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HYDROXYLATION OF ANILINE AND AMINOANTIPYRINE (1-PHENYL-2,3-DIMETHYL-AMINOPYRASOLON-5) DERIVATIVES IN LIVER ENDOPLASMATIC RETICULUM

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Abstract—The absence of correlation between the effect of aniline and aminoantipyrine derivatives on the rates of reduction reactions of cytochrome P-450 and the rate of oxidation of these substrates allows one to suppose that the reductase reaction is not the rate limiting step of hydroxylation for all substrates. K_m has been found to be directly proportional to V_{max} of hydroxylated substrates. Hence, in these reactions the K_m value is not determined by the value K_s but by the (k_{+2}/k_{-1}) ratio and K_m does not characterize the affinity of cytochrome P-450 to substrates. Calculations have been made to show that cytochrome P-450 may form two types of enzyme–substrate complexes containing one or two substrate molecules. The complex in which one molecule of cytochrome P-450 binds one substrate molecule is believed to be active.

AT PRESENT the most well-known point of view is that reduction of cytochrome P-450 or its complex with the substrate is a rate limiting step of hydroxylation.[1–3] But there exists a different opinion according to which the rate of hydroxylation correlates with the amplitude of spectral changes arizing when cytochrome P-450–substrate complex is formed and is, consequently, determined by the given reaction.[4–6] Thus in accordance with these views the rate limiting step of hydroxylation should be localized at one of the first sites of such reactions: either when the enzyme–substrate complex is formed, or when it is reduced.

To verify these concepts, we have studied the rate of hydroxylation of certain aniline and aminoantipyrine derivatives. The effect of these compounds on the NADPHA cytochrome P-450 reductase activity and the magnitude of spectral changes caused by the formation of complexes of these compounds with cytochrome P-450 was also measured. It was supposed that should correlation between the hydroxylation rate and one of the above values exist, the rate limiting step of oxidation might be elucidated.

MATERIALS AND METHODS

Chemicals. NADPH was obtained from C. F. Boehringer. Succinate (disodium salt), rotenone and albumin (V fraction) were purchased from Sigma Chemical Co. Tris (hydroxymethyl aminomethane) was obtained from Calbiochem, U.S.A. Monomethylaniline (MMA), monometylaminoantipyrine (MAP), formylaminoantipyrine

(FAP) and aminoantipyrine (AP) were synthesized at Research Institute of Chemical Pharmacology. Dimethylaniline (DMA) before being used was sublimated at 193° in vacuum, MMA—at 86°, MAP was recrystallized. *N*-oxide of DMA was synthesized from DMA in our laboratory according to the method by Chernova and Hohlov.[7] DMA, MMA and aniline were dissolved in 0·05 N HCl; *N*-oxide of DMA—in alcohol; dimethylaminoantipyrine (DAP), MAP, FAP and AP—in distilled water.

Animals. Normally fed 150–200 g male rats were used.

Microsome fractionation. Homogenate from liver tissue was obtained as described previously.[8] Mitochondria and nuclei were sedimented at 9600 *g* for 15 min at 0–4° in a High-Speed-18 (MSE) centrifuge. The microsomal fraction was isolated from supernatant at 105,000 *g* for 1 hr in a VAC-601 (Janetzki) centrifuge. Microsomal membranes were resuspended in 0·15 M KCl. Microsomal protein was measured by the method of Lowry *et al.*[9] in presence of 0·1% sodium deoxycholate with crystalline bovine albumin as the standard.

Enzyme assays. The reaction rate of the *N*-demethylation of DMA, MMA, *N*-oxide of DMA, DAP and MAP was determined by the quantity of the formaldehyde (FA) formed. One millilitre of the incubation medium contained 40 mM Tris–HCl, pH 7·4; 16 mM $MgCl_2$; 3 mM NADPH. When the rate of DMA, DAP and MAP demethylation was determined the microsomal protein content was 2·5 mg, but when MMA and *N*-oxide of DMA were used as substrates, it was 5–7 mg. The reaction was initiated by adding substrates at different concentrations. The permanently shaken mixture was incubated at 37° for 20 min (to determine the MMA demethylation rate, the incubation was carried out for 30 min). The reaction was interrupted by adding 0·25 ml of the mixture, containing equal volumes of 25% $ZnSO_4$ and $Ba(OH)_2$. After that the samples were centrifuged in a CLN-2 centrifuge at 3000 *g* for 10 min. The colour reaction was performed by the micromethod as previously described.[8] The colour reaction of the *N*-demethylation of MMA was performed by the method of Nash.[10] The rate of *N*-demethylation reaction is expressed as nmoles of FA/min per mg of microsomal protein.

The rate of *p*-hydroxylation of aniline and DMA in the *p*-position was determined by the quantity of *p*-aminophenols formed. One millilitre of the incubation mixture contained 40 mM Tris–HCl, pH 7·4; 16 mM $MgCl_2$; 3 mM NADPH; 4 mg of microsomal protein. Aniline and DMA were added to the incubation mixture at different concentrations. The incubation was carried out at 37° for 20 min with continuous shaking. The reaction was interrupted by adding 0·5 ml of 15% trichloracetic acid. The samples were centrifuged in a CLN-2 centrifuge at 3000 *g* for 10 min. The content of *p*-aminophenols was determined by adding 0·5 ml of 10% sodium carbonate and 1·5 ml of 2% phenol to 1·0 ml of the supernatant. The incubation was carried out for 30 min in a water bath at 37°. The measurements were taken on a Hitachi-124 spectrophotometer at 630 nm. The molar extinction coefficient of *p*-aminophenol was 10 cm^{-1} mM^{-1}. The rate of *p*-hydroxylation reaction is expressed as nmoles of *p*-hydroxy of DMA or aniline/min per mg of microsomal protein. The composition of the incubation medium in the enzymic hydroxylation reactions was chosen so as to ensure a zero order reaction for 20–30 min with respect to the substrates.

Cytochrome P-450 content. Microsomal cytochrome P-450 was determined by its carbon monoxide difference spectrum after reduction with $Na_2S_2O_4$.[11] Cytochrome P-450 content was measured on a SF-10 spectrophotometer by the difference in

absorbance between 450–490 nm and was calculated per mg of microsomal protein. Extinction coefficient for cytochrome P-450 was 91 cm^{-1} mM^{-1}.

A study of the substrate binding. Microsomes were diluted to 3 mg protein/ml with 40 mM Tris–HCl, pH 7·4. Measurements were carried out at room temperature. Serial additions of substrate to a cuvette containing 3·0 ml of mixture, were made and after each addition the differential spectrum was recorded between 350–520 nm using the Shimadzu MPS-50L split-beam spectrophotometer. The difference in absorbance between two predetermined wavelengths was taken. The substrates which are the derivatives of the aniline were added at a final concentrations ranging from 0·1 to 2·4 mM and the derivatives of the aminoantipyrine—from 0·2 to 4·0 mM.

Plotting results. The K_m and V_{max} values were calculated according to the method of Lineweaver–Burk.[12] K_s (the spectral dissociation constant) and $\Delta O.D._{max}$ (maximal spectral change) were calculated from Lineweaver–Burk plots and also taking into account the possibility of formation of an enzyme complex with two molecules of the substrate.[12]

NADPHA cytochrome P-450 reductase activity. The reduction rate of cytochrome P-450 was measured in anaerobic conditions in the presence of hydroxylation substrates on a Hitachi-356 spectrophotometer. The incubation mixture contained 50 mM Tris–HCl, pH 7·4; 16 mM $MgCl_2$; microsomal protein (1 mg/ml). Total volume of the incubation mixture was 3·0 ml. To ensure anaerobic conditions "sonic" submitochondrial particles, obtained by the method of Hansen *et al.*[13] (2 mg of protein), 15 mM succinate and 2·0 μM rotenone were added to the microsomal suspensions. Before the measurements the incubation medium was gassed with CO for 1 min. The reaction was initiated by adding 40 μM NADPH to the incubation medium. Measurements were carried out at 25°. The reaction rate was calculated as described by Gigon *et al.*[14]

RESULTS AND DISCUSSION

The data on determination of K_m and V_{max} with the derivatives of the aniline and aminoantipyrine used as substrates are summed up in Table 1 and Fig. 1a, b. It is

TABLE 1. THE KINETIC PARAMETERS OF THE HYDROXYLATION REACTIONS OF THE DERIVATIVES OF ANILINE AND AMINOANTIPYRINE*

Substrate	The reaction product	K_m (mM)	V_{max} (nmoles/min per mg of protein)	k_{+2}^{*} min^{-1}† (apparent)
		Aniline serie		
DMA	FA	0·66	6·2	7·0
	p-hydroxy of DMA	0·26	0·53	0·6
MMA	FA	0·50	4·8	5·3
Aniline	*p*-hydroxy of aniline	0·14	0·57	0·63
N-oxide of DMA	FA	70	17	19
		Aminoantipyrine serie		
DAP	FA	0·42	4·1	4·5
MAP	FA	0·62	5·4	6·0

* Results are given as an average from four to five experiments.

† k_{+2} min^{-1} are calculated as V_{max} of the hydroxylation reaction per nmole of cytochrome P-450. The cytochrome P-450 content in these experiments was on an average 0·9 nmoles/mg of microsomal protein.

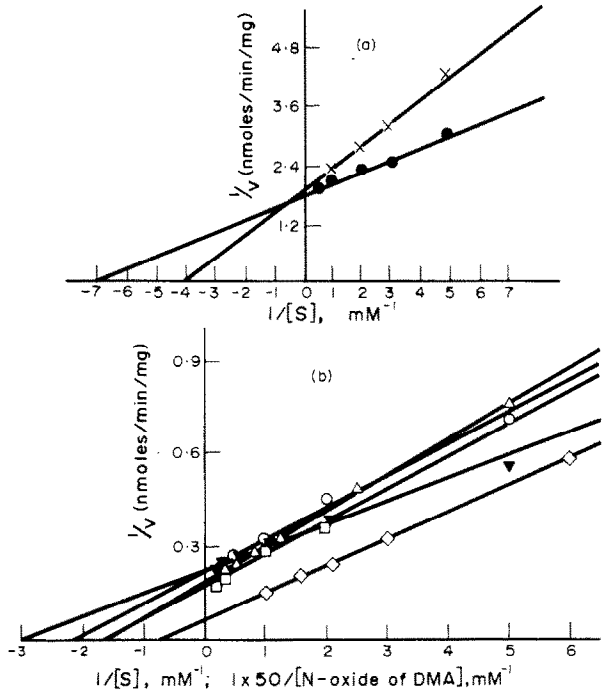

FIG. 1. The dependence of the hydroxylation rate of the derivatives of the aniline and aminoantipyrine on the substrate concentrations. (a) The rate of the *p*-hydroxylation of DMA (×) and aniline (●) is expressed as nmoles of *p*-hydroxy of DMA or aniline/min per mg of microsomal protein. (b) The rate of the *N*-demethylation of MMA (○), DMA (□), *N*-oxide of DMA (◇), DAP (▼) and MAP (△) is expressed as nmoles of FA/min per mg of microsomal protein. Results are expressed as an average from four to five experiments.

seen from these data that the rate of hydroxylation is not the same for different substrates. It ranges from 0·6 to 19 nmoles/min per nmole of cytochrome P-450. The data obtained testify to the fact that cytochrome P-450 displays a certain selectivity with respect to the substrates. It was interesting to elucidate on what factors V_{max} depends.

A comparative analysis of the rate of hydroxylation of the DMA ring and that of oxidation of its methyl group shows that it is not always reduction of the enzyme-substrate complex in the NADPH oxidation chain is the rate limiting step of hydroxylation. In fact it is clearly seen in Fig. 2a that DMA, on being bound to cytochrome P-450, accelerates the reduction of the cytochrome P-450—DMA complex in the NADPH oxidation chain. Thereby the reaction of demethylation proceeds at a much higher rate (V_{max} = 6·2 nmoles/min per mg) than that of *p*-hydroxylation (V_{max} = 0·53 nmoles/min per mg), as though in both cases the reduction rate of the cytochrome P-450–DMA complex is the same. And what is more, the retardation of this reaction when DMA is substituted by aniline (Fig. 2a) does not affect the value of the maximal rate of the *p*-hydroxylation (V_{max} = 0·57 nmoles/min per mg). Exactly the same absence of correlation is observed between the demethylation rate of the *N*-oxide of DMA and the reduction rate of the *N*-oxide of DMA–cytochrome P-450 complex (c.f. the data in Table 1 and Fig. 2a). Almost similar values of V_{max} in the demethylation reactions of DAP and MAP do not correlate with the effects of these compounds on the cytochrome P-450 reduction rate. DAP accelerates this reaction

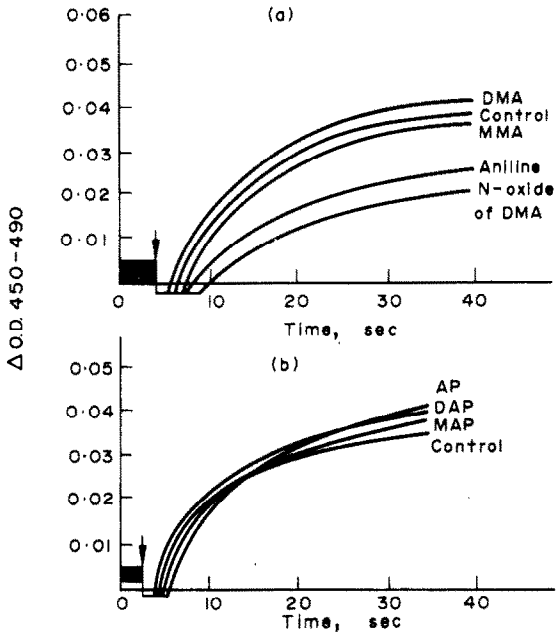

FIG. 2. Reduction of microsomal cytochrome P-450 by NADPH in the presence of the derivatives aniline (a) and aminoantipyrine (b). The reduction rate of cytochrome P-450 was measured in anaerobic conditions. The incubation mixture contained 50 mM Tris–HCl buffer, pH 7·4; 16 mM $MgCl_2$; microsomal protein (1 mg/ml). Total volume of the incubation mixture was 3·0 ml. Before the measurements the microsomal suspension was carefully gassed with CO for 1 min. Measurements were carried out at 25°. The experiment was repeated in the presence of DMA (0·7 mM); MMA (1·6 mM); aniline (1·5 mM); *N*-oxide of DMA (4 mM); AP (6 mM); DAP (2 mM); MAP (3·3 mM). The arrow indicates the moment when 40 μM NADPH was added to the incubation mixture.

whereas MAP inhibits it very little if at all (Fig. 2b). The results obtained allow a statement that the direct dependence between the hydroxylation rate and the NADPHA cytochrome P-450 reductase activity is not observed with all substrates also Schenkman[15] who investigated the temperature dependence of the hydroxylation rate of aminopyrine, ethylmorphine and aniline, revealed that reduction of the cytochrome P-450–substrate complex can not be the rate limiting step of ethylmorphine and aniline oxidation. V_{max} of hydroxylation of these substrates was respectively 7·0; 3·5; 0·7 nmoles/min per mg at 37°. At the same time aminopyrine and ethylmorphine stimulate NADPHA cytochrome P-450 reductase the same degree (V_{max} = 7·0 nmoles/min per mg) whereas aniline either does not affect this reaction if taken at a concentration close to K_m, or inhibits it at very high concentrations. With the data at our disposal there is little doubt that reduction of the cytochrome P-450–substrate complex is not the rate limiting step of hydroxylation in all cases. A very interesting phenomenon was revealed when the amplitude of the spectral changes caused by the formation of the complex of cytochrome P-450 with the substrate was measured (Fig. 3). It turned out that no typical Lineweaver–Burk plots of ΔO.D. vs the substrate concentration can be obtained. The character of deviation allowed one to suppose that cytochrome P-450 may bind successively two substrate molecules:

$$\text{cyt P-450} + S \rightleftarrows \text{cyt P-450–S} + S \rightleftarrows \text{cyt P-450–S}_2$$
(I)

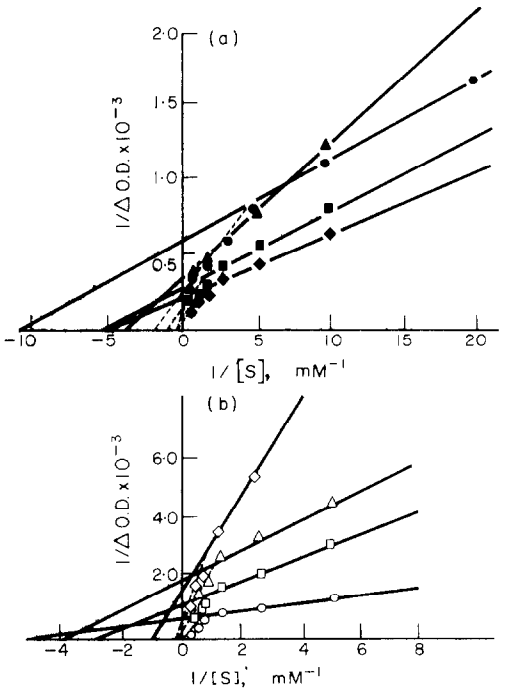

FIG. 3. The dependence of the maximal spectral change of the complex of cytochrome P-450 with the derivatives of aniline (a) and aminoantipyrine (b) on the substrate concentrations. For DMA binding, the difference in absorbance between 385–420 nm was measured (●), for MMA binding—between 415–480 nm (▲); for aniline binding—between 390–425 nm (■); for *N*-oxide of DMA binding—between 410–460 nm (◆); for DAP binding—between 385–420 nm (○); for MAP binding—between 420–484 nm (△); for FAP binding—between 380–420 nm (◇); for AP binding—between 430–480 nm (□). Maximal spectral change is expressed as ΔO.D./mg of microsomal protein × 10^{-3}. Solid lines: calculated straight lines for K_s'. Broken lines: calculated straight lines for K_s''. Results are expressed as an average from three experiments.

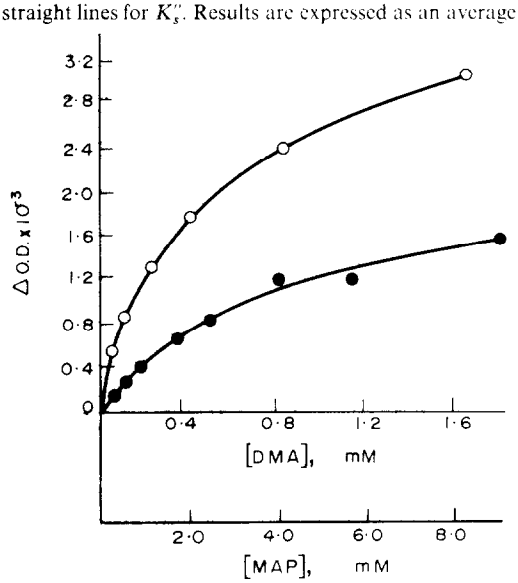

FIG. 4. The plots of $\Delta O.D._{max}$ vs the substrate concentration, obtained experimentally and calculated by equation (II). The curves given in the figure are calculated by equation (II). The points on the curves correspond to experimentally obtained values. ΔO.D. are calculated per mg of microsomal protein. DMA (○); MAP (●).

Assuming that the system is equilibrium and both enzyme-substrate complexes produce spectral changes, mechanism (I) allows the following equation to be written, which expresses the dependence of ΔO.D. upon the substrate concentration:

$$\Delta O.D._{\cdot max} = \Delta O.D._{\cdot max} \cdot P\text{-}450\text{–}S + \Delta O.D.''_{max} \cdot P\text{-}450\text{–}S_2$$

$$= \frac{\Delta O.D._{\cdot max} \cdot K''_s \cdot S}{K'_s \cdot K''_s + K''_s \cdot S + S^2} + \frac{\Delta O.D.''_{max} \cdot S^2}{K'_s \cdot K''_s + K''_s \cdot S + S^2} \quad \text{(II)}$$

where $\Delta O.D.'_{max}$ is the maximal amplitude of the spectral changes of the cytochrome P-450–S complex, $\Delta O.D.''_{max}$ is the maximal amplitude of the cytochrome P-450–S_2 complex, K'_s and K''_s are the spectral constants of the dissociation of these complexes. Usual expressions for concentrations of enzyme–substrate complexes with one and two substrate molecules are used in this equation.[12] The validity of the mechanism suggested (I) is confirmed by a good agreement of the dependence of $\Delta O.D._{\cdot max}$ calculated from the two equations system (II) upon [S] with the experimental curves. Fig. 4 presents several such curves where it is clearly seen that the obtained experimentally points coincide with the calculating values. Table 2 gives the K'_s, K''_s, $\Delta O.D.'_{max}$, $\Delta O.D.''_{max}$ values obtained from the Lineweaver–Burk curves and calculated from four equations for four pairs of the experimental points (ΔO.D., K_s) (II). The most pronounced differences are observed in the case of K'_s and $\Delta O.D.'_{max}$ is a result of the flattening of curves (I/ΔO.D., I/S) with low values of [S].

Comparing the V_{max} values of hydroxylation of the derivatives of aniline and aminoantipyrine with the values of $\Delta O.D.'_{max}$ and $\Delta O.D.''_{max}$ obtained by two methods one sees no correlation between them. A high value of $\Delta O.D.'_{max}$ is very often corresponds to a low V_{max} and vice versa. That is why it remains obscure which of the two complexes formed is active, i.e. is able to produce a hydroxylated product on being decomposed. Hence it is still unknown which of the values, $\Delta O.D.'_{max}$ or $\Delta O.D._{\cdot max}$, characterises the reaction of hydroxylation. These results do not support the concepts of some authors[16] who believe that the amplitude of the spectral changes of the complex of cytochrome P-450 with some substrates, which characterises the quantity of cytochrome P-450 bound with substrate, may be the factor determining the rate of its conversion. Good correlation between V_{max} and $\Delta O.D._{\cdot max}$ reported in these works should be qualified as being inherent only in some substrates.

Having compared the spectral changes of the complexes of cytochrome P-450 with the substrates we revealed a dependence of the chemical nature of the substituent at the nitrogen of the aminogroup of the compounds studied, upon the type of the spectral changes of the complex. Several types of spectral changes were found in the following aniline derivatives: DMA, MMA, aniline and *N*-oxide of DMA compounds which differ by the substituent at the side chain nitrogen (Fig. 5). DMA gives the spectral changes of the first type, aniline of the second type. MMA forms a complex with cytochrome P-450, the difference spectra of which is intermediate between DMA and aniline. Quite a special type of the spectral interactions is produced by the complex of cytochrome P-450 with *N*-oxide of DMA. The chemical nature of the substituent at the aniline aminogroup seems to play an important role in the formation of the spectral changes of the complex. It is apparently this group that directly interacts with Fe-heme and its ligands. A high value of $\Delta O.D._{\cdot max}$ of the com-

TABLE 2. K_s AND $\Delta O.D._{max}$ OF THE COMPLEXES OF CYTOCHROME P-450 WITH SUBSTRATES THE DERIVATIVES OF ANILINE AND AMINOANTIPYRINE*

Substrate	Results are obtained by the method of Lineweaver–Burk				Results are calculated by equation (II)			
	K'_s†	K''_s	$\Delta O.D.'_{max}$‡	$\Delta O.D.''_{max}$	K'_s	K''_s	$\Delta O.D.'_{max}$	$\Delta O.D.''_{max}$
				Aniline serie				
DMA	0·09	0·66	1·6	4·8	0·021	0·67	0·56	4·0
MMA	0·29	2·5	3·0	6·2	0·064	1·8	1·0	5·2
Aniline	0·21	1·4	3·7	8·3	0·079	1·8	1·7	9·1
N-Oxide of DMA	0·19	2·0	4·3	16·6		2·7	–	19·6
				Aminoantipyrine serie				
DAP	0·27	1·3	1·9	3·3	0·12	2·4	1·0	3·6
MAP	0·27	5·5	0·55	2·5	0·029	8·2	0·18	2·3
AP	0·39	7·1	0·71	4·2	0·016	11	0·30	5·5
FAP	1·3	33	0·70	8·3	—	18		5·6

* Results are given as an average for four to five experiments.
† The K_s value is expressed in mM.
‡ The $\Delta O.D._{max}$ value is expressed as $\Delta O.D.$/mg of microsomal protein $\times 10^{-3}$.

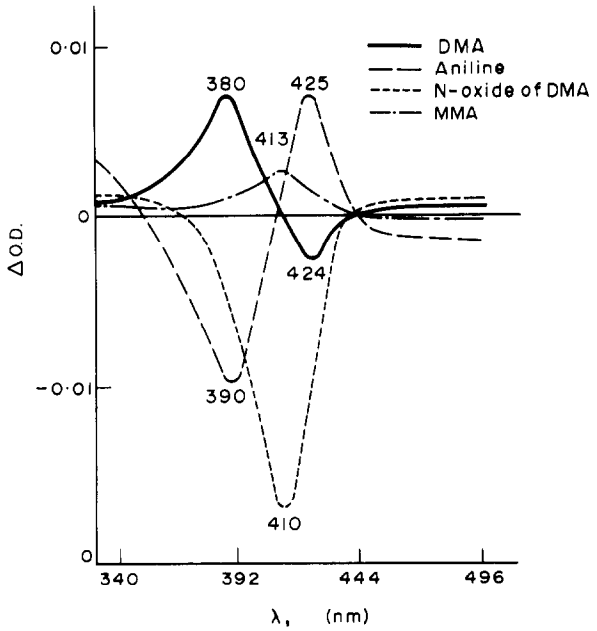

FIG. 5. Differential spectra of microsomes obtained when cytochrome P-450 binds with derivatives of aniline. The incubation mixture contained 40 mM Tris–HCl buffer, pH 7·4; microsomal protein (4 mg/ml). Total volume of the incubation mixture was 3·0 ml. Measurements carried out at room temperature. The substrates were added to microsomal suspensions at a final concentration 1·6 mM.

plex consisting of *N*-oxide of DMA and cytochrome P-450 means that the higher the polarity of the group introduced to this region, the stronger is the interaction.

Comparing K_s' and K_s'' values in the series of interconverting substrates, one may see that demethylation is not accompanied by a great increase in K_s. Comparing the affinity of cytochrome P-450 to *N*-oxide of DMA and DMA it is possible to make the conclusion that *N*-oxide of DMA cannot be an intermediate of DMA demethylation. With DMA used as substrate, the concentration of *N*-oxide formed is very low,

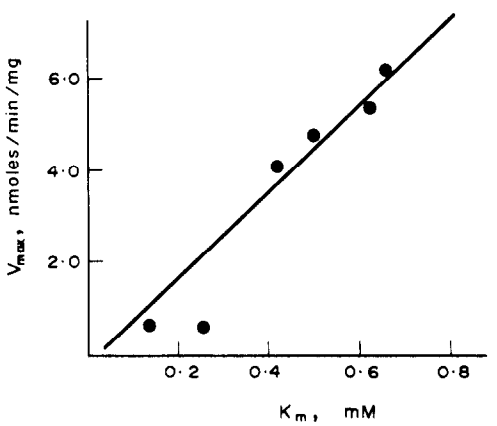

FIG. 6. The correlation between the values K_m and V_{max} in the hydroxylation reactions. (The curve drawn basing on the data of Table 1.)

and taking into account the values of K_s found, it becomes clear that this substrate cannot efficiently compete with DMA for cytochrome P-450. Similarly bearing in mind the values of the spectral constants of dissociation of DAP, MAP, AP, FAP one can hardly suppose that formylaminoantipyrine is formed as an intermediate in the course of demethylation of MAP. With *N*-oxide of DMA and FAP used as substrates we failed to calculate the values of K_s' and $\Delta O.D._{max}'$ according to equation (II). It is likely that a different mechanism of interaction of these two substrates with the cytochrome exists.

In view of the fact that cytochrome P-450 may bind both one and two substrate molecules it is not clear which of the two enzyme–substrate complexes is active. Comparing the values of K_m, K_s'' and K_s' (Tables 1 and 2) one may conclude that the active complex consists of one substrate molecule as K_m cannot be lower than K_s:

$$K_m = \frac{k_{+2} + k_{-1}}{k_{-1}} = \frac{k_{+2}}{k_{+1}} + \frac{k_{-1}}{k_{+1}} = \frac{k_{+2}}{k_{-1}} + K_s$$

Figure 6 presents the dependence of K_m on V_{max}. The correlation which is observed between the two values, i.e. the direct proportionality may be obtained only in one case, if $K_s \ll K_m$. The K_s' values fit this unequality, which is also evidence in favour of activity of the enzyme–substrate complex with one substrate molecule. How it becomes clear that the spectral changes are characterized by $\Delta O.D._{max}'$ when the active complex is formed. The directly proportional dependence between K_m and V_{max} in hydroxylation made it possible to suggest the existence of the constant value k_{+1} in these reactions.

Thus cytochrome P-450 should readily bind different substrates. It saturation occurs at very low concentrations of the substrates. The reduction of the given complex may well be the rate limiting step. In this case stimulation of NADPHA cytochrome P-450 reductase is accompanied by acceleration of hydroxylation.[8] However no unequivocal answer can be given to the question of the limiting step, as in some cases no correlation is observed between the effect of different derivatives of aniline and aminoantipyrine on the reductase reaction rate and the activity of the hydroxylating systems. In these reactions the rate limiting step is localized at the other stages of the hydroxylation mechanism i.e. the limiting step migrates in the hydroxylation chain depending on the substrates.

Thus the results obtained allow us to believe that the K_m value in the hydroxylation reaction does not reflect the affinity of cytochrome P-450 to the substrates. As a result of relevant calculations K_s was derived whose value is about 2×10^{-5}–10^{-4} M, which is somewhat less than the K_m values. This points to the possibility of oxidation of trace amounts of xenobiotics in the cell. This was not believed to be the case previously on the ground of the low affinity of cytochrome P-450 to them. The magnitude of it was suggested by the K_m or K_s values calculated in terms the stationary kinetics of the single-step reactions.

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