Biochemical Pharmacology Vol. 18, pp. 1501–1509. Pergamon Press 1969. Printed in Great Britain

N-OXYGENATION OF *N*-ALKYL- AND *N,N*-DIALKYLANILINES BY RABBIT LIVER MICROSOMES*

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(*Received* 20 *September* 1968; *accepted* 17 *January* 1969)

Abstract—The *N*-oxygenation of *N*-ethylaniline to the *N*-hydroxy derivative and of *N,N*-dimethylaniline to the *N*-oxide in rabbit liver microsomes were studied. Both oxygenating systems had a low affinity for oxygen and were not inhibited by carbon monoxide. They were not stimulated by the treatment of rabbits with phenobarbital. *p*-Chloromercuribenzoate, *N*-ethylmaleimide, and 8-hydroxyquinoline were found to affect the *N*-oxygenation of *N*-ethylaniline and of *N,N*-dimethylaniline differently.

LIVER microsomes in the presence of oxygen and NADPH oxygenate *N*-alkyl anilines to the *N*-hydroxy derivatives[2] and *N,N*-dialkyl anilines to the *N*-oxides.[3, 4] Studies by Kiese and co-workers[5–9] showed that the microsomal *N*-hydroxylation of *N*-alkyl anilines differs from other microsomal hydroxylations like C-hydroxylation of anilines or de-alkylation of *N*-alkylanilines in the affinity of the system for oxygen and carbon monoxide, in the influence of SH-reagents and in other respects. This paper reports the results of experiments which were carried out in order to study the relationship of the microsomal *N*-oxygenation of *N,N*-dialkyl anilines to the *N*-hydroxylation of *N*-alkylanilines.

METHODS

Microsomes were prepared according to Von Jagow, Kampffmeyer, and Kiese.[10] They were used 24 hr after the preparation had begun, except the experiments in which the effect of storage was tested. The suspensions of microsomes used in the experiments contained $1{\cdot}2 \times 10^{-4}$ M NADP, $4{\cdot}8 \times 10^{-3}$ M glucose-6-phosphate, $1{\cdot}2 \times 10^{-2}$ M nicotinamide, 6×10^{-3} M magnesium chloride, 40 Bücher-Units glucose-6-phosphate dehydrogenase per ml, and 10^{-3} M substrate (anilines). For the experiments with acetanilide as substrate the microsomes were suspended in 0·1 M Tris buffer pH 7·4 with a protein content of about 7 mg per ml of suspension. For all other experiments the microsomes were suspended in 0·1 M phosphate pH 7·4 with a protein content of about 3 mg per ml of suspension. Unless the effect of low oxygen pressures or of carbon monoxide was tested, the suspensions were incubated with the substrate for 40 min under air at 37°. Experiments with low oxygen pressures or mixtures of oxygen and carbon monoxide were carried out in round vessels with a flat bottom of about 80 cm² and a capacity of about 100 ml. Estimations of the oxygen uptake by microsome suspensions and the low affinity of NADPH oxidase

* The results were briefly reported at a meeting of the Deutsche Pharmakologische Gesellschaft in Mainz on March 10 to 13, 1968.[1]

for oxygen[11, 12] prompted us to abandon the technique used by Kampffmeyer and Kiese[8] to eliminate oxygen diffusion as a rate limiting factor. Instead on the area of 80 cm^2 7 ml of microsome suspension was shaken with a frequency of 100 per min in a water bath of 37°. The microsome suspensions were equilibrated for 15 min with the gas mixtures by passing 20–25 l. of the mixture through the vessel containing the microsome suspension without substrate. Then the substrate was injected through the gas outlet and the stopcocks were closed. The reaction mixtures were incubated in the dark for 10 min with aniline and the *N*-alkylanilines, and for 30 min with acetanilide. All mixtures of nitrogen and oxygen and mixtures of carbon monoxide with an oxygen content of 2 per cent were prepared in steel flasks. Mixtures of carbon monoxide with a higher content of oxygen were obtained by mixing the gases flowing with controlled velocities. The oxygen content of the mixtures was checked after they had passed the reaction vessels.

The content of nitrogen, oxygen, and carbon monoxide in the gas mixture was determined in the Beckman gas chromatograph GC 2 with a column G 70020 molecular sieve 5 Å heated to 40°. Helium was used as carrier with a pressure of 20 psig at the entrance.

The product of *N*-hydroxylation was determined according to Herr and Kiese[13] after being oxidized to nitrosobenzene. Ziegler and Pettit's[3] method was used for determining *N,N*-dimethylaniline-*N*-oxide and Kérékjartó and Staudinger's[14] method for *p*-acetylaminophenol.

Formaldehyde was measured according to Nash.[32]

Gornall, Bardawill, and David's[15] method was used for determining protein concentrations.

Microsome suspensions with 10–20 mg protein per ml were used for determining spectral changes caused by substrates. The content of cytochrome P-450 in the microsomes was measured according to Omura and Sato.[16]

Young rabbits of about 1·3 kg weight were used for experiments in which the induction of *N*-oxygenating microsomal enzymes was studied. A group of three animals was injected subcutaneously (s.c.) with 50 mg phenobarbital sodium per kg ten times in 2 weeks. Another group of three animals was injected with a corresponding amount of isotonic sodium chloride solution. A day after the last injection microsomes were prepared from the livers of the rabbits.

RESULTS

(1) *The determination of* N-*oxide formation*

Pettit and Ziegler[17] as well as Machinist *et al.*[18] found that *N,N*-dimethylaniline-*N*-oxide is demethylated by pig liver microsomes. They discuss the possibility of the *N*-oxide being an intermediate in the dealkylation of *N,N*-dimethylaniline.

If there were a rapid decay of *N,N*-dimethylaniline-*N*-oxide in microsome suspensions, the *N*-oxide concentration observed after incubating *N,N*-dimethylaniline with microsomes would not correctly indicate the rate of *N*-oxide formation. We therefore determined the rate of *N*-oxide decay under the conditions of our experiments. The results summarized in Table 1 show that the decay of *N*-oxide is slow and, therefore, almost negligible in the determination of *N*-oxide formation.

We also measured the demethylation of *N,N*-dimethylaniline-*N*-oxide and found that the amount of formaldehyde produced from *N*-oxide is in good agreement with

TABLE 1. DECAY OF *N,N*-DIMETHYLANILINE-*N*-OXIDE AND FORMATION OF FORMALDEHYDE IN SUSPENSIONS OF MICROSOMES PREPARED FROM VARIOUS TISSUES

I	II	III		IV	V	
Microsomes from	Decrease in *N,N*-dimethylaniline-*N*-oxide concentration μM	μM Formaldehyde produced with *N,N*-dimethylaniline-*N*-oxide	without	μM Formaldehyde produced from *N,N*-dimethylaniline *N*-oxide	% Decrease in *N,N*-dimethylaniline-*N*-oxide concentration calculated from II	IV
Pig's corpus luteum	0	29·1	26·8	2·3	0	1
Pig's liver	10·0	54·1	41·0	13·1	6	7
Rabbit's liver	4·0	14·3	9·6	4·7	2	3

The microsomes were incubated with 0·18 mM *N,N*-dimethylaniline-*N*-oxide for 40 min at 37°. The data are the means of two to three experiments.

the decrease in *N*-oxide concentration. In these experiments it was observed that the microsomes of the tissues studied produce formaldehyde without *N*-oxide being added. This formaldehyde production, which was also observed by Wilson,[19] may simulate a decay of *N*-oxide.

(2) *The affinity of the oxygenating systems for substrates*

(a) *Oxygen.* Kampffmeyer and Kiese[8] observed that the *N*-hydroxylation of *N*-ethylaniline in rabbit liver microsomes has a lower affinity for oxygen than the *N*- and C-hydroxylations of aniline, the oxygen pressure for half-maximal velocity being 20 Torr and the apparent Michaelis constant $K_m = 3 \times 10^{-5}$ M.

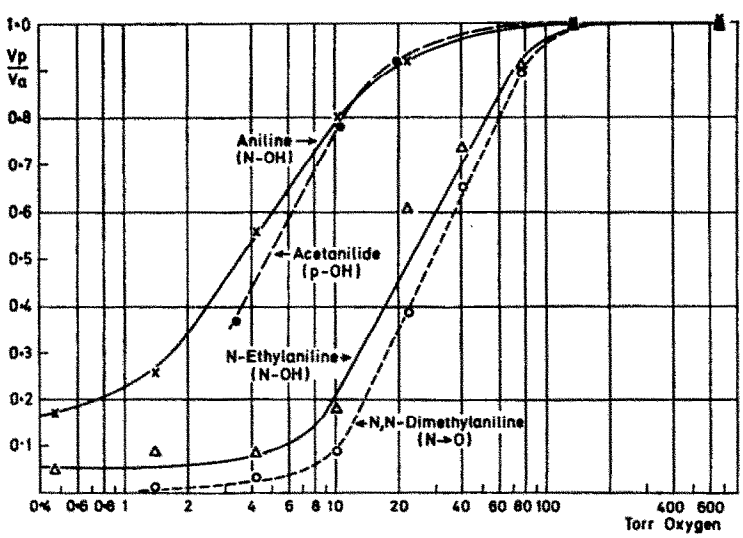

FIG. 1. *N*-Oxygenation of aniline, *N*-ethylaniline, *N,N*-dimethylaniline, and *p*-hydroxylation of acetanilide by rabbit liver microsomes at various oxygen pressures. The ordinate shows the quotient of the oxygenation rate under the oxygen pressure of the abscissa (V_P) divided by the rate under air (V_a). The symbols indicate the means of three to six experiments.

The results presented in Fig. 1 show that the formation of *N,N*-dimethylaniline-*N* oxide from *N,N*-dimethylaniline also has a low affinity for oxygen, the oxygen concentration for half-maximal reaction rate being 4×10^{-5} M versus 3×10^{-5} M for the *N*-hydroxylation of *N*-ethylaniline and 5×10^{-6} M for the *N*-hydroxylation of aniline.

With all 3 *N*-oxygenations studied the reaction velocities increased with the oxygen pressure to a maximum value which did not decrease on further increase of the oxygen pressure up to 666 Torr. An optimal oxygen pressure in a narrow range of low oxygen pressures which Kerékjártó and Staudinger[14] observed with the *p*-hydroxylation of acetanilide did not show up.

Since the inhibition by higher oxygen pressures might be a quality which distinguishes the C-hydroxylation from the *N*-oxygenation of arylamines we also studied the *p*-hydroxylation of acetanilide at various oxygen pressures. Except for the temperature, which was 25° in Kerékjártó and Staudinger's[14] and 37° in our experiments, our experiments were similar to Kerékjártó and Staudinger's. The data reproduced in Fig. 1 do not hint at a maximum velocity at low oxygen pressures. By means of a Lineweaver–Burk plot K_M was determined to be 9×10^{-6} M.

(b) *Arylamines.* Reaction velocities of the *N*-oxygenation of aniline, *N*-ethylaniline, and *N,N*-dimethylaniline were measured with concentrations of the substrates varying from 10^{-4} to 10^{-3} M. Apparent Michaelis constants calculated from Lineweaver–Burk plots are shown in Table 2.

TABLE 2. SPECTRAL CHANGES PRODUCED BY THE ADDITION OF ANILINE, *N*-ETHYLANILINE OR *N,N*-DIMETHYLANILINE TO RABBIT LIVER MICROSOMES AND APPARENT DISSOCIATION CONSTANTS (K_S) CALCULATED FROM ABSORBANCE DATA

	Absorption nm	Absorption nm	K_S M	K_M M
Aniline	423	—	4×10^{-2}	7×10^{-3}
N-Ethylaniline	—	417	3×10^{-4}	4×10^{-3}
N,N-Dimethylaniline	—	420	2×10^{-4}	7×10^{-4}

K_M is the apparent Michaelis constant derived from kinetic data. The figures are the means of three experiments.

Narasimhulu *et al.*,[20] Remmer *et al.*,[21] Imai and Sato,[22, 23] as well as Schenkman *et al.*[24] observed spectral changes in liver microsomes upon addition of various substances which may be hydroxylated by microsomal enzymes. The spectral changes caused by some arylamines after the addition to rabbit liver microsomes are listed in Table 2. *N*-Ethylaniline and *N,N*-dimethylaniline produced a difference spectrum not of the aniline type but of the hexobarbital type with minima at 417 and 420 nm.

The apparent dissociation constants for *N*-ethylaniline and *N,N*-dimethylaniline, listed in Table 2, are similar to one another and different from the constant for aniline. Table 2 also shows the apparent Michaelis constants. There is a small difference between K_S and K_M for *N,N*-dimethylaniline and a more substantial difference between the constants referring to the binding of *N*-ethylaniline.

(3) *The effect of some substances on microsomal* N-*oxygenations*

(a) *Carbon monoxide.* The effect of carbon monoxide was tested with various oxygen pressures and various ratios of carbon monoxide to oxygen. The activities of the oxygenating enzyme under the respective oxygen pressure may be seen in Fig. 1.

The results of the experiments, presented in Table 3, show that the *N*-oxygenation of *N*-ethylaniline and *N*,*N*-dimethylaniline was not inhibited by carbon monoxide. Ziegler and Pettit[4] studied the effect of carbon monoxide on the *N*-oxygenation of *N*,*N*-dimethylaniline in pork liver microsomes and did not observe an inhibiting effect. They used ratios of carbon monoxide to oxygen from 2 to 9, but they do not

TABLE 3. *N*-OXYGENATION OF *N*-ETHYLANILINE AND *N*,*N*-DIMETHYLANILINE AND *p*-HYDROXYLATION OF ACETANILIDE BY RABBIT LIVER MICROSOMES IN THE PRESENCE OF CARBON MONOXIDE

$\frac{p_{CO}}{p_{O_2}}$	p_{O2} Torr	Phenylhydroxylamine from *N*-ethylaniline	*N*,*N*-Dimethylaniline-*N*-oxide from *N*,*N*-dimethylaniline	*p*-Acetaminophenol from acetanilide	
		n	*n*	*n*	K_G
1·6	15	1·45 ± 0·16	1·58 ± 0·13	—	—
2·3	16	—	—	0·74 ± 0·03	6·56
4·9	16	—	—	0·51 ± 0·04	5·10
9·1	66	1·18 ± 0·11	1·25 ± 0·04	—	—
9·7	15	1·41 ± 0·29	1·96 ± 0·23	—	—
13·8	16	—	—	0·32 ± 0·06	6·49
42·2	15	1·40 ± 0·25	1·19 ± 0·06	—	—
					6·05

n indicates the ratio between the reaction rates in the presence and in the absence of carbon monoxide; K_G is the distribution constant (Warburg[25]). The data are the means of four to five experiments.

mention the oxygen pressure used in the experiments. The effect of carbon monoxide on the *N*-hydroxylation of aniline had to be studied more thoroughly. The results will be published in another paper.

Experiments with acetanilide confirmed that the *p*-hydroxylation of this substrate is inhibited by carbon monoxide (see Table 3). The distribution constant of the enzyme reacting with carbon monoxide was calculated according to Warburg as being $K_G = 6$. This may be compared with $K_G = 3$ found by Kerékjártó and Staudinger[14] with rabbit liver microsomes at 25°.

(b) *Other substances.* In earlier experiments,[6, 7] several substances were found to affect the microsomal *N*-hydroxylation of *N*-ethylaniline differently from other hydroxylations. Some of these substances were chosen for a comparison of their effect on the *N*-oxygenation of *N*-ethylaniline and *N*,*N*-dimethylaniline. The results summarized in Table 4 show that *p*-chloromercuribenzoate and *N*-ethylmaleimide inhibited the *N*-oxide formation and stimulated the *N*-hydroxylation of *N*-ethylaniline. 8-Hydroxyquinoline slightly inhibited the *N*-hydroxylation of *N*-ethylaniline but not the *N*-oxide formation. The other substances did not affect these reactions differently.

TABLE 4. THE EFFECT OF SOME SUBSTANCES ON THE *N*-OXYGENATION OF ANILINE AND *N*-ALKYLANILINES BY RABBIT LIVER MICROSOMES

		Phenylhydroxylamine from aniline	Phenylhydroxylamine from *N*-ethylaniline	*N,N*-Dimethylaniline -*N*-oxide from *N,N*-dimethylaniline	P
p-Chloromercuribenzoate	10^{-3}M	− 43 ± 5·1	+ 21 ± 6·5	− 48 ± 3·9	0·001
N-Ethylmaleimide	5×10^{-3}M	− 48 ± 3·8	+ 22 ± 5·5	− 12·4 ± 2·2	0·0027
8-Hydroxyquinoline	10^{-3}M	− 51 ± 3·5	− 15 ± 3·5	− 0·6 ± 1·0	0·01
2,4-Dichlorophenol	2×10^{-3}M	− 17 ± 0·9	+ 30 ± 6·8	+ 8·9 ± 2·9	0·05
Semicarbazide	10^{-2}M	− 31·6 ± 3·2	− 40·6 ± 2·3	− 41 ± 4·3	0·475
o-Phenanthroline	10^{-3}M	− 53·5 ± 2·4	− 8·2 ± 6·3	+ 2·1 ± 2·9	0·10

The figures, which are the means of three to five experiments, show percent change in activity caused by the substance added. P indicates the probability of the *N*-oxygenation of *N*-ethylaniline and *N,N*-dimethylaniline being equally affected by a substance.

(4) *Influence of hydrogen ion concentration on the oxygenating rates*

For determining the oxygenating rates at various pH values the liver microsomes were suspended in 0·1 M phosphate pH 5·5–7·5, in 0·1 M tris buffer pH 7·5–9·0, and 0·1 M borate —K Cl pH 9·0–10·5 according to Atkins and Pantin.[26] The results are shown in Fig. 2. The velocities of the oxygenation of *N*-ethylaniline and *N,N*-dimethylaniline were found to be most rapid at pH around 8·5. The *N*-hydroxylation of aniline, however, proceeds with maximum speed at pH 7·5.

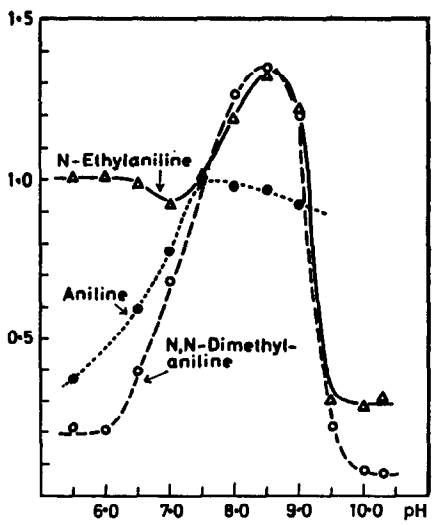

FIG. 2. *N*-Oxygenation of aniline, *N*-ethylaniline and *N,N*-dimethylaniline by rabbit liver microsomes at various hydrogen ion concentrations. The symbols indicate the means of three experiments. In order to eliminate the differences in the activity of various microsome preparations, the activity found at pH 7·5 was taken to be 1·0.

(5) *Influence of storage of microsomes on* N-*oxygenating activities*

Rabbit liver microsomes suspended in a small volume of 0·1 M phosphate solution pH 7·4 were kept in the refrigerator at 2–3°. After various periods of time samples were taken for testing the oxygenating activities. These were found to decrease steadily with time, the inactivating reaction being of the first order. The rate of decrease was not uniform with all oxygenations. As an average of three experiments the following half-lives were found: *N*-Hydroxylation of aniline 300 hr, *N*-oxygenation of *N,N*-dimethylaniline 150 hr, *N*-hydroxylation of *N*-ethylaniline 100 hr.

(6) *Induction of microsomal oxygenating enzymes by treatment of rabbits with phenobarbital*

In a study of the stimulation of some microsomal oxygenations by treatment of young rabbits with phenobarbital, Lange[27] observed that in liver microsomes the *N*-hydroxylation of aniline increased to nearly 20-fold its rate found in the microsomes of untreated animals. The rate of the *N*-hydroxylation of *N*-ethylaniline, however, was not increased. Induction experiments, therefore, offered another possibility to investigate the relationship between the *N*-oxygenations of *N*-alkyl- and *N,N*-dialkylanilines.

The results of our experiments are summarized in Table 5. The data referring to the *N*-hydroxylation of aniline and the content of cytochrome P-450 are in fair agreement with Lange's[27, 28] results. When the figures in Table 5 are compared with Lange's,

TABLE 5. THE EFFECT OF PHENOBARBITAL TREATMENT OF RABBITS ON THE *N*-OXYGENATING ACTIVITY OF LIVER MICROSOMES

	Phenylhydroxylamine				*N,N*-Dimethylaniline-*N*-oxide from *N,N*-dimethylaniline		Cytochrome P 450 nMol/mg	
	from aniline		from *N*-ethylaniline					
	μg/ml	P	μg/ml	P	μg/ml	P	protein	P
Controls	0·53 ± 0·08	0·0005	2·3 ± 0·7	0·025	14·3 ± 3·2	0·025	1·18 ± 0·22	0·005
Phenobarbital treated rabbits	7·3 ± 0·93		0·82 ± 0·17		5·7 ± 1·1		2·32 ± 0·08	

Young rabbits were treated for 2 weeks with 50 mg phenobarbital sodium per kg daily. The figures are the means of seven to nine experiments.

the difference in the experimental conditions for determining the oxygenating activities must be taken into account. Lange presents the activities observed with the microsomes obtained from 1 g of liver. As a consequence of the phenobarbital treatment the yield of microsomal protein increased to twice the value observed with untreated animals. Our figures for oxygenating activities always refer to the same amount of microsomal protein no matter what the yield of microsomes was. The data presented in Table 5 also confirm Lange's observation that the *N*-hydroxylation of *N*-ethylaniline is not stimulated by the treatment of the rabbits with phenobarbital. In our experiments, the *N*-hydroxylation of *N*-ethylaniline per mg microsomal protein decreased by 65 per

cent as a consequence of the phenobarbital treatment. Similarly the phenobarbital treatment of the rabbits did not stimulate but diminished the *N*-oxygenation of *N,N*-dimethylaniline by 60 per cent.

DISCUSSION

The results of our experiments demonstrate a close relationship between the microsomal *N*-oxygenation of *N*-alkylanilines and *N,N*-dialkylanilines. The affinity of the enzyme system for oxygen is low; carbon monoxide does not inhibit the reactions; the spectral changes caused in microsomes are similar; treatment of rabbits with phenobarbital does not stimulate the reaction in the microsomes. There are, however, differences in the effect of substances reacting with SH-groups like *p*-chloromercuribenzoate or *N*-ethylmaleimide. They inhibit the *N*-oxygenation of *N,N*-dimethylaniline but not of *N*-ethylaniline. Presently, the causes of such differences are not known.

The failure of carbon monoxide to inhibit the *N*-oxygenations studied in this paper seems to hint at a mechanism in which cytochrome P-450, the carbon monoxide binding hemoprotein in the microsomes, is not involved. This view may be supported by the observation of Heinze *et al.*[29] that microsomes prepared from corpora lutea of pig ovaries rapidly *N*-oxygenate *N*-ethyl- and *N,N*-dimethylaniline, although the P-450 content is very low. This, certainly, proves that cytochrome P-450 is not a rate limiting factor in the *N*-oxygenation of *N*-alkylarylamines in rabbit liver microsomes.

The *N*-oxygenation of *N*-alkyl and *N,N*-dialkylanilines is not the only microsomal oxygenation with extremely low or no sensitivity to carbon monoxide. Gillette[30] found that the sulfoxidation of diaminodiphenyl sulfide by mouse liver microsomes is not inhibited by carbon monoxide.

In experiments with rats, Arrhenius[31] found that feeding a vitamin E-deficient diet or a protein free diet as well as treatment with 2-aminofluorene, 2-acetyl-aminofluorene, or prednisolone stimulated the microsomal formation of *N,N*-dimethylaniline-*N*-oxide from *N,N*-dimethylaniline. The *N*-hydroxylation of *N*-alkylaniline was not studied. These results are not necessarily at variance with ours. Little is known of species differences in the stimulation of microsomal hydroxylations by various substances. But there is a body of evidence for certain specificities in the stimulation of microsomal hydroxylations by substances like phenobarbital and hydrocarbons or others.

Acknowledgements—The authors are grateful to Miss Renate Böhm and Mrs. Renate Malangrè for competent technical assistance.

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