The Effect of Magnesium Ions on the Dimethylaniline Oxidation Rate and Electron Transfer in Liver Microsomal Fraction

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1. Reactions of *N*-demethylation, *p*-hydroxylation and *N*-oxidation of one substrate, i.e. dimethylaniline, have been used to show that the activating effect of Mg^{2+} takes place only in the first two reactions. 2. An increase in V_{max} of *N*-demethylation of dimethylaniline is accompanied by an increase in K_m . In the *p*-hydroxylation of dimethylaniline V_{max} increases whereas K_m does not change. A comparison of the changes in the K_m values of these reactions with the change in K_s shows that in both cases K_m does not characterize the affinity of cytochrome *P*-450 for dimethylaniline. 3. The rate-limiting site of *N*-demethylation and *p*-hydroxylation of dimethylaniline, as well as the total rate of NADPH oxidation in the presence of dimethylaniline, is between cytochromes b_5 and *P*-450. Addition of Mg^{2+} to the incubation medium changes the hydrophobic environment of phosphatidylcholine in the membrane, the process being accompanied by a sharp increase in the fluorescence quantum yield of 8-anilino-naphthalene-1-sulphonate.

Magnesium ions are capable of accelerating electron-transfer reactions in liver microsomal fraction (Fouts & Pohl, 1971; Peters & Fouts, 1970). Peters & Fouts (1970) have shown that an activation of electron transfer in the chain is accompanied by an increase in the rate of hydroxylation of both the substrates of the first (benzphetamine) and the second (aniline) types. With benzphetamine, the change takes place for both K_m and V_{max} , whereas with aniline only V_{max} changes. On the grounds of the data obtained the conclusion may be made that the stimulating effect of Mg^{2+} depends on the chemical structure of the oxidized substrate. An alternative explanation would be that the mechanism of the activating effect of Mg^{2+} does not depend on the chemical structure of the substrate, but is determined by the type of hydroxylation. With benzphetamine it is a side chain of the heterocyclic molecule that is hydroxylated, and with aniline it is an aromatic ring.

To differentiate between these two possibilities, the effect of Mg^{2+} on dimethylaniline hydroxylation has been studied in different reactions: *N*-demethylation, *p*-hydroxylation and *N*-oxidation. In addition, the use of dimethylaniline as the only substrate for different types of hydroxylation makes it possible to solve the question of whether the three reactions mentioned above have the same or different rate-limiting sites in the electron-transfer chain.

Materials and Methods

Materials

Chemicals. NADPH was obtained from C. F. Boehringer; 8-anilino-naphthalene-1-sulphonate (am-

monium salt), EDTA (disodium salt), rotenone and succinate (disodium salt) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Dimethylaniline was sublimed at 193°C *in vacuo* and was dissolved in 0.05M-HCl. Cytochrome *c* was obtained from Schuchardt, Munich, Germany. 2,6-Dichlorophenol-indophenol and potassium ferricyanide were analytical grade (Sojuzkhimreaktiv, Moscow, U.S.S.R.).

Buffers. Tris buffer was obtained from Calbiochem., Los Angeles, Calif., U.S.A. It was adjusted with HCl to pH 7.4.

Animals. Male albino rats, 150–200g, were starved overnight and killed by decapitation. The liver was washed with 1.15% KCl in 0.1mM-EDTA and removed. The tissue was minced and then homogenized in 3 vol. of the KCl solution in a Dounce homogenizer (glass with a Teflon pestle). Homogenates were centrifuged for 15 min at 9600g in a MSE High Speed 18 refrigerated centrifuge. The microsomal fraction was isolated by centrifugation of the 9600g supernatant fraction at 105000g for 1 h in a VAC-601 (Janetzki) centrifuge. The microsomal pellets were resuspended in the KCl solution with EDTA. Microsomal protein content was measured by the method of Lowry *et al.* (1951) in the presence of 0.1% sodium deoxycholate with crystalline bovine serum albumin as the standard.

Enzyme assays

The rate of *N*-demethylation of dimethylaniline is determined by the quantity of formaldehyde formed.

The incubation medium contained microsomal fraction (2.0mg of protein), 40mM-Tris buffer, 16mM-MgCl₂, 3mM-NADPH and dimethylaniline at a concentration of 0.2–2.0mM. Total volume of the incubation medium was 1ml. The permanently shaken mixture was incubated for 20min at 37°C in air. The reaction was interrupted by adding 0.25ml of a mixture of equal volumes of 25% ZnSO₄ and Ba(OH)₂, after which the samples were centrifuged for 5min in a CLN-2 centrifuge (U.S.S.R.) at 3000g. The colour reaction was performed by the micro-method. To 240μl of supernatant, 120μl of Nash reagent was added in a concentration fourfold that used by the author (Nash, 1953). The samples were incubated at 37°C for 45min in a water bath. The measurements were taken in the microcuvette of a SF-4A spectrophotometer (U.S.S.R.) at 412nm. The calibrating curve was made with formaldehyde as a standard, the content of which was determined beforehand titrimetrically.

The rate of *p*-hydroxylation of dimethylaniline was determined by the quantity of the *N*-dimethyl-*p*-aminophenol formed. The incubation medium contained microsomal fraction (4.0mg of protein), 40mM-Tris buffer, 16mM-MgCl₂, 3mM-NADPH and dimethylaniline at a concentration of 0.2–1.5mM, in a total volume of 1ml. The incubation was carried out at 37°C for 20min with continuous shaking. The reaction was interrupted by the addition of 0.5ml of 15% trichloroacetic acid. After centrifugation at 3000g for 10min, 1.0ml of the supernatant was added to 0.5ml of 10% Na₂CO₃ solution, followed by 1.5ml of 2% phenol in 0.2M-NaOH (Hilton & Sartorelli, 1970). The samples were incubated in a water bath at 37°C for 30min. The content of *N*-dimethyl-*p*-aminophenol was measured in a Hitachi-124 spectrophotometer at 630nm and calculated according to the calibrating curve, which was obtained with *p*-aminophenol used as a standard (Jagow *et al.*, 1965).

The rate of *N*-oxidation of dimethylaniline was determined by the quantity of *p*-nitrosodimethylaniline formed in the subsequent nitrosation of the enzymically derived dimethylaniline *N*-oxide. The content of the components of the incubation mixture was the same as with demethylation of dimethylaniline. The reaction was stopped by adding 0.5ml of 0.9M-HClO₄. The extraction into ether and the colour reaction was performed as described by Ziegler & Pettit (1964). The molar extinction coefficient of *p*-nitrosodimethylaniline was 8.2×10^3 litre·mol⁻¹·cm⁻¹ (Ziegler & Pettit, 1964).

The activity of NADPH-cytochrome *c* reductase was determined kinetically at 30°C in a Hitachi-124 spectrophotometer in a 1cm thermostatically controlled cuvette at 550nm. The incubation medium contained microsomal fraction (10μg of protein/ml), 100μM-NADPH, 330μM-NaCN, 50μM-cytochrome *c* and 40mM-Tris buffer, in a total volume of 3ml. The

molar extinction coefficient of cytochrome *c* (reduced minus oxidized) is 18.5×10^3 litre·mol⁻¹·cm⁻¹ (Dallner, 1963).

NADPH-2,6-dichlorophenol-indophenol reductase activity was measured in the thermostatically controlled 1cm cuvette at 30°C for 2–3min in a Hitachi-124 spectrophotometer at 600nm. The incubation medium contained microsomal fraction (15μg of protein/ml), 100μM-NADPH, 40μM-2,6-dichlorophenol-indophenol and 40mM-Tris buffer, in a total volume of 3ml. The reaction was initiated by adding NADPH. The molar extinction coefficient of oxidized 2,6-dichlorophenol-indophenol is 21×10^3 litre·mol⁻¹·cm⁻¹ (Dallner, 1963).

NADPH-ferricyanide reductase activity was measured in a thermostatically controlled 1cm cuvette at 30°C for 2–3min in a Hitachi-124 spectrophotometer at 420nm. Incubation medium contained microsomal fraction (150μg of protein/ml), 100μM-NADPH, 330μM-potassium ferricyanide and 40mM-Tris buffer, in a total volume of 3ml. The molar extinction coefficient of oxidized ferricyanide is 1.02×10^3 litre·mol⁻¹·cm⁻¹ (Dallner, 1963).

NADPH-oxidase activity was recorded fluorimetrically. The incubation medium contained 40mM-Tris buffer and 33μM-NADPH, in a total volume of 3ml. The reaction was initiated by adding 3mg of microsomal protein. The measurements were made at 30°C in a thermostatically controlled cuvette of a EF-3M fluorimeter (U.S.S.R.) supplied with an automatic recording device. Maximum transmittance of the excitation filter was at 366nm and of the recording filter was at 420nm.

Oxygen consumption

The rate of oxygen consumption was measured in a LP-60 polarograph (Lab. Instr. Praha) with a fixed exposed platinum electrode. Incubation medium contained microsomal fraction (2.0mg of protein) and 40mM-Tris buffer. Total volume of the incubation medium was 1ml. The reaction was initiated by adding 1mM-NADPH. The measurements were made at 30°C for 2–3min.

NADPH-cytochrome b₅ and P-450 reductases

The measurements were made in anaerobic conditions with and without 1.3mM-dimethylaniline and 16mM-MgCl₂. The incubation mixture contained microsomal fraction (1.0mg of protein/ml) and 50mM Tris buffer, in a total volume of 3ml. To ensure anaerobic conditions the 'sonic' submitochondrial particles (Hansen & Smith, 1964) (2.0mg of protein), 15mM-succinate and 2μM-rotenone were added to the incubation medium. Before measuring NADPH-cytochrome *P*-450 reductase activity, the microsomal

suspension was gassed with CO for 1 min. The reaction was initiated by adding $30\mu\text{M}$ -NADPH to the incubation medium. The measurements were made in a Hitachi-356 split-beam dual-wavelength recording spectrophotometer. The reduction rate of cytochrome b_5 was measured at 424–475 nm and of cytochrome P -450 at 450–475 nm (Orrenius *et al.*, 1969). The record was made at the rate of 240 mm/min. The reaction rate was calculated as described by Gigon *et al.* (1969).

The content of cytochromes b_5 and P -450 was determined by the method of Omura & Sato (1964) in a split-beam SF-10 spectrophotometer (U.S.S.R.).

Dimethylaniline binding with cytochrome P -450

The spectral changes arising when dimethylaniline interacts with cytochrome P -450 were recorded in a Hitachi-356 spectrophotometer (Remmer *et al.*, 1968). The incubation mixture contained microsomal fraction (3.0 mg of protein/ml) and 40 mM-Tris buffer, in a total volume of 3 ml. Difference spectra of type I were obtained by adding dimethylaniline, at a final concentration of 0.2–1.4 mM, to the microsomal suspensions. Extinction was measured as the difference between the trough at 420 nm and the peak at 385 nm.

Plotting of results

K_m , V_{max} , K_s (the spectral constant of the dimethylaniline binding with cytochrome P -450) and ΔE_{max} (maximal spectral change of the complex of cyto-

chrome P -450 with dimethylaniline) were determined by the method of Lineweaver–Burk (Dixon & Webb, 1966). When K_m and V_{max} were calculated the quantity of the substrate utilized during the incubation was taken into account. For this purpose, instead of the final concentrations of the substrates the $([S_0] + [S_t])/2$ values were used, where $[S_0]$ is the final concentration of the substrate in the incubation mixture at the beginning of the reaction, and $[S_t]$ is the final concentration of the substrate in the incubation medium when the reaction was interrupted.

8-Anilinonaphthalene-1-sulphonate–microsomal fraction fluorescence was measured in an EF-3M fluorimeter in a thermostatically controlled cuvette at 30°C. The incubation mixture contained microsomal fraction (0.3 mg of protein/ml), 50 mM-Tris buffer and 8-anilinonaphthalene-1-sulphonate at a final concentration of 5–25 μM , in a total volume of 3 ml. The excitation wavelength was 366 nm and the relative intensity of fluorescence was taken at 480 nm.

Results

The use of dimethylaniline as substrate makes it possible to study the three types of reactions occurring in liver microsomal fraction, shown in Scheme 1.

Fig. 1 shows the results for the effect of different concentrations of Mg^{2+} on the rate of N -demethylation and p -hydroxylation of dimethylaniline. Maximal stimulation of the reaction rates was observed at a MgCl_2 concentration of 15 mM and higher. However, MgCl_2 produced different stimulating effects on the

Scheme 1. Oxidation of dimethylaniline in rat liver microsomal fraction

Fig. 1. Effect of different concentrations of MgCl_2 on the rate of *N*-demethylation and *p*-hydroxylation of dimethylaniline

The incubation mixture for *N*-demethylation (●) consisted of microsomal fraction (2.0 mg of protein), 40 mM-Tris buffer, 3 mM-NADPH, 6 mM-dimethylaniline and MgCl_2 at different concentrations, in a total volume of 1 ml. The incubation mixture for *p*-hydroxylation (○) consisted of microsomal fraction (4.0 mg of protein), 40 mM-Tris buffer, 3 mM-NADPH, 1.5 mM-dimethylaniline and MgCl_2 at different concentrations, in a total volume of 1 ml. Experimental details are given in the Materials and Methods section.

rates of *p*-hydroxylation and *N*-demethylation (2.2- and 1.5-fold increases respectively). Higher concentrations of MgCl_2 were required for maximal stimulation of *p*-hydroxylation of dimethylaniline than for stimulation of *N*-demethylation.

To study the mechanism of the stimulating action of Mg^{2+} , the effect of 16 mM- MgCl_2 on the kinetic parameters of *N*-demethylation and *p*-hydroxylation of dimethylaniline was investigated. It is seen from the data given in Fig. 2 and Fig. 3 that Mg^{2+} at a concentration of 16 mM increase, on average 2.4-fold, $V_{\max}$ of demethylation of dimethylaniline and increase 2.9-fold the $V_{\max}$ of *p*-hydroxylation of dimethylaniline. At the same time, K_m increases only with demethylation (1.5-fold). The difference between the absolute values of K_m for *N*-demethylation and *p*-hydroxylation of dimethylaniline (0.67 and 0.28 mM respectively) seems to favour the conclusion that with *N*-demethylation the k_{+2}/k_{+1} value is great and affects the value of K_m .

When the *N*-oxidation of dimethylaniline was examined it was found that Mg^{2+} does not accelerate the formation of the *N*-oxide (Table 1). This should be taken to mean that either Mg^{2+} has no activating effect on NADPH-specific flavoprotein or *N*-oxidation of dimethylaniline is accomplished by a specific flavoprotein, which does not belong to this chain, and on which Mg^{2+} does not produce an activating effect.

Thus when dimethylaniline is used as substrate Mg^{2+} causes an increase in $V_{\max}$ of *N*-demethylation and *p*-hydroxylation and does not affect the rate of *N*-oxidation.

To elucidate the mechanism of the activating effect, the action of Mg^{2+} on dimethylaniline binding with cytochrome *P*-450 and on the electron-transfer rate at different sites of the NADPH oxidation chain has been studied. The results on the effect of Mg^{2+} on the spectral constant for dimethylaniline binding with cytochrome *P*-450 and on the magnitude of the maximal spectral change of the complex of cytochrome *P*-450 with dimethylaniline are given in Fig. 4. It emerged that addition of Mg^{2+} to the incubation mixture causes some decrease (1.5-fold) in the spectral constant of the binding and an increase in $\Delta E_{\max}$ (1.3-fold).

A study of the effect of Mg^{2+} on the electron-transfer reactions has revealed that these ions considerably stimulate the NADPH oxidation rate in the presence of dimethylaniline in the incubation mixture (2.2-fold according to ΔO_2 and 2.4-fold according to ΔNADPH) (Table 2). At the same time, Mg^{2+} and dimethylaniline added separately also produce an activating effect on NADPH oxidation. However, the coefficient of stimulation in each case is much less. The stimulating effect of dimethylaniline is retained against the background of Mg^{2+} . The data obtained

Fig. 2. Effect of Mg^{2+} on *N*-demethylation of dimethylaniline

Assays were done in the absence ($\blacklozenge$, $\blacktriangle$, $\blacksquare$, $\bullet$) and in the presence ($\diamond$, $\triangle$, $\square$, $\circ$) of 16mM- $MgCl_2$. K_m (mM) and V_{max} (nmol of formaldehyde/min per mg of microsomal protein) in the absence of $MgCl_2$ were 0.45 ± 0.017 and 2.8 ± 0.15 respectively and in the presence of 16mM- $MgCl_2$ were 0.67 ± 0.050 and 6.8 ± 0.37 respectively. K_m and V_{max} are Lineweaver-Burk plots and are average values of four experiments $\pm$ S.E.M.

Fig. 3. Effect of Mg^{2+} on the reaction of *p*-hydroxylation of dimethylaniline

Assays were done in the absence ($\blacklozenge$, $\blacktriangle$, $\blacksquare$, $\bullet$, $\blacktriangledown$) and in the presence ($\diamond$, $\triangle$, $\square$, $\circ$) of 16mM- $MgCl_2$. K_m (mM) and V_{max} (nmol of *N*-dimethyl-*p*-aminophenol/min per mg of microsomal protein) in the absence of $MgCl_2$ were 0.28 ± 0.013 and 0.23 ± 0.003 respectively and in the presence of 16mM- $MgCl_2$ were 0.28 ± 0.015 and 0.66 ± 0.06 respectively. K_m and V_{max} are Lineweaver-Burk plots and are average values from four or five experiments $\pm$ S.E.M.

Table 1. *Effect of MgCl₂ on the rate of N-oxidation of dimethylaniline*

The incubation medium contained microsomal fraction (4.5mg of protein), 40mM-Tris buffer, 16mM-MgCl₂, 3mM-NADPH and 6mM-dimethylaniline, in a final volume of 1 ml. The reaction was stopped by adding 0.5ml of 0.9M-HClO₄. For further details see Ziegler & Pettit (1964). Activity of *N*-oxidase on dimethylaniline is expressed as nmol of *N*-oxide of dimethylaniline/min per mg of microsomal protein.

Preparation no.	Rate of <i>N</i> -oxidation	
	MgCl ₂ absent	16mM-MgCl ₂
1	3.2	3.2
2	4.0	3.8
3	4.0	3.9

on acceleration of NADPH oxidation by Mg²⁺ and dimethylaniline show that these effects have an additive character. The NADPH oxidation rate in the presence of Mg²⁺ and dimethylaniline is the sum of the NADPH oxidation rates obtained with dimethylaniline and Mg²⁺ added separately. Hence the efficiency of Mg²⁺ with electron-transfer reactions in the NADPH oxidation chain is to be studied in the presence of a hydroxylation substrate.

The results on the effect of Mg²⁺ on the activity of the first components of the NADPH-flavoprotein oxidation chain are summarized in Table 3. By measuring the activity of the NADPH-specific flavoprotein in the reductase reactions with the help of different electron acceptors, it has been shown that neither Mg²⁺ and dimethylaniline separately, nor in combination, activates the reductase reactions; moreover, with NADPH-ferricyanide reductase activity Mg²⁺ are even responsible for a sharp inhibiting effect.

Fig. 4. *Effect of Mg²⁺ on the binding of dimethylaniline with cytochrome P-450*

The incubation mixture contained microsomal fraction (3.0mg of protein/ml) and 40mM-Tris buffer, in a total volume of 3 ml. Dimethylaniline was added at a final concentration of 0.2–1.4mM to microsomal suspensions. The measurements were made at room temperature. Extinction was measured as the difference between the trough at 420nm and the peak at 385nm and values are calculated per mg of microsomal protein. Assays were done in the absence (◆, ▲, ■, ●) and in the presence (◇, △, □, ○) of 16mM-MgCl₂. *K_s* (mM) and $\Delta E_{\max}$. (ΔE /mg of microsomal protein) in the absence of MgCl₂ were 0.69 ± 0.025 and 0.0048 ± 0.00008 respectively and in the presence of 16mM-MgCl₂ were 0.48 ± 0.007 and 0.0062 ± 0.0001 respectively.

Results for the effect of Mg^{2+} on the initial and terminal sites of the NADPH oxidation chain are shown in Table 4. According to the concepts developed in this laboratory, one of the cytochrome b_5 functions is to act as an intermediate between flavo-protein and cytochrome $P-450$ (Archakov *et al.*, 1969a). It is seen from the data in Table 4 that Mg^{2+} accelerates the reduction of cytochrome b_5 in the NADPH oxidation chain. At the same time, dimethylaniline produces a much weaker action, if any, on the reaction rate in the absence of Mg^{2+} and does not affect it, should Mg^{2+} be present. Table 4 demonstrates the stimulating action of Mg^{2+} on the rate of the NADPH–cytochrome $P-450$ reductase

reaction. It is clearly seen that here the stimulation coefficient (2.3) agrees well with the stimulation coefficient of dimethylaniline hydroxylation by Mg^{2+} (2.6). Addition of dimethylaniline in the absence of Mg^{2+} produces much less stimulation of the cytochrome $P-450$ reduction rate than when $MgCl_2$ is present in the incubation medium. The efficiency of

Table 2. *Effect of dimethylaniline and Mg^{2+} on the NADPH oxidation rate*

Experimental details are given in the text (see the Materials and Methods section). Dimethylaniline was added to the incubation medium at a final concentration of 1 mM and $MgCl_2$ was 16 mM. The rate of NADPH oxidation is expressed as nmol of NADPH ($\Delta NADPH$) or of O_2 (ΔO_2)/min per mg of microsomal protein. Results are average values from six experiments $\pm$ S.E.M.

Experimental conditions	$\Delta NADPH$	ΔO_2
Free oxidation of NADPH	2.0 ± 0.17	4.5 ± 0.4
With dimethylaniline	2.7 ± 0.27	6.9 ± 0.6
With dimethylaniline plus Mg^{2+}	7.1 ± 0.71	15.0 ± 1.0
Free oxidation of NADPH	2.1 ± 0.04	5.3 ± 0.9
With Mg^{2+}	4.7 ± 0.22	9.0 ± 1.2
With Mg^{2+} plus dimethylaniline	6.7 ± 0.42	13.0 ± 1.3

Table 4. *Reduction of cytochromes b_5 and $P-450$ in the presence of dimethylaniline and Mg^{2+}*

The incubation mixture contained microsomal fraction (1.0 mg of protein/ml) and 50 mM-Tris buffer, in a total volume of 3 ml. The reaction was initiated by adding $30 \mu M$ -NADPH. The NADPH–cytochrome b_5 reductase reaction was recorded at $20^\circ C$ and the NADPH–cytochrome $P-450$ reductase reaction at $25^\circ C$. Dimethylaniline was added to the incubation medium at a final concentration of 1.3 mM and $MgCl_2$ was 16 mM. For further experimental details see the Materials and Methods section. Results are average values from six experiments $\pm$ S.E.M. and are expressed as k (min^{-1}) values, the apparent rate constants of the reduction of cytochromes b_5 and $P-450$, calculated as the initial rates of the reduction of these cytochromes/nmol of cytochromes b_5 and $P-450$.

Experimental conditions	k (min^{-1})	
	Cytochrome b_5	Cytochrome $P-450$
Control	37 ± 4.3	7.8 ± 1.3
With dimethylaniline	41 ± 5.2	9.2 ± 1.3
With Mg^{2+}	58 ± 7.1	12 ± 1.4
With Mg^{2+} plus dimethylaniline	58 ± 7.1	21 ± 1.5

Table 3. *Effect of dimethylaniline and Mg^{2+} on NADPH-reductase activities of microsomal fraction*

Experimental details are given in the text (see the Materials and Methods section). Dimethylaniline was added to the incubation medium at a final concentration of 1 mM and $MgCl_2$ was 16 mM. Enzyme activities are expressed as nmol of the reduced acceptor/min per mg of microsomal protein. Results are average values from six experiments $\pm$ S.E.M.

Experimental conditions	Enzyme activities		
	NADPH–cytochrome c reductase	NADPH–ferricyanide reductase	NADPH–2,6-dichlorophenol-indophenol reductase
Control	150 ± 8.0	140 ± 8.8	42 ± 2.4
With dimethylaniline	130 ± 6.7	110 ± 6.4	33 ± 1.9
With dimethylaniline plus Mg^{2+}	110 ± 6.5	0	35 ± 2.6
Control	150 ± 5.3	130 ± 12.6	40 ± 3.9
With Mg^{2+}	120 ± 9.6	0	38 ± 4.1
With Mg^{2+} plus dimethylaniline	100 ± 7.1	0	29 ± 1.8

Fig. 5. Effect of Mg^{2+} on the quantum yield of 8-anilino-naphthalene-1-sulphonate fluorescence and on the binding of 8-anilino-naphthalene-1-sulphonate by rat liver microsomal fraction

The incubation mixture contained microsomes (0.3 mg of protein/ml), 50 mM-Tris buffer and 8-anilino-naphthalene-1-sulphonate at a final concentration of 5–25 μM , in a total volume of 3 ml. Excitation wavelength was 366 nm and the relative intensity of fluorescence was read at 480 nm. Fluorescence intensity is given per mg of microsomal protein: ●, without $MgCl_2$; ○, plus 16 mM- $MgCl_2$. The results are Lineweaver-Burk plots and are an average of ten experiments.

Fig. 6. Effect of Mg^{2+} and dimethylaniline on the 8-anilino-naphthalene-1-sulphonate fluorescence

For experimental conditions see Fig. 5 and the Materials and Methods section. 8-Anilino-naphthalene-1-sulphonate was added (at the first arrow) to microsomal suspensions at a final concentration of 7.5 μM , $MgCl_2$ was 16 mM and dimethylaniline was 1 mM.

the joint action of dimethylaniline and Mg^{2+} is much higher than just a sum of the effects observed on their being added separately.

The accelerating effect of Mg^{2+} on the NADPH-cytochrome *P*-450 reductase reaction may be a result of the significant conformational changes in the membrane, which are produced by Mg^{2+} and revealed by means of a fluorescent probe (8-anilino-naphthalene-1-sulphonate) (Fig. 5). Addition of 16 mM- $MgCl_2$ to the incubation mixture causes a considerable increase in the fluorescence quantum yield of 8-anilino-naphthalene-1-sulphonate and does not affect the binding constant of the fluorescent dye with the membrane. This effect of Mg^{2+} is retained if dimethylaniline is present in the incubation medium (Fig. 6).

Discussion

According to the data of Peters & Fouts (1970), the stimulating effect of Mg^{2+} is not the same in *p*-hydroxylation of aniline and *N*-demethylation of benzphetamine. To elucidate the mechanism of the Mg^{2+} effect, *N*-demethylation, *p*-hydroxylation and *N*-oxidation of one and the same substrate (dimethylaniline) have been studied. Such an approach made it possible to show that only *N*-demethylation and *p*-hydroxylation of dimethylaniline could be stimulated by Mg^{2+} . The absence of this effect for *N*-oxidation of dimethylaniline proves that either this effect does not depend on the NADPH-specific flavoprotein of the NADPH oxidation chain, where the reaction of *N*-oxidation of dimethylaniline might be localized (Archakov *et al.*, 1969b), or some other flavoprotein that is not sensitive to Mg^{2+} takes part in this reaction (Masters & Ziegler, 1971).

In *N*-demethylation of dimethylaniline, an increase in both V_{max} and K_m was observed, and for *p*-hydroxylation of dimethylaniline V_{max} increased whereas K_m remained unchanged. Thus a different effect of Mg^{2+} on *N*-demethylation and *p*-hydroxylation, which was first reported by Peters & Fouts (1970), also takes place when dimethylaniline is used as the only substrate. Hence this effect is determined by the mechanism of *p*-hydroxylation and *N*-demethylation, and may be realized owing to different functional groups of cytochrome *P*-450 participating in these reactions. A comparison of the kinetic parameters of *p*-hydroxylation and *N*-demethylation of dimethylaniline proves that in *N*-demethylation the K_m value is affected by the dissociation constant of the enzyme-substrate complex (k_{+2}). The K_m value for *N*-demethylation of dimethylaniline (0.45 mM without $MgCl_2$ and 0.67 mM with $MgCl_2$) is much higher than that of *p*-hydroxylation of dimethylaniline (0.28 mM).

A decrease in K_s under the action of Mg^{2+} , from 0.70 mM to 0.48 mM, is directly opposite to the increase of K_m for *N*-demethylation of dimethylaniline

and does not correlate with the absence of changes in K_m in *p*-hydroxylation of dimethylaniline. Thus an increase in the affinity of cytochrome *P*-450 for dimethylaniline under the action of Mg^{2+} followed by the changes in K_i is not accompanied by the same K_m changes in both reactions. The results obtained allow the conclusion to be made that both in *N*-demethylation and *p*-hydroxylation of dimethylaniline, the K_m value does not characterize the affinity of cytochrome *P*-450 for dimethylaniline. The coefficient of stimulation of dimethylaniline hydroxylation by Mg^{2+} (2.6) is in a good agreement only with the Mg^{2+} stimulation coefficient of the NADPH-cytochrome *P*-450 reductase (2.3).

Other reactions characterizing electron-transfer rate in the NADPH oxidation chain were not stimulated either by Mg^{2+} or by dimethylaniline (NADPH-flavoprotein reductase) (Table 3), and they were not stimulated by dimethylaniline if Mg^{2+} were present in the incubation medium (NADPH-cytochrome b_5 reductase) (Table 4). From the results obtained we may conclude that the rate-limiting site of NADPH oxidation in the presence of dimethylaniline, and of dimethylaniline hydroxylation is between cytochromes b_5 and *P*-450. Measurements of the apparent rate constants of reduction of cytochromes b_5 and *P*-450 by NADPH show that the rate constant of reduction of the former is much higher than that of the latter, and consequently electron transfer from cytochrome b_5 to cytochrome *P*-450 is the slowest reaction in the NADPH oxidation chain. When the incubation medium contains no dimethylaniline, Mg^{2+} produce a stimulating effect of approximately equal magnitude on reduction of cytochromes b_5 and *P*-450 (1.6-fold) and on NADPH oxidation rate (1.9-fold). However, as reduction of cytochrome *P*-450 is much slower than that of cytochrome b_5 we may suppose that in this case the limiting site is again between cytochromes b_5 and *P*-450.

The stimulating effect of Mg^{2+} on the electron transfer in the NADPH-cytochrome *P*-450 oxygenase system may be due to Mg^{2+} affecting the conformational state of membrane lipoprotein complexes. We have shown that when 8-anilino-naphthalene-1-sulphonate is used as a fluorescence probe, addition of Mg^{2+} to the incubation medium causes an increase in the quantum yield of the fluorescence of the dye without changing the number of 8-anilino-naphthalene-1-sulphonate-binding sites. According to the data of Eling & DiAugustine (1971), 8-anilino-naphthalene-1-sulphonate in the membranes of endoplasmic reticulum binds first of all with phos-

phatidylcholine, hence addition of Mg^{2+} affects the environment of phosphatidylcholine, increasing the degree of its non-polarity. Such an effect of Mg^{2+} may be the main reason for the increase in the reduction rate of cytochrome *P*-450 in the electron-transfer chain. It has been shown in a reconstructed system that it is phosphatidylcholine that is necessary for cytochrome *P*-450 to be reduced by NADPH-specific flavoprotein (Coon *et al.*, 1971).

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