, affording one of the many pieces of evidence against such a mechanism in monooxygenase-catalysed hydroxylations.

Metabolic studies of hexobarbitone (XX) have recently been summarized in an 'in depth' and comprehensive review by Bush & Weller (1972). They show the hydroxylation of the cyclohexenyl ring to be specific for the 3′-position in all animals studied. The alternate allylic position (6′), and the other positions, have never been found to undergo hydroxylation. It is thus clear that electronic factors are predominant in the 3′-hydroxylation. Steric factors however may also play a certain role in the lack of 6′-hydroxylation. This is indicated by the human metabolism of heptabarbitone (XXI); as expected, the 3′-position is the major site of metabolism. However, in contrast to hexobarbitone, the metabolite resulting from the hydroxylation of the alternate allylic position (7′-hydroxyheptabarbitone) has been tentatively identified in human urine; its formation is explained by the greater conformational mobility of the seven-membered ring as compared to the cyclohexenyl ring (Gilbert, Millard & others, 1974).

Regioselective hydroxylation of allylic positions is also demonstrated by the metabolism of tetrahydrocannabinols. Consistently, the major site of oxidative attack in these compounds is found to be *C*-11, but this allylic position is not relevant to the present discussion, as opposed to allylic positions in the cyclohexenyl ring. In a variety of species and conditions, (—)-Δ^8-THC (X) was found to yield three metabolites resulting from *C*-7-oxidation, namely 7α-hydroxy-, 7β-hydroxy- and 7-keto-Δ^8-THC (e.g. Mechoulam, Varconi & others, 1972; Mechoulam, Ben-Zvi & others, 1973). Oxidation at *C*-10, the alternate allylic position, has never been detected to the best of our knowledge; hindrance factors may be implicated. In (—)-Δ^9-THC, oxidation of the cyclohexenyl ring occurs specifically at the allylic 8-position, resulting in the production of 8α-hydroxylated, 8β-hydroxylated and 8-keto derivatives (e.g. Mechoulam & others, 1972; Ryrfeldt, Ramsey & others, 1973; Ben-Zvi, Burstein & Zikopoulos, 1974; Jones, Widman & others, 1974; Widman & others, 1975).



XX



XXI

4. OXIDATION OF AROMATIC RINGS

4.1. Mechanistic aspects

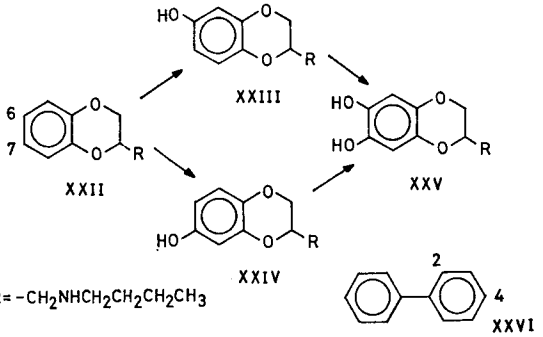
The main oxidative route in the metabolism of aromatic rings involves the formation of arene oxides as intermediates; an authoritative review has been published on the subject by Daly, Jerina & Witkop (1972). In the case of naphthalene, its 1,2-oxide is considered to be an obligatory intermediate for all naphthalene metabolites. Naphthalene-1,2-oxide thus: a) isomerizes to 1- and 2-naphthol, b) is hydrated to a *trans*-dihydrodiol and c) yields a glutathione conjugate (Jerina, Daly & others, 1969).

In the primary mechanism of arene oxidation, aspects of regioselectivity are apparent. Keeping naphthalene as an example, the addition of an activated oxygen atom appears to occur specifically at the 1,2-aromatic bond. Also, non-enzymic isomerization of 1,2-naphthalene oxide generates almost exclusively 1-naphthol, and only a few percent of 2-naphthol (Jerina & others, 1969).

Direct hydroxylation of aromatic rings (i.e. not involving an intermediate epoxide) has, however, also been characterized unambiguously in some cases and must therefore be viewed as a second if less important oxidative pathway. Thus, butamoxane (XXII) has been shown to undergo two consecutive hydroxylations when metabolized to the catechol derivative XXV. Interestingly, the direct hydroxylation mechanism in rat liver microsomes yields the 6-hydroxylated and 7-hydroxylated metabolites (XXIII and XXIV, respectively), in a ratio of approximately 2 : 1, thus showing intrinsic regio-

selectivity for this route also (Murphy, Bernstein & McMahon, 1974).

Both direct hydroxylation and formation of arene oxide intermediates appear to be involved in the metabolism of chlorobenzene by rat liver preparations. *Ortho-*, *meta-* and *para*-chlorophenol are all



formed as metabolites, the regioselectivity of the overall oxidative reaction varying markedly with the hepatic preparation used and the nature of pretreatment with enzyme inducers (Table 4); the most striking results are the low percentages of *m*-isomer produced, the strong *o*-inducing effect of 3-methylcholanthrene and the lack of selectivity of phenobarbitone induction. Three metabolic pathways have been characterized, each one leading to one of the isomeric metabolites. Thus, the formation and rearrangement of intermediate 4-chloro- and 3-chlorobenzene oxides give rise respectively and specifically to *p*- and *o*-chlorophenol, while the formation of *m*-chlorophenol appears to result from a direct hydroxylation reaction (Selander, Jerina & Daly, 1975a; Selander, Jerina & others, 1975b). The authors of these studies are investigating whether three separate monooxygenases are involved, or if the three regioisomeric products reflect competitive oxidations of the substrate in the active site of one monooxygenase.

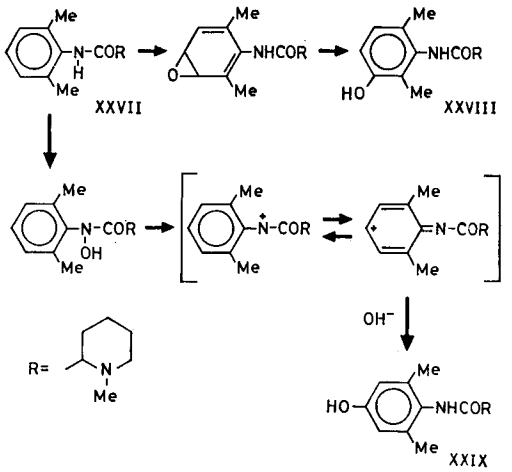
Table 4. *Regioselective oxidation of chlorobenzene by rat liver preparations* (Selander & others, 1975a,b).

Preparation	% chlorophenol formed		
	ortho-	*meta-*	*para-*
Perfused liver			
normal	40	20	40
PB	46	10	44
3-MC	89	2	9
Postmitochondrial supernatant, normal	28	13	59
Microsomes			
normal	18	7	75
PB	32	6	62
3-MC	59	6	35
Soluble haemoproteins			
normal	60	0	40
PB (P-450)	58	2	40
3-MC (P-448)	85	2·5	13

PB = phenobarbitone. 3-MC = 3-methylcholanthrene.

Differences in the regioselectivity of monooxygenase reactions carried out using hepatic preparations from animals pretreated with phenobarbitone or 3-methylcholanthrene also occur in the metabolism of biphenyl (XXVI). While liver microsomes from phenobarbitone-induced rats and a reconstituted cytochrome P-450 system yielded the 2-hydroxylated and 4-hydroxylated metabolites in a 1 : 20 ratio, this ratio was 1 : 2 in microsomes from 3-methylcholanthrene-induced animals and a cytochrome P-448 system (Burke & Mayer, 1975).

Besides the formation of arene oxides and the direct arene hydroxylation discussed above, a third oxidative mechanism does exist for the metabolism of some aromatic substrates. This mechanism has been characterized for aromatic amides. Its initial step involves *N*-hydroxylation, followed by enzymic formation of a resonance-stabilized nitrenium ion and addition of a nucleophile (here an hydroxyl anion) to an electron-deprived position in the ring (Sternson & Gammans, 1975). Such a mechanism appears to be involved in the metabolism of mepivacaine (XXVII); two phenolic metabolites of this drug have been characterized, namely, the 3′-hydroxy (XXVIII) and the 4′-hydroxy metabolite (XXIX). In man, the former metabolite was initially excreted in higher



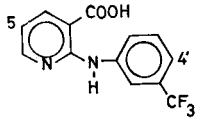
urinary concentrations, but by the end of the collection period (24 h) the proportions were reversed. All the evidence obtained was consistent with the

hypothesis that the 3′-hydroxy metabolite (XXVIII) arises from an epoxide intermediate, while its 4′-hydroxy isomer is generated *via* an *N*-hydroxy intermediate by the mechanism discussed above (Meffin & Thomas, 1973). Also, the different time courses of excretion of the two metabolites are presumably due to the differences in mechanisms and enzymes involved in their formation.

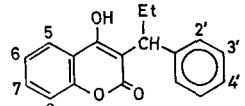
4.2. Bi- and polycyclic systems

The literature contains many examples of monophenyl derivatives metabolized to regioisomeric phenols, but a lengthy list of such examples would be meaningless here. On the other hand, compounds containing two or more aromatic rings either fused or not, may result in complex but interesting examples of regioisomerism.

Niflumic acid (XXX) is metabolized by two oxidative pathways generating respectively the 5-hydroxy and the 4′-hydroxy derivatives, the structures of which have been determined unambiguously (Cohen, Weliky & others, 1974). In man and the dog, these two metabolites are of major and approximately equal importance (Lan, Chando & others, 1973). *In vitro* studies using rat liver microsomes have shown at least two enzymes to be active; indeed, the extent of hydroxylation of the two aromatic rings varied independently under the influence of various inducers and inhibitors. Thus, phenobarbitone pretreatment increased 4′-hydroxylase activity 50 %, whereas the 5-hydroxylase activity was increased by 200 % (Lan, Chando & Schreiber, 1975).



XXX

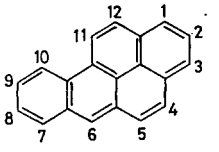


XXXI

Phenprocoumon (XXXI) also contains two non-fused aromatic rings undergoing metabolic hydroxylation; four out of the seven possible monophenolic metabolites have been shown to be produced by the rat. When liver microsomes were used, quantities of these four isomeric metabolites were found in the decreasing order 4′-6-8-7, whereas in the faeces (the major route of excretion) and in urine the order was 6-4′-7-8. Consistently, the positions 4′ and 6 (*para* to a lactonic oxygen) are the major sites of hydroxylation, while the 7- and 8-positions are minor, and the 5-, 2′- and 3′-positions apparently remain unattacked (Haddock, Trager & Pohl, 1975).

Regioisomerism in the metabolism of naphthyl derivatives is exemplified by two β-blocking drugs, propranolol and pronethalol, which are 1-naphthyl and 2-naphthyl derivatives, respectively. 4-Hydroxylation of propranolol has been recognized for several years as a significant route of metabolism in man and other animal species (e.g. Walle & Gaffney, 1972). Ring oxidation thus appeared regiospecific, until a positional isomer of 4-hydroxypropranolol was recently identified in rat and man, although not in the dog (Walle, Morrison & Tindell, 1974). The actual position of hydroxylation remains undetermined for this minor metabolite, although the authors suggest either the 2- or 3-position.

The metabolism of pronethalol tells a comparable story. 7-Hydroxylation has long been recognized as a major metabolic route, and the sole pathway of ring hydroxylation (Schreiber, 1970). But again new findings contradict this apparent regiospecificity. In the mouse, minor amounts of 6-hydroxypronethalol and of two unidentified positional isomers were detected alongside large amounts of 7-hydroxypronethalol. In the rat, a major metabolite was found to be a hydroxypronethalol claimed not to be identical with the 7-isomer on the basis of insufficient evidence (Stillwell & Horning, 1974).



XXXII

Polycyclic aromatic hydrocarbons afford interesting examples of regioisomeric metabolism which are of toxicological significance. Consider for instance benzo[a]pyrene (XXXII), a potent carcinogen known to generate many oxygenated metabolites. Most metabolic studies have been undertaken using liver preparations from rats pretreated with 3-methylcholanthrene; this treatment increases the yield, but does not affect the nature of the products (Sims, 1967). The discussion to follow summarizes results from four excellent representative papers based on the same experimental conditions (liver preparations from 3-MC-treated rats) but using various analytical techniques (t.l.c., column chromatography, h.p.l.c.) (Sims, 1967; Sims, 1970; Grover, Hewer & Sims, 1972; Selkirk, Croy & Gelboin, 1975). Three dihydrodiol derivatives of benzo[a]pyrene have been

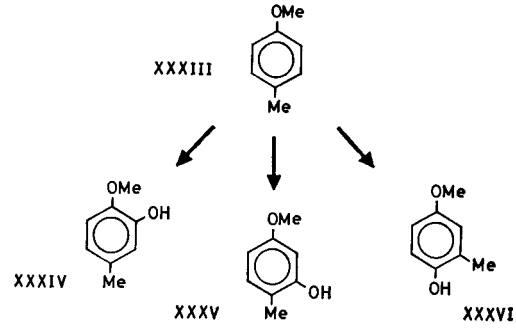
characterized consistently; they are the 4,5-, 7,8-, and 9,10-dihydrodiols, generated as secondary metabolites. A minor metabolite, the 1,2-dihydrodiol is also known. These metabolites prove the formation of the corresponding epoxides as intermediates. In the presence of an epoxide hydrase inhibitor, the K-region epoxide (4,5-oxide) is sufficiently stable to be characterized, as opposed to the other epoxides. Interestingly, the 11,12-dihydrodiol and thus the 11,12-epoxide have not been characterized, suggesting the second K-region to be of little or negligible metabolic importance. Oxidation at positions other than the 1,2-, 4,5-, 7,8- and 9,10-bonds is indicated by the characterization of two monophenols, the 3-hydroxylated product (a major metabolite) and the 9-hydroxylated products, and of the 1,6-, 3,6- and 6,12-quinones. It is thus apparent that besides the four aromatic bonds just mentioned, positions 3, 6 and 12 are also oxidized either via an epoxide or by direct hydroxylation, Of the twelve peripheral positions of benzo[a]pyrene, eleven have been shown up to now to experience oxidative attack by either route. There is little doubt that identification of further metabolites may indicate oxidation at other sites, in particular at 'valley' positions (see also below).

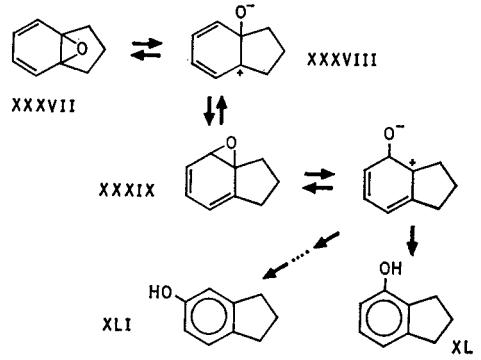
4.3. Intramolecular migrations following epoxidation

The chemistry and biochemistry of epoxides is of immense complexity and richness. So far we have discussed the regioisomeric aspects of their formation and their 'straightforward' products of transformation (e.g. phenols and dihydrodiols). However, it has become apparent in recent years that epoxides may also undergo rearrangement reactions involving intramolecular migrations, thereby considerably increasing the number of potential metabolites and of regioisomeric products. The now well-known NIH shift, and the recently characterized oxygen walk, will therefore be discussed here.

Some years ago, workers at NIH discovered that the *p*-hydroxylation of phenyl derivatives labelled with deuterium or tritium at the *p*-position occurs with a partial retention of the label. The latter can either migrate to the *m*-position (NIH shift) or be lost during the proton-catalysed rearrangement of the intermediate epoxide to a phenol; the mechanistic aspects of this reaction have been discussed (Daly & others, 1972; Testa & Jenner, 1976). The relative importance of migration (retention) and loss, among other factors, is a function of the migrating group and of the ring substituent; as a rule, substituents which cannot readily ionize by proton loss contribute to a high degree of migration, whereas substituents able to ionize by proton loss govern a high degree of label loss (Daly, Jerina & Witkop, 1968).

Migrating groups, however, are not limited to hydrogen isotopes and include halo- and alkyl-substituents of greater importance in medicinal chemistry. The model substrate *p*-methylanisole (XXXIII), when incubated with liver microsomes from phenobarbitone-induced male rabbits, yielded as relevant metabolites the two expected isomeric phenols XXXIV and XXXV, together with significant



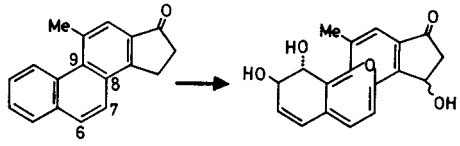


amounts of a third phenol resulting from migration of the methyl group (XXXVI) (Dünges, 1973). The three metabolites are regioisomers, and this example nicely illustrates how the NIH shift indeed increases the number of potential regioisomers generated by a metabolic pathway.

To account for the chemical rearrangement of 8,9-indane oxide (XXXVII) to 4-indanol (XL), a mechanism coined 'oxygen walk' has been postulated on the basis of careful kinetic studies. This mechanism involves opening of the arene epoxide giving a zwitterion (XXXVIII) which collapses to a regioisomeric oxide (XXXIX), finally yielding 4-indanol (XL). A second step in this walk around the ring accounts for the minor isomeric product 5-indanol

(XLI) (Bruice, Kasperek & others, 1973). That such a mechanism is indeed operative in the chemical rearrangement of metabolically produced arene oxides is apparent in the metabolism of the carcinogen 15,16-dihydro-11-methylcyclopenta [a] phenanthren-17-one (XLII).

In the rat, the major urinary metabolite was proven to be 8,9-epoxy-1α, 2β, 15ξ-trihydroxy-11-methyl-8,9-secogona-3,5,7,9,11,13-hexaen-17-one (XLIII), a cyclodecene derivative including an internal epoxy bridge. Formation of the metabolite



XLII XLIII

XLIII by direct insertion of oxygen is considered improbable; several lines of evidence, (e.g. the critical role of the 11-methyl group) suggest the metabolite to arise by an oxygen walk type of rearrangement from a reactive intermediate, probably the K-region 6,7-epoxide (Coombs & Crawley, 1974).

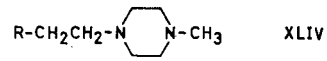
It is evident that the latter example is not directly relevant to regioisomerism. It is meant however to lead the reader into speculating the number of yet unknown regioisomeric metabolites of polynuclear aromatic hydrocarbons that might be formed.

5. METABOLISM OF NITROGEN-CONTAINING FUNCTIONAL GROUPS

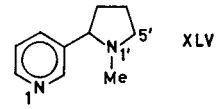
5.1. N-*oxidation*

In the metabolism of compounds containing several tertiary amino groups, regioselectivity or even regiospecificity is the general rule. Two difficulties, however, warrant caution in interpretation of metabolic data. The first difficulty arises in determining the structure of the *N*-oxides : unambiguous synthesis of authentic compounds, and unambiguous chromatographic and spectrometric techniques are required; this difficulty appears greater for *N*-oxides than it is for *C*-oxidized metabolites. The second difficulty results from *N*-oxide reduction, a metabolic route whose importance varies greatly with the substrate, but which must always be kept in mind since it may seriously alter the overall regioselectivity of *N*-oxidation.

Steric factors are active in directing *N*-oxidation. For example, *N*-diethyl tertiary amines do not form *N*-oxides, as opposed to the *N*-dimethyl analogues. In substituted piperazine derivatives (XLIV) having approximately equally basic nitrogens, only the less



XLIV



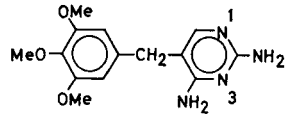
XLV

hindered nitrogen (methylated nitrogen) has been found to be *N*-oxidized (see Testa & Jenner, 1976).

Electronic factors as described by pKa values are of primary importance in governing *N*-oxidation; only in a few exceptions do they appear to be overshadowed by steric or other factors. As a general rule in polybasic molecules, the amino group forming an *N*-oxide is the most basic one, i.e. the group having the highest pKa value. This is understandable in terms of both *N*-protonation and *N*-oxidation being electrophilic processes involving the nitrogen lone pair of electrons.

Nicotine (XLV) contains two tertiary basic centres: the weakly basic pyridine nitrogen, and the strongly basic pyrrolidine nitrogen. The latter is specifically oxidized in human and laboratory animals to form significant amounts of nicotine-1′-*N*-oxide (Beckett, Gorrod & Jenner, 1971; Beckett, Jenner & Gorrod, 1973; Jenner, Gorrod & Beckett, 1973). On the other hand, cotinine (5′-oxo-nicotine) differs from the parent drug in that the pyrrolidine nitrogen is non-basic due to the adjacent carbonyl group (amide nitrogen). The weakly basic pyridine nitrogen becomes the most basic centre in the molecule. In agreement with the above rule, the *N*-oxide resulting from cotinine biotransformation is cotinine-1-*N*-oxide (Dagne & Castagnoli, 1972). Apparently complete regioselectivity (regiospecificity) is indicated when the two basic centres have markedly differing basicities.

A fascinating yet poorly understood example is provided by trimethoprim (XLVI). Both the 1- and the 3-*N*-oxide occur as minor urinary metabolites; in man, they are excreted in approximately equal amounts, whereas the 1-*N*-oxide strongly predominates in rat urine, and the 3-*N*-oxide in the urine of dog (Brooks, de Silva & D'Arconte, 1973; Sigel & Brent, 1973). In such an example the pKa parameter is of no help, and more elaborate electronic parameters such as charge densities would be needed to



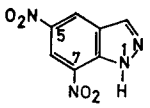
XLVI

understand the biochemical reactivity of the two heterocyclic nitrogen atoms.

Besides substrate-related factors, enzymic factors can be postulated to contribute to the regioselectivity of *N*-oxidation. A potent theory recently presented (Gorrod, 1973) may shed some light at this point of the discussion. From a global consideration of nitrogen oxidation (formation of *N*-oxides and of hydroxylamines), it is suggested that primary, secondary and tertiary amines of the basic type (pKa 8-11, group I) are oxidized by a FAD-dependent enzyme system, whereas non-basic nitrogen-containing groups (group III) are oxidized by a cytochrome P-450-dependent system. Weak bases (pKa 1-7, group II) are substrates for both systems. Tertiary amines yielding *N*-oxides belong to groups I and II; it is apparent that at least for the pyridine- and pyrimidine-type *N*-oxides discussed above, both enzymic systems could be operative.

5.2. Nitro reduction

A discussion of metabolic nitro reduction is rendered difficult by the various enzymic systems involved (liver microsomal and cytoplasmic reductases, gut microflora reductases) and by the discrepancies occurring between the *in vivo* and *in vitro* situations. However, a structural element leading to regioselective nitro reduction in polynitro aromatic compounds has been characterized (see Testa & Jenner, 1976). It is apparent that a nitro group forming an intramolecular hydrogen bond is reduced with a high degree of selectivity. Such is the case for 5,-7-dinitroindazole (XLVII); specific reduction of the 7-nitro group to yield the 7-amino derivative



XLVII

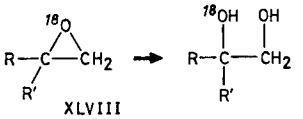
was found to occur in mouse and rat *in vivo*. Incubation with rat gut microflora resulted in the same specificity of reduction, but no selectivity was apparent when 1-methyl-5,7-dinitroindazole was the substrate; there is no possibility for intramolecular hydrogen bonding in the latter compound (Woolhouse, Kaye & others, 1973).

The reason for the facilitation of nitro reduction by an intramolecular hydrogen bond is not clear; it is likely however that this effect is an indirect one, the hydrogen bond stabilizing the intermediate nitroso and hydroxylamino groups towards oxidation.

6. HYDRATION REACTIONS

Enzymic hydration reactions represent a little explored metabolic route; their importance, however, should not be underestimated, and we believe their further study will result in fundamental advances within the next few years.

A recent report based on ^{18}O-tracer studies conclusively shows that the hydration of 1-substituted oxiranes (XLVIII) by rat liver microsomes is a highly



XLVIII

regioselective process involving *C*(2)-O bond cleavage. The experimental results were consistent with the proposal that epoxide hydrase activates a water molecule by means of a general base catalysis; the less hindered oxirane carbon is then the site of nucleophilic attack (Hanzlik, Edelman & others, 1976).

There is also indirect evidence in the metabolism of butyrophenones that carbon-carbon double bond hydration is indeed a regioselective or specific process (see Testa & Jenner, 1976). Direct proof however awaits the isolation of metabolic intermediates.

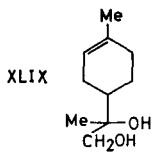
7. PHASE II REACTIONS

In phase II reactions, the possibility exists that two functional groups in a molecule may be competing sites for the binding of a conjugating moiety. Specific analytical methods are required to unambiguously assign the correct site of binding. A few relevant cases have appeared in the literature, but up to now they can only be considered as isolated examples allowing nothing more than poorly founded generalizations.

The major reaction of inactivation and detoxication of chloramphenicol in man is by the direct formation of a glucuronide; any factor which decreases the importance of this reaction may result in greatly increased toxicity. In this context, the actual binding site of glucuronic acid is a relevant factor. Two possible sites for the introduction of the glucuronic acid moiety are apparent in the molecule, namely, the secondary alcoholic group adjacent to *C*-1, and the *C*-3-adjacent primary alcoholic group. Recently, the glucuronide moiety has been unequivocably placed on the 3-position, that is on the primary alcoholic group, by the use of mass spectrometry (Thompson, Gerber & others, 1973).

Steric factors may be operative in selecting a primary alcohol, and this is also indicated in the metabolism of (+)-limonene in rabbits. A major metabolite of this compound is *p*-menth-1-ene-8,-9-diol (XLIX); the latter was shown to be excreted mainly as the 9-glucuronide, that is to say with the glucuronide moiety attached to the primary alcoholic group (Kodama, Noda & Ide, 1974).

XLIX



Electronic factors as assessed by the acidity of the hydroxyl group may also be operative in the formation of *O*-glucuronides. Some examples in the literature tend to suggest phenolic groups to be preferred binding sites as compared to alcoholic groups. An unambiguous and interesting example is provided by 4′-hydroxyfenoprofen, a metabolite of fenoprofen containing a phenolic and a carboxylic group. This metabolite is excreted in minor amounts in the urine of subjects dosed with the drug. Its glucuronide however is a major metabolite accounting for almost half the administered dose; this conjugate was found to be the acyl glucuronide, and not the ether glucuronide (Rubin, Warrick & others, 1972). Binding to the more acidic group is thus indicated in this example.

Enzymic factors such as the multiplicity and heterogeneity of the enzymes involved are also to be considered when discussing the regioselectivity of conjugation reactions. So far, it is generally believed that hepatic UDP-glucuronyl transferase is heterogenous. However, a recent critical examination of all available evidence casts serious doubts on this belief, and indicates that all studies up to now have failed to demonstrate multiple forms of glucuronyl transferase (Mulder, 1974). The above reported examples of regioselective glucuronidation would then be fully accounted for by the properties of the substrates.

On the other hand, the dominating influence of enzymic factors has been eloquently demonstrated in the regioselectivity of the conjugation reactions mediated by catechol-*O*-methyl transferase (COMT). The cytoplasmic catechol-*O*-methyl transferase activity from rat liver was resolved by gel filtration into two enzymes present in relative amounts of about 5 : 1. The two enzymes differ in several properties such as pH optimum and thermal stability; the minor form (COMT-B) has an estimated molecular weight of 45 500 and may be a dimer of the major form (COMT-A, molecular weight 23 000). The two enzymes also markedly differ in the regioselectivity of *m*- and *p*-*O*-methylation of catechols; thus, COMT-A at pH 8·0 methylates dihydroxybenzoic acid and dopamine with *meta*: *para* ratios of 5·6 and 2·7, respectively, while the corresponding values for COMT-B are 11·7 and 5·8. At pH 8, COMT-B displays *meta* : *para* methylation ratios which are twice those shown by COMT-A. Also, the regioselectivity of COMT-B mediated methylation is very pH-dependent as opposed to that displayed by COMT-A (Marzullo & Friedhoff, 1975).

The metabolism of dopamine further shows that the regioselectivity of one metabolic reaction may be markedly influenced by the regioselectivity of a competing reaction. The regioselectivity of COMT for the *m*-phenolic group is paralleled by the selectivity of a sulphotransferase for the same position. High oral doses of L-dopa to Parkinsonian patients results in the urinary excretion of dopamine 3-*O*-sulphate and 4-*O*-sulphate in an approximate 20 : 1 ratio; when tracer amounts of the drug were administered, this ratio decreased to 3 : 1 (Bronaugh, Hattox & others, 1975). These findings suggest that at low doses the methylating route efficiently competes with sulphate formation for the *m*-position, whereas at higher doses the methylating enzymes are saturated. The true regioselectivity of sulphate formation is therefore apparent at high dosage levels only.

8. CONCLUSION

When discussing any particular aspect of a scientific field, it is convenient as a first step to consider it independently of all other related aspects in the field. Such has been our approach in defining, characterizing and discussing regiochemical aspects of drug metabolism. Such an approach however is valid only as a first approximation. Another step must follow, in which such factors as electronic and solubility properties (Hansch, 1972), chemical structure in the broadest sense, regioisomerism and stereoisomerism (Jenner & Testa, 1973) should be considered together to unravel the multiplicity of their interrelationships. In so doing, a higher level of complexity will be reached which will significantly improve our global understanding of drug metabolism, and more generally of the interactions between living systems and foreign compounds.

Studies designed specifically to unravel the involvement of regioselectivity in the metabolism of

xenobiotics are few. As a first attempt to examine the role of regioselectivity, this review has revealed that some general rules are apparent in the mechanisms of drug metabolism, if not fully understood. Until these rules are hardened into clearly definable predictive statements, it is hoped that the content of this article might be of value in assisting in metabolic studies of novel molecules.

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