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COMMENTARY

APPLICATION AND INTERPRETATION OF KINETIC ANALYSES FROM THE MICROSOMAL DRUG METABOLIZING OXYGENASES

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Dedicated to Prof. Dr. Manfred Kiese on the occasion of his 65th birthday

1. *Kinetic constants*

1.1. K_M *and* V_{max}. Application of kinetic analysis to drug metabolism has created a need for the introduction of new expressions which are of practical value for both, those who have a fundamental interest in drug metabolism and those who just want to employ the kinetic tools available to solve their particular problems. Therefore, besides the classical kinetic constants V_{max}, K_M, and K_I the spectral dissociation constants A_{max} or Δ O.D.$_{max}$ and K_S and the I_{50}-value have been introduced.

The attempt of determining V_{max} and K_M of special metabolic pathways of various (drug) substrates by employing suspensions of microsomal membranes as the enzyme source and various xenobiotics as substrates of lipophilic character raises the question of a generalized applicability of equations derived for purified, soluble systems for a multicomponent enzyme system 'buried' in a membrane (see 5, Limitations of Applicability) and creates special problems which are discussed in 4, Essentials for Kinetic Analysis.

With one exception [1], however, the observed enzyme activities, like for example, *O*- and *N*-dealkylation, *C*-oxygenation of aromatic nuclei, of cycloalipathic and alipathic compounds, *N*- and *S*-oxygenation could be analyzed by employing equations based on the Michaelis Menten theory of enzyme action [2]. Nevertheless, numerical values for V_{max}, K_M, and K_I should not be taken as absolute, since among other reasons, they are found to vary occasionally from one laboratory to the other. Considering the relative significance of the numerical values, most authors have preferred to use the expressions *app.* V_{max} and *app.* K_M.

V_{max} then, which is usually expressed in nmoles or μmoles of an oxygenation product formed per min per mg microsomal protein and rarely expressed in E(nzymatic) U(nits), reflects the rate of breakdown of the enzyme substrate complex after the insertion of oxygen has taken place and represents the maximum velocity constant. K_M, the Michaelis constant, which is normally expressed in μM or mM can be looked at as the reciprocal affinity of the enzyme for the substrate in respect to a special metabolic pathway of the drug employed. K_M is distinguished from K_S, the dissociation constant of the enzyme substrate complex, which describes equilibrium conditions between enzyme, substrate, and enzyme substrate complex. If the rate of breakdown of the complex into enzyme and oxygenated product(s) is so slow that it can be neglected, K_M becomes K_S. Furthermore, K_M describes the ease with which the enzyme substrate complex is formed, and it also represents the substrate concentration for which any observed oxygenation velocity is one-half the maximal velocity. The aforementioned exception was reported by Nebert and Gelboin [1] who studied hydroxylation of benzo(a)pyrene by the microsomal fraction from hamster fetus cell cultures. They observed a linear dependence of the *app.* K_M for benzo(a) pyrene hydroxylation from microsomal enzyme concentration and explained this unusual result with an example of substrate depletion (Mutal Depletion System, see [3] due to binding of substrate to nonspecific components of the microsomal membranes.

1.2. *Cytochrome P-450 characteristics.* On addition of various (drug) substrates to microsomal suspensions characteristic changes of the cytochrome P-450 absorption are observed (among them: type I-, type II-, and the reverse type I-binding spectrum) which were assumed to reflect complex formation between (drug) substrate and the terminal oxygenase, until recently it was shown that the type I spectral change was rather due to an increase in electronegativity or polarity of the sixth heme ligand effected by type I substrates. (Drug) substrate and the properties of the microsomal enzyme source determine the type of the binding spectrum. Since the spectral changes depend upon the concentration of free substrate in microsomal suspensions, and the reversibility of the complex formation has been demonstrated by washing experiments, Michaelis Menten kinetics have been applied to measure maximal spectral changes (A_{max} or Δ O.D.$_{max}$) and the spectral dissociation constant K_S by plotting the reciprocal spectral changes versus reciprocal substrate concentrations.

A comparison of the spectral changes obtained with aminopyrine, hexobarbital, and aniline with the microsomal *N*-demethylation of aminopyrine, the metabolism of hexobarbital, and the *p*-hydroxylation of aniline suggested that at least for type I drug sub-

Abbreviations: PB = phenobarbital (C.A., 5-ethyl-5-phenyl-2,4,6-(1H,3H,5H)-pyrimidinetrione); 3-MCh = 3-methylcholanthrene (C.A., 1,2-dihydro-3-methyl-benz(j)-aceanthrylene); 3-MMAB = 3-methyl-4-methyl-aminoazobenzene (C.A., N,2-dimethyl-4-(phenylazo)-benzenamin); SKF 525-A (C.A., α-phenyl-α-propyl-benzeneacetic acid 2-(diethylamino)ethylester).

strates spectral changes (expressed as A_{max} and K_S) parallel enzyme activity (expressed as V_{max} and K_M). However, a number of reports on *N*-demethylation of ethylmorphine, of (+)- and (−)-amphetamine and benzphetamine, of (+)- and (−)-propoxyphene, carbinoxamine and methorphane, and *N*-oxygenation of *N*-ethylaniline and *N,N*-dimethylaniline which have appeared since, have shown that either no binding spectrum was observed at all or no correlation between K_S and K_M or A_{max} and V_{max} was found. Recent papers have drawn the attention of the investigators of drug metabolism to the often ignored fact that most drug substrates are oxygenated in various positions of the molecule, V_{max} and K_M being different for each specific oxygenation reaction. In order to find a correlation between K_S and K_M, the kinetic constants of all these oxygenations would have to be determined to find out which of these is correlated with the optical dissociation constants. This is not feasible presently, because, among other reasons, many of the primary oxygenation reactions of drug substrates are still unknown. Thus, the results which indicated a correlation between K_S and K_M seem to be rather accidental and not universally applicable.

2. *Estimation of the kinetic constants*

2.1. V_{max} *and* K_M. The numerical values for K_M and V_{max} are obtained from pairs of v (rate of a specific oxygenation) and S (Substrate concentration) either by graphical procedures or by computer programs (on the basis of regression analysis or iterative fitting, for which FORTRAN programs are available) using initial estimates for V_{max} and K_M from graphical methods, as recommended by Wilkinson [4] or Cleland [5].

Among graphical procedures, V_{max} and K_M are determined by a direct linear plot of v versus S according to Eisenthal and Cornish Bowden [6]. Different lines are drawn each corresponding one observation of v and S. These lines intersect at a common point, provided there exists a one enzyme one substrate relation and the experimental error of all v values is small. The coordinates of that point provide the values for V_{max} and K_M. If, for several reasons, there is no common intersection point, the coordinates of each intersection point are drawn and the median of each series of v and S values taken as the best estimate for V_{max} and K_M. Other graphical procedures include linear plots derived from transformations of the Michaelis Menten equation, among them Lineweaver Burk plots, where $1/v$ is plotted versus $1/S$, Hanes plots, where S/v is plotted versus S, or Hofstee plots, where v is plotted versus v/S.

Plotting the experimental data according to Lineweaver Burk is the most frequently used graphical procedure for determining V_{max} and K_M. Yet, it has some disadvantages which have been discussed by Dowd and Riggs [7]. It has been criticized that the experimental points are concentrated near the left-hand side of the graph and that the values of v for very small substrate concentrations, which often cannot be determined with the required accuracy, have such an important influence on the curve. The author has experienced that in certain cases Lineweaver Burk plots are not sensitive enough to detect a slight, but nevertheless distinct curvature in the kinetic curve, and this is certainly true for Hanes plots, as can be seen by comparing Fig. 1D with 1B or 1C. Instead, plotting pairs of v and S according to Hofstee will give a more uniform distribution of the experimental points. The Hofstee plot is also recommended as a method for estimating V_{max} and K_M from unweighted points and in all cases when the error of v is large and constant, or large and variable, although a disadvantage is that any error in v will cause an oblique displacement of the corresponding point. However, in all cases where the kinetic data reveal a two enzyme one substrate relation, the Hofstee procedure of plotting experimental data is superior over the Lineweaver Burk procedure, because the kinetic constants of the two participating enzyme activities can be evaluated more accurately. This is illustrated by Figs. 1A, 1B, 1C, and 1D, which show kinetic curves obtained by plotting kinetic data from microsomal (ω-1)-hydroxylation of 4-chloropropionanilide according to Eisenthal and Cornish Bowden, Lineweaver Burk, Hofstee and Hanes, respectively.

2.2 A_{max} *and* K_S. The numerical values for A_{max} or Δ O.D.$_{max}$, the maximal spectral change ($A_{max} = \Delta A$ (absorption) per mg of protein per ml suspension) and for K_S (mM), the spectral dissociation constant, have been estimated graphically by plotting pairs of A versus S according to Lineweaver Burk or Hofstee.

2.3. K_I *and* I_{50}. If a competitive or a noncompetitive inhibition pattern is observed of a special oxygenation reaction due to the interference of a second (drug) substrate, the inhibition constant K_I can be estimated graphically according to Dixon by plotting $1/v$ versus inhibitor concentration [I] at two different fixed substrate concentrations. The intersection point of the two linear kinetics on the left of the vertical axis provides the numerical value for K_I. It can also be determined from the position of the intersection point of a series of lines each representing the oxygenation rate at a fixed inhibitor concentration. Whereas K_I is independent on substrate concentration, I_{50} describing the inhibitor concentration causing a reduction of enzyme activity by 50% depends on the (drug) substrate whose special oxygenation reaction is inhibited. The relationship between K_I and I_{50} has been discussed in more details by Cheng and Prussoff [8], Tipton [9], and Chou [10].

3. *Aims of kinetic analyses*

3.1. *Abnormal graphical plots.* Aims of kinetic analyses are to study the functioning of enzymes under *in vitro* conditions, that is to establish a relation between the rate of any specific oxygenation of a (drug) substrate v and its concentration S. The kinetic constants which are obtained from pairs of v and S characterize both, the participating enzyme(s) as well as the specific metabolic pathway, provided the assay procedure is specific enough. The condition for establishing a relationship is that drug oxygenation by microsomal cytochrome P-450 follows Michaelis Menten theory of enzyme action. However, investigation of drug metabolism *in vitro* is mostly not aimed at determining the absolute values of the kinetic constants (see 5.0) obtained with a particular substrate

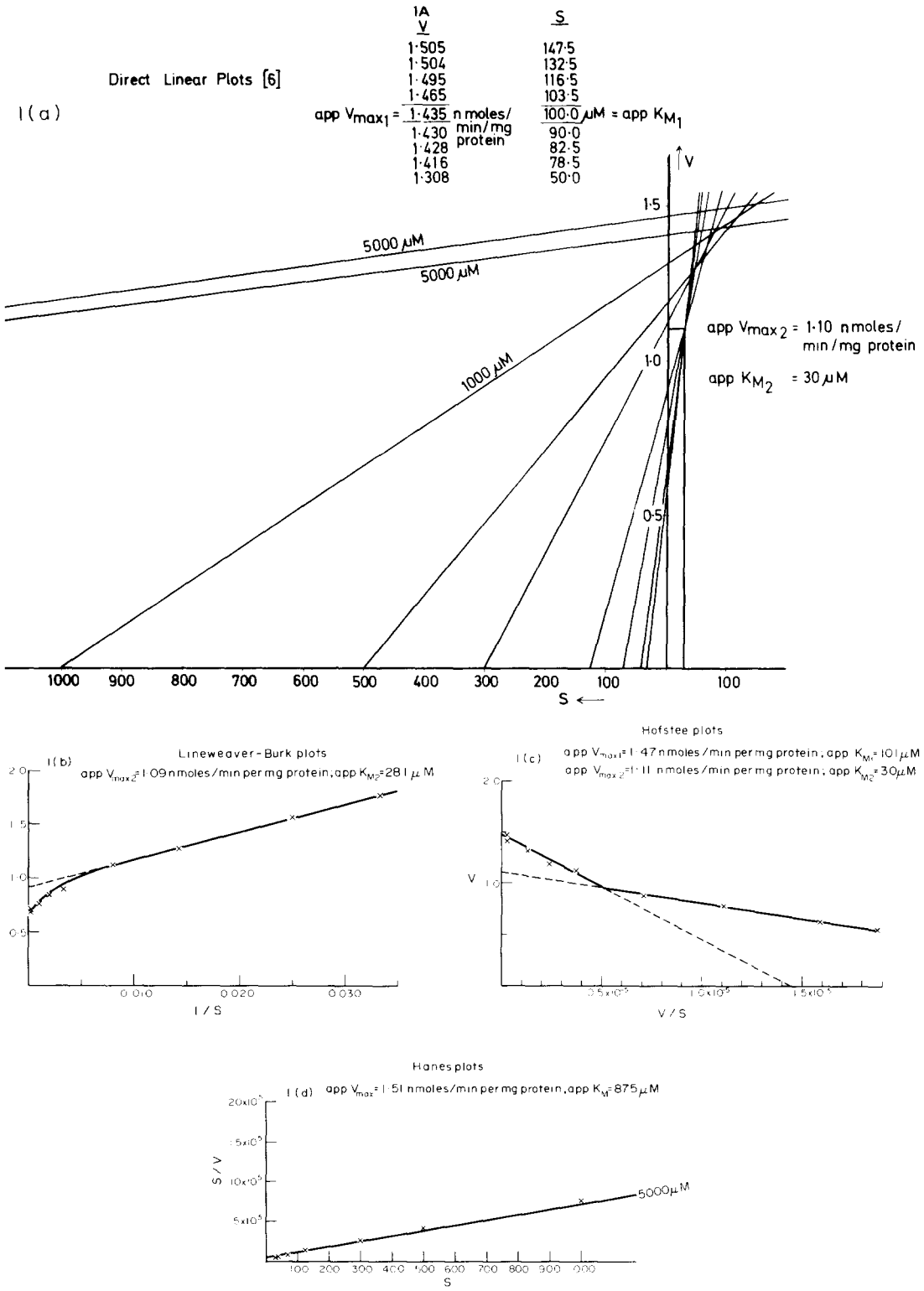

Fig. 1A–D. Kinetic curves for (*o*-1)-hydroxylation of 4-chloropropionanilide (the curve for *o*-hydroxylation is not shown) obtained from pairs of v and S in a single experiment with hepatic microsomes from untreated rabbits are shown in figures 1A, 1B, 1C, and 1D, corresponding to plots by Eisenthal and Cornish Bowden, Lineweaver Burk, Hofstee, and Hanes, respectively. Kinetic analyses were performed with 3 mg microsomal protein per ml incubation fluid; the relationship between specific activity of *o*- and (*o*-1)-hydroxylation and microsomal protein content was linear under the conditions used for 1–4 mg of protein. v is expressed as nmoles of 4-chlorolactanilide formed per min per mg of microsomal protein, S in μM.

or enzyme preparation, but to estimate alterations in specific enzyme activities and affinities, as they are observed following a repeated administration of certain drugs (induction processes), or if a second (drug) substrate modifies enzyme activity or (and) affinity by either activation or inhibition.

Direct linear plots of v versus S permit the detection of allosteric or cooperative effects. Whereas in most cases the kinetic curve has a hyperbolic shape, in cases of allosterism or cooperativity, sigmoidicity of the curve is observed. When double reciprocal plots of v and S (Lineweaver Burk) were used to establish a relation between v and S, mostly a linear function was observed. Sometimes, however, kinetics have been obtained which were linear at low and curved downward at high substrate concentrations or linear at high and curved upward at low substrate concentrations.* Although there are many reasons for a curvature at high substrate concentrations, it was explained by most authors as an indication for the presence of a second enzyme acting on the same substrate (a two enzyme one substrate relation) in microsomal membranes. A variety of reasons may explain an upward curvature of Lineweaver Burk plots at low substrate concentrations, they are: an irreversible binding of substrate to an inhibitor, further metabolization of the oxygenation product, an interference of endogenous substrates of the microsomal membranes with oxygen binding, or a limitation in the diffusion of oxygen through the microsomal membranes.

3.2. *One enzyme or many?* Application of two substrate kinetic analysis [11] to drug metabolism has contributed to settle the long-discussed problem, whether or not 3-MCh treatment causes qualitative changes of the cytochrome P-450 moiety, that is, the formation of a second enzyme system besides that which is present in normal livers. Two-substrate kinetic analyses are performed to determine whether or not two similar oxygenation reactions are catalyzed by a single nonspecific oxygenase or by many specific oxygenases. Sladek and Mannering [12] performed two-substrate kinetics of *N*-demethylation with ethylmorphine and 3-methyl-4-methylaminoazobenzene as substrates to determine whether PB-treatment or 3-MCh-treatment of rats causes the enhanced formation of the same or another oxygenating system in microsomal membranes (quantitative or qualitative differences). In the first part of the kinetic analysis the concentration of 3-MMAB was kept constant and that of ethylmorphine was varied. Formaldehyde formation from either substrate was used to determine the sum of both velocities. In the second part of the kinetic analysis the concentration of ethylmorphine was varied in the absence of 3-MMAB. When the reciprocal velocity of formaldehyde formation was plotted versus reciprocal ethylmorphine concentration, hyperbolic curves were obtained in the presence of 3-MMAB with liver microsomes from untreated and PB-treated rats, but not from 3-MCh-treated rats. These curves intersected a similarly plotted linear curve obtained in the absence of 3-MMAB if normal or PB-stimulated, but not when 3-MCh-stimulated liver microsomes were employed. With 3-MCh stimulated microsomes both kinetic curves did not intersect nor merge, an indication for the involvement of two different enzyme systems in the *N*-demethylation of ethylmorphine and 3-methyl-4-methylaminoazobenzene in 3-MCh-stimulated liver microsomes.

Two-substrate kinetics of *N*-demethylation with morphine and ethylmorphine as substrates [13] have also contributed to understand alterations in enzyme activity due to repeated administration of PB, especially to understand the classic issue of drug metabolism, whether only quantitative or qualitative changes occur in the drug metabolizing enzyme system after repeated administration of PB.

3.3. *Kinetic analysis and mechanism of oxygenation.* Kinetic analysis can be an appropriate tool for analyzing and understanding the complex mechanism of drug oxygenation, if the rates of all discrete oxygenation reactions of a substrate which really occur, are measured and not simply one. This is not feasible at present, because not all specific metabolic pathways are known for that particular substrate under investigation and because it certainly is a time consuming task to work out sensitive and specific assay procedures for the estimation and to estimate several different products at a time. Archakov *et al.* [14] and a few authors before have drawn the attention of the investigators of drug metabolism to the often neglected fact that most (drug) substrates are not metabolized by a single specific oxygenation reaction, but by a variety of distinct oxygenations, each generating a defined derivative of the substrate, and that the primary oxygenation products may be further metabolized. *N,N*-dimethylaniline, for example, may serve as substrate for *N*-demethylation, *N*-oxygenation, and *p*-hydroxylation. In addition, *N*-methylaniline, *N,N*-dimethylaniline-*N*-oxide, and aniline being oxygenation products themselves, may also serve as substrates for further *N*-demethylation, *N*-oxygenation, or *p*-hydroxylation. Archakov *et al.* have found that in this complex reaction, where starting material, primary and secondary metabolites can serve as substrates, all discrete oxygenations differ in I_{max} and K_M, and that even for *N,N*-dimethylaniline as substrate the kinetic constants for the three specific oxygenation reactions (*N*-demethylation, *N*-oxygenation, and *p*-hydroxylation) are different. Table 1 contains these results, and similar results were obtained from other authors with different substrates.

When they measured the velocity with which microsomal cytochrome P-450 is reduced by NADPH in the presence of *N,N*-dimethylaniline or amidopyrine and their oxygenation products, they found that *N,N*-dimethylaniline increased the reduction rate of the cytochrome P-450 complex, but that the *N*-demethylation proceeded at a much higher rate than either *N*-oxygenation or *p*-hydroxylation, and that aniline, which decreased the rate of reduction was *p*-hydroxylated with the same velocity as *N,N*-dimethylaniline. These data allowed them to deduce that reduction of the cytochrome P-450-substrate complex is not the rate-limiting step in drug oxygenation.

4. *Essentials for kinetic analyses*

In the early experiments on mixed function oxygenation the velocity of drug oxygenation was deter-

* Kinetics curved upward at high substrate, indicative of substrate inhibition, will not be discussed here.

Table 1.

Enzyme source	Substrate	Specific metabolic pathway	V_{max} (nmoles min mg protein)	*app.* K_M (mM)	References
Rat liver microsomes	*N,N*-dimethylaniline	*N*-demethylation	1·93	0·128	[15]
	N,N-dimethylaniline	*N*-oxygenation	0·75	0·093	
	N,N-dimethylaniline-*N*-oxide	*N*-demethylation	13·0	33·0	
Rat liver microsomes	*N,N*-dimethylaniline	*N*-demethylation	6·2	0·66	[14]
	N,N-dimethylaniline	*p*-hydroxylation	0·53	0·26	
	N,N-dimethylaniline-*N*-oxide	*N*-demethylation	17·0	70·0	
	N-methylaniline	*N*-demethylation	4·8	0·50	
	Aniline	*p*-hydroxylation	0·57	0·14	
Rabbit liver microsomes	Aniline	*p*-hydroxylation		0·53	[16]
	Aniline	*N*-hydroxylation		5·8	
Rat liver microsomes	Amidopyrine	*N*-demethylation	4·1	0·42	[14]
	Nor-amidopyrine	*N*-demethylation	5·4	0·62	
	4-aminophenazone	*N*-demethylation	n.d.	n.d.	
Rabbit liver microsomes	4-Chloropropion-anilide	*o*-hydroxylation	0·066	0·085	[17]
		(*o*-1)-hydroxylation	$\begin{cases} 1·256 = V_{max_1} \\ 0·686 = V_{max_2} \end{cases}$	$\begin{matrix} 0·466 = K_{M_1} \\ 0·019 = K_{M_2} \end{matrix}$	
Rat liver microsomes	[^{14}C]Griseofulvin	4-*O*-demethylation	0·30	0·22	[18]
		6-*O*-demethylation	0·32	0·33	

mined by measuring the oxygen consumption during the incubation period after it had been established that one oxygen atom of the O_2-molecule is inserted in the substrate and the other is reduced and utilized as an acceptor for the protons released during the oxygenation cycle. A second method was to correlate the decline in NADPH concentration during the incubation period with drug oxygenation. Both methods are now obsolete, not only because of lack of specificity, but also because oxygen as well as NADPH are also consumed in the absence of substrate as well as in the presence of such substrates as perfluoro-*n*-hexane, which stimulate NADPH oxidation but are not oxygenated themselves (so-called uncouplers). In both cases an accumulation of H_2O_2 is observed which effects *i.a.* lipid peroxidation. Kamataki and Kitagawa [19] have shown that lipid peroxidation can alter the kinetic constants of a variety of drug oxygenations and that addition of 100 μM EDTA effectively inhibits lipid peroxidation. However, addition of EDTA was reported to decrease enzyme activities in few cases.

Furthermore, NADPH may be metabolized by enzyme(s) residing in the microsomal fraction (nucleotide pyrophosphatase) which is prominent only in rat liver microsomes.

Perfluoro-*n*-hexane was recognized as an uncoupler of mixed function oxygenation because attempts have failed to prove oxydative displacement of fluorine. Obviously, the C–F bonds in perfluoro-*n*-hexane resisted the attack by the oxygenase. It is noteworthy that C–F bonds do not generally resist the attack by oxygenase. Gottlieb *et al.* [20] have shown that fluorine is not only oxydatively displaced from aromatic *p*-fluoro compounds, like *p*-fluorophenylaniline or 4-fluoroaniline, but also from alipathic compounds as *trans*-4-fluoroproline, which is catalyzed by proline hydroxylase from guinea pig granuloma minces. Thus proline hydroxylase, one of the mixed function oxygenases, resembles other (aromatic) oxygenases in its mode of action.

For determining the velocity of drug oxygenations today, most investigators chose the rate of formation of the oxygenated substrate, or if it is unstable as is the case with aromatic hydroxymethylether or hydroxymethyl-methylamines, with its decomposition products.

4.1. *Lack of sensitivity and specificity of the assay procedure.* Some investigators have preferred to determine the amount of formaldehyde produced from xenobiotics with more than one *N*-methyl group, like for example amidopyrine or *N,N*-dimethylaniline, instead of estimating the *N*-demethylation product(s), that would have been more specific. Because 3 moles of formaldehyde can be generated from amidopyrine, at least three different compounds may serve as substrates for *N*-demethylation, namely amidopyrine itself, Nor-amidopyrine, and 4-aminophenazone (desdimethylamidopyrine), each being distinguishable in affinity (K_M) and reaction rate (V_{max}). This has been proved for the first and second stage of *N*-demethylation of amidopyrine and the corresponding stages of *N,N*-dimethylaniline (see Table 1). 4-Amino-phenazone may be further *N*-demethylated to 4-amino-1-phenyl-3-methyl-pyrazol-5-one, a biochemical reaction which was shown to proceed *in vivo* but has not yet been measured *in vitro*. Only few, because of lack of specific assay procedures, determined the residual substrate. This method, which suffers from a lack of specificity, has been applied for the metabolism of diphenylhydantoin, for the hydroxylation of benzo(a)-pyrene, and for the metabolism of hexobarbital. It was only recently that a more specific assay procedure has been introduced for the metabolism of hexobarbital, which permits to measure the time course of hydroxylation in 3′-position. The method of studying drug metabolism by measuring the rate of disappearance of substrate also suffers from a lack of specificity.

It is therefore recommendable to find and use a sensitive and specific assay procedure which permits the determination of that product with sufficient accuracy, whose formation rate is to be measured.

4.2. *Assay conditions*

4.2.1. *Initial velocity.* Chief factors which determine the initial velocity are: Enzyme concentration, expressed in mg of (microsomal) protein per ml incubation mixture, substrate concentration, pH, temperature, the presence of a second substrate, the specific activity of the enzyme source (depending on the grade of induction of drug-metabolizing enzymes), and lipid peroxidation, which however, is species-dependent and is highest in rats. If the experimental conditions have been worked out under which the initial velocity of a special oxygenation reaction to be studied is linear by determining v versus t, v is then assayed on variation of S. Substrate concentrations to be used vary from $1.3 \times K_M$ to $3 \times K_M$ as recommended by Wilkinson [4] or $1.5 \times K_M$ to $5 \times K_M$ as recommended by Cleland [5].

4.2.2. *Enzyme concentrations.* Enzyme source. Some authors have observed higher oxygenation rates with the 9000 g supernatant fraction of liver homogenate as the enzyme source than with microsomes (105,000 g pellet). However, since the 105,000 g supernatant contains a variety of enzymes for which the primary oxygenation products formed by microsomes are substrates, the determination of actual concentrations of primary oxygenation products with the 9000 g supernatant might be misleading, and the same holds for hepatocytes being employed as enzyme source. Investigators of microsomal enzyme kinetics have used microsomal protein concentrations of 1–4 mg per ml incubation fluid. Although it was recommended to choose 2 mg or less [28], a general recommendation concerning the protein concentration cannot be given at present. If a (drug) substrate is oxygenated in various positions of the molecule and the rates of all these or at least some of them are to be determined, then one probably finds that the V_{max}- as well as the K_M-values vary considerably, so that with a given protein concentration of 1 mg per ml, the kinetic data for a rapid oxygenation reaction could be determined, but not for a slow, because of lack of sensitivity of the assay procedure. In this case the experiments may be carried out with 2 or 3 mg ml, provided a linear relationship is established between the oxygenation rate and the concentration of microsomal protein, that is, if the specific activity, expressed in μmoles or nmoles product formed per mg protein per minute, is independent on protein concentration. The finding that the specific activity of a special oxygenation reaction is greater with 1 mg protein ml than it is with 3 mg, reflects a limitation in the capacity of the method of estimation due to an exhaustion of substrate or of the cofactors required for mixed function oxygenation. Investigators of kinetic analysis may experience pitfals if they compare specific activities of different microsomal enzyme preparations, for example, controls versus PB- or 3-MCh-stimulated microsomes without having established this linearity with each specific preparation before. However, if the limitation is caused by the presence of inhibitors of NADPH-cytochrome c reductase formed from NADPH by the action of an enzyme (pyrophosphatase) present in rat liver microsomes, then a dependence of K_M (NADPH) for benzo(a)pyrene hydroxylation on protein concentration is observed. It was recommended therefore, that protein concentration should not exceed 0·1 mg ml [21]. In another case, the explanation for a linear relationship between the K_M for benzo(a)pyrene hydroxylation, effected by the microsomal fraction of hamster fetus cell cultures, and the microsomal protein concentration was to assume nonspecific binding of substrate to nonspecific enzyme site(s) or other membrane components [1].

4.2.3. *On the use of nicotinamide for measuring rate constants.* In 1941 Mann and Quastel [22] detected that the breakdown of NADPH or $NADP^+$ in homogenates of rat brain or rat liver due to the presence of a NADPH-nucleotidase, could be inhibited by the addition of 0·1% nicotinamide. Without to determine whether this additive affects drug oxygenation unless NADPH is the limiting factor in the incubation fluid, nicotinamide was added in the early experiments on mixed function oxygenation in concentrations ranging from 8 mM to 50 mM. Schenkman *et al.* [23] who studied the effect of nicotinamide on the rate constants for the *N*-demethylation of amidopyrine and *p*-hydroxylation of aniline with concentrations from 5 to 50 mM, and Sasame and Gillette [24] who studied its effects on the rate constants for the *N*-demethylation of amidopyrine and ethylmorphine and *p*-hydroxylation of aniline with concentrations ranging from 6·6 mM to 40 mM, found that nicotinamide even in such low concentrations as 5 mM can alter the *app.* K_M for a substrate and give rise to an inhibition pattern depending on the substrate and the enzyme source. In addition, Schenkman *et al.* found that the presence of 50 mM of nicotinamide did not significantly protect NADPH so that enzyme activity decreased during the incubation period. Cohen and Estabrook [25] confirmed the inhibitory effect of nicotinamide on the *N*-demethylation of amidopyrine but demonstrated the stabilizing effect of 10 mM nicotinamide on the concentration of NADPH in the NADPH-regenerating system. The presence of 10 mM nicotinamide in microsome suspensions prepared from the livers of rats enabled them to measure initial rates of *N*-demethylation. Parli and Mannering [26] studying the *N*-dealkylation of morphine, ethylmorphine, 3-MMAB, and SKF 525-A employing rat liver microsomes and concentrations of 4 and 50 mM nicotinamide, found some inhibition of the *N*-dealkylation of 3-MMAB and SKF 525-A as substrates even with the lowest concentration of nicotinamide. However, the effect of 12 mM nicotinamide on the ω- and (ω-1)-oxygenation of 4-chloropropionanilide by PB-stimulated rabbit liver microsomes appears complex: whereas (ω-1)-oxygenation is slightly inhibited, ω-oxygenation is activated [17]. Netter and Illing [27] who studied NADH oxidation rates with rat liver microsomes confirmed the results of Cohen and Estabrook that nicotinamide is necessary to maintain a constant level of NADPH but that the lowest effective concentration was 0·4 mM. Evidence has been presented by several authors that nucleotide pyrophosphatase is far less active in corresponding enzyme preparations from rabbits, guinea pigs, dogs, or mice as it is in rats, so that a generalized employment of nicotinamide does not seem necessary nor desirable.

4.2.4. *Substrate concentration.* Some investigators have used a relatively short range of substrate concentrations (25–100 μM for example) to estimate the kinetic constants as if they could be certain that only

one enzyme is involved in that specific oxygenation reaction studied. This certainty, however, does not exist if the microsomal fraction or the 9900 *g* supernatant fraction of liver or adrenal cortex homogenate is used as enzyme source in oxygenation reactions. Short range plots may show linearity, but if it is only a part of a curved kinetic, it would have little meaning; if only a short range of low substrate concentrations is used, the part of the kinetic where substrate activation (due to the action of a second enzyme) is likely to be observed and which then may be curved downward, is missing. It may also be difficult to detect the true shape of a kinetic curve with short range plots, for, if its curvature is only small, it would not be detected.

Although the lower limit of the substrate concentration normally is the sensitivity of the assay method, the postulate of the Michaelis Menten theory holds, that even at low S values the concentration of substrate should be much higher than that of the enzyme in order to enable still the formation of the enzyme–substrate complex, otherwise too small values for v are obtained.

The upper limit of substrate concentration usually is the solubility and (or) the homogenous distribution of the substrate in the incubation mixture. As one is dealing mostly with lipophilic organic molecules, their solubility in aqueous systems is limited, and at high concentrations the problem of an even distribution may arise.

Factors which affect an even distribution of substrate in the incubation mixture are the shaking rate of the flasks and agitation, whose efficiency may be increased by the addition of glass marbles [28], but also the properties of the enzyme source, for example of microsomal preparations. It was observed by the author that the distribution of substrate was much better in suspensions with PB-stimulated than with controls or 3-MCh-stimulated rabbit liver microsomes, probably due to the increased content of phospholipids in this enzyme source. However, a high shaking rate is not only required for an even distribution of substrate, but also to improve oxygen supply in those monooxygenations, in which the velocity increases with increasing substrate concentration.

4.2.5. *Standardisation.* Investigators using enzyme preparations from laboratory animals for their kinetic studies should try to perform kinetic analyses under standardised conditions, that is, beginning with age and sex of the animals (sex differences are relevant only in rats and mice), the preparation of the enzyme source and the performance of the assay itself should be carried out always in the same manner within the same time period. If this is ignored, results within one series of experiments as well as results from various laboratories cannot be compared. The considerable variation of the kinetic constants among different laboratories, for example, the variation of K_M for aniline *p*-hydroxylation (14·0–200 μM) or of K_M for benzo(a)pyrene hydroxylation (2·95–60·6 μM) may, at least, partly be due to the lack of uniformity of assay performance. The requirement of standardisation not only for the experiments, but also for feeding, holding and inducing the animals stems from our present knowledge on the alterations of V_{max} and K_M during postnatal development and maturation in laboratory animals, on changing the periods of feeding (starvation) and the diet, on differences in the kinetic constants between male and female rats and mice, on differences in enzymic activity within 24 hr (circadian rhythms) and within a year (seasonal rhythms), and on the decrease of some, but not all enzyme activities during storage at low temperatures.

5. *Limitation of applicability*

5.1. *Composition of microsomal enzymic complex.* If the microsomal fraction (105,000 *g* sediment) or the 9900 *g* supernatant fraction of liver or kidney cortex homogenate prepared from various vertebrata is used as enzyme source for studying mixed oxygenation *in vitro*, one should be aware of the heterogeneity of the enzyme system one is dealing with. Hepatic microsomes are artefacts that are formed by destruction of the endoplasmic reticulum and are composed of rough and smooth microsomes as well as fragments from the cell membrane, of lysosomes and peroxisomes, and they are probably contaminated from membranes from the Golgi apparatus, plasma membrane, and disrupted mitochondria. The microsomal fraction is composed of phospholipids, protein, hemin, steroids, and ribonucleic acid, and their particle size is 60–200 μm in diameter. Microsomes have been shown to contain not only the components of the electron transport chains for mixed function oxygenase system with the oxygen-activating terminal oxygenase cytochrome P-450 integrated in a membrane, but also enzymes with hydrate epoxides, dehydrogenate certain carbinols, reduce ketones, nitro compounds, *N*-hydroxy derivatives of aryl- or alkylamines or *N*-arylacetamides, and azo compounds, deacylate esters or amides (esterases), and glucuronidate amines, phenols, alcohols, hydroxamic and carboxylic acids. In addition, microsomes contain enzymes, such as glucose-6-phosphatase, NADH-semidehydroascorbate-oxidoreductase, and 3-ketosteroid-Δ^4-dehydrogenase which are not directly involved in mixed function oxygenation. A variety of soluble enzymes have been shown to reside in the 105,000 *g* supernatant fraction, so that the number of enzyme components is still much higher in such preparations, if the 9900 *g* supernatant is used as the enzyme source.

5.2. *Problem of kinetic constants measurements with non-purified enzymes.* What do kinetic constants really mean if they have been obtained not from single, purified enzyme entities in solution, but from unsoluble membrane-bound multicomponent enzyme systems? Can the observed activities be analyzed and described by the use of Michaelis–Menten equations derived for single, soluble purified enzymes?

It was argued that not the absolute value of the kinetic constants is relevant, but that they can serve only as indicators of changes in enzyme activity, not definers of change. If for example, alterations of enzyme activity are observed due to various induction processes or due to activation or inhibition by the interference of a second drug, then characteristic changes of the kinetic constants are observed. Thus, K_M can merely reflect a rate limiting step, but it is interesting to know when a rate limiting step is shifted from one reaction site to another [29].

5.3. *Immobilized enzymes as a model.* Recent experiments on the kinetic behaviour of enzymes immobi-

lized in artificial membranes have elucidated some of the rules which control heterogeneous enzyme kinetics. Results obtained with such systems can serve as a model for analyzing the kinetic behaviour of the multicomponent enzyme system present in microsomes.

Boguslaski *et al.* [30] using urease integrated into a membrane, analyzed the factors which determine rate constants. They found that the formation of the enzyme substrate complex is complicated by interphase diffusion and mass transport of reactants and products, and that a critical factor is the thickness of the membrane. Three models were designed to test the influence of diffusion and mass transport on the rate constant: a membrane covered sensor, a membrane separating two solutions of different concentration, and a membrane immersed into a solution. Among them, the immersed membrane seemed to be the most promising and widely applicable for the determination of rate constants.

Sundaram *et al.* [31] discussed five main reasons why enzymes in solution behave differently as compared to those attached to solid supports: since kinetic behaviour depends on enzyme conformation, free enzymes in solution may undergo conformational changes, whereas solid-supports may be hindered more or less, depending on the chemical nature of the membrane. The formation rate of the enzyme–substrate complex depends on the dielectric constant of the medium, for the concentration in the environment of a membrane-bound enzyme is different from that of a free enzyme. There will be partitioning of the substrate between the solution and the membrane: the concentration of substrate in the vicinity of the membrane-bound enzyme may be different from that of the free enzyme in solution. Diffusion may play an important role for the reaction within the membranes: reactions in which free enzymes in solution are involved are usually not diffusion controlled. However, if the thickness of the membrane is lower than 1 mm, or if the reaction is very slow, diffusion will not be the rate-limiting step. At low substrate concentrations, reaction rates will be less for the immobilized enzyme, if the substrate is less soluble in the membrane than in solution, and, besides, *app.* K_M will be greater. Conversely, if the substrate is more soluble in the membrane, reaction rates will be greater and the *app.* K_M smaller.

5.4. *Differences between membrane-bound and soluble enzymes.* Hervagault *et al.* [32] studied the kinetic behaviour of two enzymes, xanthine oxidase and uricase, both in solution and when bound to a protein membrane. As substrate served xanthine, a competitive inhibitor of uricase, for which uric acid, formed by the action of xanthine oxidase, is a substrate. Uric acid is transformed by uricase into allantoin. Experiments on the kinetic behaviour of uricase in solution and immobilized in a membrane were first performed in the absence of xanthine oxidase with increasing amounts of xanthine. It turned out that the immobilized uricase was less sensitive to inhibition by xanthine than the free enzyme in solution. The effect of a second enzyme was investigated in the presence of xanthine oxidase in the membrane. Whereas the instantaneous activity of uricase in solution was determined only by the concentration of substrate and inhibitor, the activity of membrane-bound uricase was additionally determined by local concentrations of substrate and inhibitor, modulated by the second enzyme. When the effect of the concentration of the second enzyme in the membrane on uricase activity was studied, it turned out that, above a critical concentration of xanthine oxidase, uricase activity was higher in the presence of the inhibitor xanthine than without it due to the modulation of the concentrations inside the 'tertiary structure' of the membrane. Due to modulation of the relative concentrations of substrate and metabolites inside the membrane is the fact that, provided the concentration of xanthine oxidase is high enough, the activity of the membrane-bound uricase is higher than that of the free enzyme in the bulk solution.

5.5. *Application.* A comparison of the kinetic properties of free enzymes in solution with that of the immobilized bienzyme system showed that, at least for a fixed ratio of xanthine oxidase to uricase, the maximum enzyme activities are the same in both cases. It was due to the high initial velocity of the bienzyme that during the first 2 hr three times more allantoin was formed than by the free enzymes in solution.

The thickness of the microsomal membrane is far below the critical value, and the immersed membranes were recognized as an appropriate model to eliminate interphase diffusion and the transport of reactants and metabolites as the rate-limiting step. As for enzyme conformation, evidence has been presented that among others, a binding site exists in microsomal membranes which displays a sigmoidal behaviour on binding (drug) substrates [33]. This is in agreement with the findings of Hlavica [34, 35] that conformational changes of the CO-sensitive cytochrome P-450 may play an important role in the *N*-oxygenation of aniline or *N,N*-dimethylaniline by rabbit liver microsomes. Thus, conformational changes of the enzymes effecting mixed function oxygenation do not seem to be hindered by integration into a protein membrane.

Although there is no conclusive evidence today that the observed kinetic constants (*app.* V_{max}, *app.* K_M) reflect the actual kinetic behaviour of the enzymes participating in mixed function oxygenation, the results of the aforementioned papers suggest that microsomal membranes constitute a system applicable for the determination of rate constants.

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