Kinetic Studies on the Control of Ethylmorphine N-Demethylase. The Role of Ethylmorphine Activation of Reduced Nicotinamide Adenine Dinucleotide Phosphate–Cytochrome P-450 Reductase†

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ABSTRACT: The addition of the substrate ethylmorphine to hepatic microsomes stimulates the activity of the NADPH–cytochrome P-450 reductase. The degree of stimulation is affected by the order of addition so that when the NADPH and substrate are added together, there is only 28% the stimulation as when the substrate is added before, suggesting that the stimulatory process takes a few hundred milliseconds to reach completion. The ethylmorphine N-demethylase activity for four pools of male rats gave straight lines on the double-reciprocal plots with an average K_m of 0.25 mM ($\pm$0.01) (SEM of pools) and a V_m of 9.5 nmol of HCHO formed per minute per mg of protein. The maximal stimulation of the NADPH cytochrome P-450 reductase was 8.7 nmol/min per mg of protein with two sites having K_s's of 0.029 $\pm$ 0.006 and 1.0 $\pm$ 0.2 mM and a combined site of 0.15 $\pm$ 0.02 mM. The type I spectral site gave a K_s of 0.07 $\pm$ 0.006 mM. In three pools of female rats the N-demethylase gave a K_m of 0.64 $\pm$ 0.04 mM with a V_m of 2.3 $\pm$ 0.2 nmol of HCHO formed per min per mg of protein. There was only a single site for the stimulation of the reductase with a K_s of 0.067 $\pm$ 0.008 mM and a V_m of 2.47 $\pm$ 0.09 nmol/min per mg of protein. The type I binding site gave a K_s of 0.05 $\pm$ 0.01 mM. These data indicate that there are separate activation and catalytic sites for ethylmorphine. Further there are two activation sites in the male rat. In the male rat none of these sites seem to be related to the type I spectral binding site. The good stoichiometry between the maximal activity for the N-demethylase and the stimulation of the reductase would indicate a coupling between the transport of the first electron to reduce the cytochrome P-450 and the formation of product. Finally there would appear to be activation sites with sufficiently high affinity for the substrate so that the activation process may be significant in the in vivo metabolism of the drug.

P resent evidence would suggest that the rate-limiting step of those mammalian mixed-function oxidases which contain cytochrome P-450 is the reduction of this cytochrome by the appropriate reductase (Holtzman et al., 1968; Schenkman, 1972). It would, therefore, appear to be the optimal step for controlling the overall rate of hydroxylation since small changes in the rate of this reaction will have a more direct and proportional effect on the rate of the overall reaction than will changes in any other. This concept has received support from the studies of Gigon et al. (1968, 1969) in which they demonstrated that type I substrates, as ethylmorphine and hexobarbital (Remmer et al., 1966), activated the NADPH–cytochrome P-450 reductase. Further, they found that this increase in the reductase activity on the addition of ethylmorphine was, for a one-electron transfer, stoichiometric with the total N-demethylase activity. These results suggested a scheme for the hepatic, microsomal mixed-function oxidases in which (1) the presence of the substrate is one factor in controlling the flux of electrons to the cytochrome P-450, and (2) the increased flux of electrons through the microsomal chain is tightly coupled to the metabolism of the substrate.

This model would suggest that varying the concentration of the substrate would have a dual effect on the rate of N-de-

methylation, the first being on the rate of activation and the second on the rate of the actual catalysis. Such a scheme would appear to be best described as a second-order extension of the usual Michalis–Menten equation (cf. Chapter IV, p 60, in Dixon and Webb, 1964). Yet neither have we, nor to our knowledge any other investigators, observed any nonlinearity in innumerable kinetic plots of this N-demethylase activity suggesting that the activation and catalysis sites have significantly different affinity constants. The apparent K_m for the N-demethylation is determined by the site which has a dissociation constant in the range of the substrate concentrations used.

In view of these considerations we have investigated the kinetic properties of the activation site and find, as recently reported, that it (1) has a high-affinity constant, (2) is almost fully saturated at the apparent K_m for the N-demethylase, (3) is active at 2 $\times$ 10^{-5} M so is probably significant in vivo, (4) shows some sex differences without totally accounting for the differences in kinetic constants, and (5) has an affinity constant close to that for the type I binding site (Holtzman and Rumack, 1971a,b).

Methods

Both the male (180–200 g) and female (160–180 g) rats used in these experiments were fed, Sprague-Dawley rats obtained from Charles River, Inc. The animals were sacrificed by cervical fracture. Several livers were chilled, homogenized in three milliliters of KCl–Tris (150–20 mM; pH 7.4) per g of liver wet weight, and centrifuged at 9000g for 15 min in a Sorval RC-2B centrifuge with an SS-34 rotor. The supernatants were pooled and centrifuged at 165,000g (av) for 38 min in a Beckman
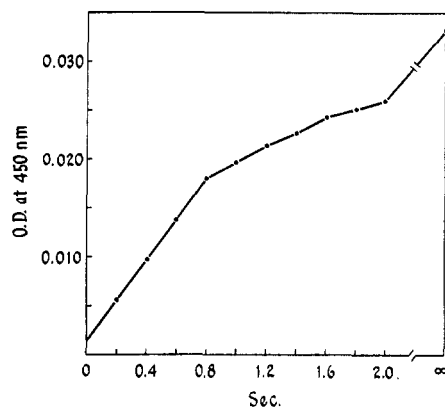
FIGURE 1: Determination of NADPH–cytochrome P-450 reductase activity in a stop-flow apparatus. Preparations and assays given in the text.

TABLE II: The Effect of Varying Concentrations of Ethylmorphine on the NADPH–Cytochrome P-450 Reductase Activity of Hepatic Microsomes from Male Rats.[a]

Ethylmorphine Concn (mM)	No. of Determinations	nmol of P-450 Reduced/min × mg of Protein	Difference from 0 mM Ethylmorphine
0	12	11.2 ± 0.1[b]	
0.02	6	13.3 ± 0.3	2.1
0.05	6	14.7 ± 0.3	3.5
0.10	3	15.6 ± 0.3	4.4
0.20	3	17.2 ± 0.8	6.0
0.50	3	18.9 ± 0.5	7.7
1.00	3	19.4 ± 0.7	8.2

[a] Assays were run on a single pool of hepatic microsomes. Preparations and assays are described in the text. [b] Values are average ±SEM.

L2-65B centrifuge with a 50 Ti rotor. The pellets were resuspended to give the equivalent of 2 g of liver/ml of KCl–Tris and the protein concentration was determined (Sutherland et al., 1949). Each pool of rats was treated as a single animal for the purposes of statistical analysis.

Ethylmorphine N-demethylase was essentially determined as previously described (Holtzman et al., 1968). In this assay varying volumes (2–40 μl) of ethylmorphine-HCl (150 mM) were added with a Hamilton PB 600 dispenser to 20-ml serum vials which were then placed in a 37° shaking water bath. An incubation mixture of NADP (0.33 mM), glucose 6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (0.67 units/ ml) was dissolved in KCl–Tris buffer and brought to 37° in a water bath. Just before zero time the microsomal suspension (1 mg of protein/ml) was added to the incubation mixture and 3 ml of the complete mixture was added to each vial. The vials were incubated for 10 min under air and the reaction was terminated by the addition of 1 ml of 5% $ZnSO_4$ followed by 0.5 ml of $Na_2B_4O_7 \cdot 10H_2O$ (saturated) and 1.5 ml of $Ba(OH)_2$ (saturated). After centrifugation, the formaldehyde was determined in the clear supernatant with acetylacetone in 4 M ammonium acetate (Nash, 1953).

The NADPH–cytochrome P-450 reductase activity was determined essentially as previously described (Holtzman and Carr, 1972). In this assay, 3 ml of a microsomal suspension containing 3 mg of protein/ml of KCl–Tris was placed in an Aminco anaerobic cuvet, the appropriate concentration of ethylmorphine was added, and the mixture was gassed for 5

min with carbon monoxide which had been deoxygenated by passage through alkaline dithionite (Holtzman et al., 1968). The plunger was filled with 10 μl of a NADPH solution (100 mg/ml), the cap was greased with Apeizon N, and the cell was capped. The gas phase above the microsomal suspension was gassed for another minute, the gas vents were closed, and the cuvet was warmed to 37° for 7 min in an Aminco-Chance spectrophotometer. The reductase activity was taken as the initial rate of change in the absorbancy of $\epsilon_{450-490}$ using an extinction coefficient of 91,000 for cytochrome P-450 (Omura and Sato, 1964a,b).

The affinity of the type I binding site was determined by placing a cuvet containing 3 ml of a microsomal suspension (3 mg of protein/ml of KCl–Tris) into each side of an Aminco-Chance spectrophotometer in the split-beam mode. The cells were allowed to warm to 37° for 5 min. A solution of ethylmorphine (60 mM) was added stepwise to the sample side with a Hamilton PB-600 dispenser and equal volumes of water were similarly added to the reference side. Difference spectra were taken between 350 and 500 nm after each addition.

The NADPH–cytochrome P-450 reductase activity was also determined in an Aminco Morrow stop-flow apparatus at 450 nm. The samples were gassed for 7 min at 37° with carbon monoxide, anaerobically removed, and placed in the stop-flow, and activity was determined. The output from the photomultiplier tube was directly fed to Tektronic storage oscilloscope. An offset voltage was provided by a battery box and the absorbancy was calculated from a Polaroid photograph of the oscilloscope pattern.

All of the above data were plotted as double-reciprocal plots and the kinetic constants were determined by inspection and confirmed by computer analyses (Holtzman and Carr, 1972).

Results

Since the rate of reduction is very rapid some question has been raised as to whether the Aminco anaerobic cuvet used in conjunction with the standard recording system on the Aminco-Chance spectrophotometer is sufficiently rapid to obtain values close to the true initial NADPH–cytochrome P-450 reductase activity. In this system the combined dead and recovery times are 0.8–1 sec. During this time as much as 20%

TABLE I: The Effect of the Order of Addition on the Stimulation by Ethylmorphine of the NADPH–Cytochrome P-450 Reductase of Hepatic Microsomes from a Male Rat.[a]

Ethylmorphine Concn (mM)	Time Added	nmol of P-450 Reduced/min × mg of Protein	Difference from 0 mM Ethylmorphine
0		9.70 ± 0.09[b]	
1	Before NADPH	14.10 ± 0.63	4.49
1	With NADPH	10.89 ± 0.01	1.28

[a] Preparations and assays given in text. [b] Values average of three runs ± SEM.

TABLE III: The Kinetic and Spectral Parameters for Ethylmorphine of Hepatic Microsomes from Male Rats.[a]

Activity	Site	K (mM)	V_{max}
Ethylmorphine N-demethylase		0.286	$11.4 \dfrac{\text{nmol of HCHO}}{\text{min} \times \text{mg of protein}}$
Cytochrome P-450 reductase activation (Δ_{red})	I	0.0370[b]	$6.1 \dfrac{\text{nmol of reduced}}{\text{min} \times \text{mg of protein}}$
	II	1.50	6.9
	I + II	0.11	9.3
Type I binding ($\Delta_{387-423}$)		0.59	0.047 (OD)

[a] All assays were run on the same pool of hepatic microsomes as used in Table II. Preparations and assays are given in the text.
[b] Site I is estimated from the Δ_{red} with concentrations of ethylmorphine from 0.020 to 0.100 mM. Site I + II from ethylmorphine concentrations of 0.1–1.0 mM and site II is taken from the plot of the difference.

of the cytochrome P-450 may be reduced. But as can be seen in Figure 1, when the assay is performed in a stop-flow apparatus, there is little or no deviation from linearity in this time interval, supporting the use of the anaerobic cuvette for this assay. The validity of the use of this arrangement is further verified by the stoichiometry observed between the stimulation and the N-demethylase activity which is much too close and reproducible to be a fortuitous result (Gigon et al., 1968, 1969; Holtzman and Rumack, 1971a,b; Holtzman and Carr, 1972).

An initial study on the effect of ethylmorphine on the NADPH–cytochrome P-450 reductase indicates that the order of addition of the drug and the NADPH has a significant effect on the stimulation of the reduction. In this experiment there was a marked stimulation when the ethylmorphine was added to the microsomes before the NADPH, but much less stimulation when it was placed in the plunger and added with the NADPH (Table I). These data clearly indicate that the activation process is not instantaneous, but rather takes at least a few hundred milliseconds. Although this observation may be of some significance in the study of the physicochemical mechanism of this reaction, it does not affect the study of the in vitro or in vivo metabolism of the drug, since in such studies the drug is present at all times and the sites will reach a steady-state level of activation, as it did here when the ethylmorphine was added before the NADPH.

The data in Table II indicate that with increasing concentrations of ethylmorphine there is a consistent increase in the activity of the NADPH–cytochrome P-450 reductase in microsomes from male rats. The activation of the reductase was taken as the difference in activity at a given concentration of ethylmorphine less the activity with no ethylmorphine present. Since all the reductase kinetics were determined from the difference between the stimulated and unstimulated activities, the unstimulated value is important in determining the kinetic constants, as well as the shape of the curve. This activity was, therefore, determined a large number of times for each experiment to achieve a high degree of precision (coefficient of variation is 1.25%). This apparent stimulation is not due to a change in the ϵ_{450}, since in our studies the final optical density was the same in the presence and absence of ethylmorphine as has been previously noted (Gigon et al., 1969).

A double-reciprocal plot of the stimulation of the reductase activity vs. the ethylmorphine concentration indicates the presence of two activation sites for microsomes from male rats (Figure 2 and Table III). The higher affinity site (I) has a K_s of 0.037 mM, while the lower affinity site (II) has a K_s of 1.5 mM.
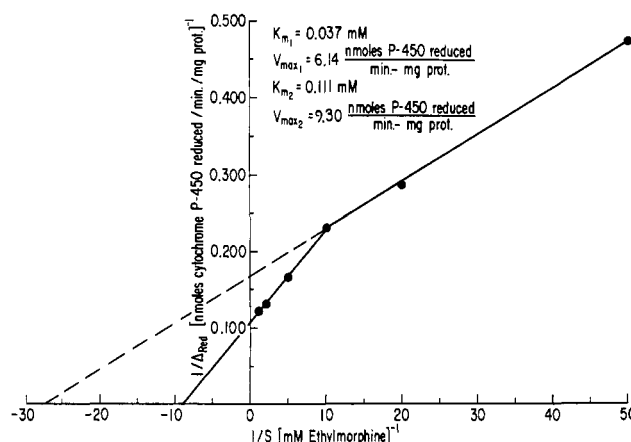

FIGURE 2: Double-reciprocal plot of the stimulation by ethylmorphine of the NADPH–cytochrome P-450 reductase of hepatic microsomes from male rats. Preparations and assays are described in the text.

The latter site was determined by subtracting the effect of the higher affinity site from the overall reductase stimulation and plotting the inverse of the difference (Table III).[1] The combined activity of the two sites gives a K_s of 0.11 mM. This value is clearly significantly lower than the K_m for the N-demethylase which was observed in the same preparation as 0.28 mM. In this particular preparation the V_m for the activation was about 20% lower than the N-demethylase activity (Table III), but as will be apparent from the compilation of several experiments, this is the exception rather than the rule and most values were within 10% of each other.

To our surprise, different workers have used different portions of the difference spectrum to determine the affinity of the type I binding site, but none to our knowledge have pub-

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[1] The kinetics of activation in the male rat were assumed to be the linear combination of two sites, A^I and A^{II}, so that $Am = Am^I + Am^{II}$. Klotz and Hunston (1971) have shown that the y intercept is Am (their eq 10). The x intercept is a combination association constant for the two sites (I + II in Tables III and VI which they give as $Ka^I Ka^{II}$. $(Am^I + Am^{II})/(Ka^I Am^{II} + Ka^{II} Am^I)$ (their $(K)_0$). The slope of the curve as $[s] \rightarrow 0$ is $1/(Am^I Ka^I + Am^{II} Ka^{II})$ (eq 16, Klotz and Hunston, 1971). Since $Am^I Ka^I \gg Am^{II} Ka^{II}$, the slope is essentially that due to the high-affinity site (I) and this, on extrapolation, is used to calculate the Ks^I and Am^I. For higher $[s]$ $Am^{II} = Am - Am^I$ the parameters of which again can be estimated by a standard double-reciprocal plot of $1/Am^{II} = 1/(Am - Am^I)$.

TABLE IV: The Effect of Varying Concentrations of Ethylmorphine on the NADPH–Cytochrome P-450 Reductase Activity of Hepatic Microsomes from Female Rats.[a]

Ethylmorphine Concn (mM)	No. of Determinations	nmol of P-450 Reduced/ min × mg of Protein	Difference from 0 mM Ethylmorphine
0	11	7.1 ± 0.08[b]	
0.02	5	7.8 ± 0.10	0.7
0.05	6	8.2 ± 0.2	1.1
0.10	2	8.7 ± 0.2	1.6
0.20	3	9.0 ± 0.1	1.9
0.50	2	9.6 ± 0.2	2.5
1.00	3	9.5 ± 0.2	2.4

[a] Assays were run on a single pool of hepatic microsomes. Preparations and assays are given in the text. [b] Values are average ±SEM.

TABLE V: The Kinetic and Spectral Parameters for Ethylmorphine of Hepatic Microsomes from Female Rats.[a]

Activity	K (mM)	V_{max}
Ethylmorphine N-demethylase	0.64	2.3 $\dfrac{\text{nmol of HCHO}}{\text{min} \times \text{mg of protein}}$
Cytochrome P-450 reductase activation (Δ_{red})	0.062	2.6 $\dfrac{\text{nmol of reduced}}{\text{min} \times \text{mg of protein}}$
Type I binding ($\Delta_{390-425}$)	0.04	0.019 (OD)

[a] All assays were run on the same pool of hepatic microsomes as used in Table IV. Preparations and assays are given in the text.

$K_s = 0.0586$ mM
$Abs_{max} = 0.0469$

$\Delta 450-424$
$\Delta 387-450$
$\Delta 387-424$

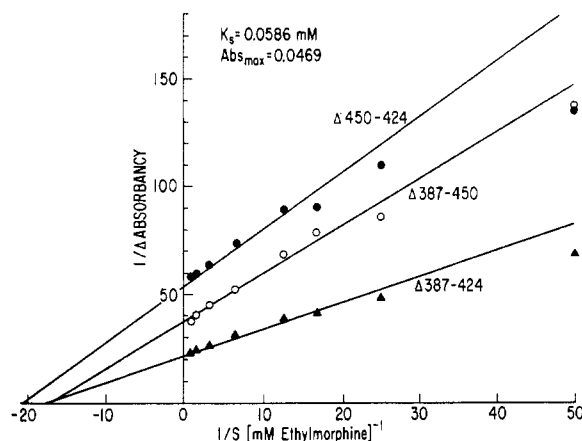I/ABSORBANCY
I/S [mM Ethylmorphine]$^{-1}$

FIGURE 3: Double-reciprocal plots of the spectral differences seen on the addition of ethylmorphine to hepatic microsomes from male rats. Preparations and assays are described in the text.

lished data showing the equivalence of using the peak (390 nm) vs. an isosbestic point (the most stable being 450 nm), the trough (425 nm) vs. the isosbestic point, and the peak vs. the trough. Clearly, if two are equivalent, then the third must also be so. These different methods could well give different results if there is more than one site and if one causes a distortion of the Soret peak. Figure 3 indicates that they all give approximately the same K_s of 0.059 mM and therefore all are apparently equivalent in this case. We have found such is not always the case, as for example with the type II site in microsomes from phenobarbital-induced animals (Rumack and Holtzman, 1971).

Like the male, the female rat shows increasing activity of the NADPH–cytochrome P-450 reductase with increasing concentrations of ethylmorphine (Table IV). Yet, unlike the male, when this stimulation is plotted against ethylmorphine concentration in a double-reciprocal plot, the curve indicates only a single activation site with a K_s of 0.062 mM (Table V). Since the K_s is tenfold lower than the apparent K_m for the N-demethylase of 0.64 mM, this clearly indicates that the activation site cannot determine the K_m for the overall reaction in the female. Yet, there is a close correlation between the max-

TABLE VI: The Kinetic and Spectral Parameters for Ethylmorphine of Hepatic Microsomes from Male and Female Rats.

Activity	Sex	Site	K (mM)	V_{max}
Ethylmorphine N-demethylase	M[a]		0.25[c] ± 0.01	9.5 ± 0.9 $\dfrac{\text{nmol of HCHO}}{\text{min} \times \text{mg of protein}}$
	F[b]		0.64 ± 0.04	2.3 ± 0.2
Cytochrome P-450 reductase activation (Δ_{red})	M	I	0.029[d] ± 0.006	4.1 ± 0.7 $\dfrac{\text{nmol of reduced}}{\text{min} \times \text{mg of protein}}$
		II	1.0 ± 0.2	7.6 ± 0.8
		I + II	0.15 ± 0.02	8.7 ± 0.5
	F		0.067 ± 0.008	2.47 ± 0.09
Type I binding ($\Delta_{390-425}$)	M		0.071 ± 0.006	0.044 ± 0.001 (OD)
	F		0.05 ± 0.01	0.016 ± 0.003

[a] Four pools of livers from 180- to 220-g male rats. [b] Three pools of livers from 160- to 200-g female rats. [c] Values are average of the pools ±SEM. [d] Site I is estimated from the Δ_{red} with concentrations of ethylmorphine from 0.020 to 0.10 mM. Site I + II from ethylmorphine concentrations of 0.1–1.0 mM. Site II is taken from the plot of the difference.

imal activity for the N-demethylase and the reductase (Table V).

As in the case of the male rats, all three methods of estimating the affinity of the type I site for ethylmorphine yield approximately the same K_s of 0.044 mM.

In a series of experiments from four pools of male rats and three pools of female rats, it is readily apparent that in both sexes the maximal values for the ethylmorphine N-demethylase and the NADPH–cytochrome P-450 reductase activation are extremely close (Table VI). Further, in the male, and even more so in the female, the K_s for the activation site is lower than the K_m for the overall reaction.

Discussion

In these studies the marked difference between the K_s of the substrate for the activation of the reductase and the K_m of the overall demethylation in microsomes obtained from both sexes of rats clearly indicates that there are separate activation and catalytic sites. This is in distinction to the studies on cytochrome P-450$_{cam}$ in which the two sites appear to be the same (Tyson *et al.*, 1972; Peterson and Ishimura, 1971). There can be little doubt, especially in the female, that the two sites are quite distinct and not just different manifestations of the same site, otherwise one would expect them to have the same affinity constants. Further, there are activation sites in both sexes which have sufficient affinity for the substrates, so that they could be significant in the disposition of drugs in the intact animal. Since in both sexes the K_s for the activation site is considerably lower than the apparent K_m for the overall N-demethylase, it would appear that the latter is determined primarily from the dissociation constant for the catalytic site.

It is unclear as to what relationship the type I binding site bears to any of the sites studied here. In the female, the similarity between the K_s's of the activation site and the spectral binding site would suggest that they are one and the same. Yet this could be fortuitous, especially in view of the value of the parameters obtained for the male where there is a marked disparity between the parameters observed for the activation sites and the type I binding site. Although it would appear to be a convenient hypothesis to assume that the type I binding site is merely an alternative manifestation of the activation site, this correlation would appear to hold up well only in the female rat, suggesting that this site may actually represent a third set of binding sites in the microsomes. Diehl *et al.* (1970) have presented evidence for cyclohexane which appears to suggest that the two sites are the same in the male rat for this substrate. It may well be that the type I site has different significance for different substrates.

A final interesting aspect of this work is the confirmation of the stoichiometry between the maximal stimulation of the reductase by the substrate and the maximal activity of the N-demethylase. Although microsomes do not exhibit good respiratory control (Holtzman and Erickson, 1972) as measured by the substrate stimulation of oxygen consumption, the correlation between the stimulation of the transport of the first electron needed to reduce cytochrome P-450 and the activity of the N-demethylase would suggest that this step is well coupled to the final metabolism of the substrate when NADPH is the sole source of reducing equivalents.[2] Such is not the case when NADH is also added since then there is greater N-demethylase activity than can be accounted for on the basis of the stimulation of the reductase step (Hildebrand and Estabrook, 1971). At this time we would be loath to speculate from the present fragmentary evidence on the mechanism whereby such a tight coupling could occur between two separate sites, the actions of which, from the available kinetic data, would appear to be separated in both space and time.

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[2] The term "respiratory control" usually refers to the 3 :1 stoichiometry of ATP formation to increased O_2 consumption by mitochondria. We wish to extend this concept to the microsomes to refer to the possible stoichiometry of substrate hydroxylation to the increase in O_2 consumption. Work in progress would suggest that microsomes exhibit variable respiratory control with ratios of oxygen consumption to product formation of 1 :1 to 3 :1 (Holtzman and Erickson, 1972).