MOLECULAR PHARMACOLOGY, 8, 481–489

Inhibition by Deuterated Water of the Mixed-Function Oxidases of Hepatic Microsomes of the Male Rat

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(Received March 9, 1972)

SUMMARY

Ethylmorphine *N*-demethylase, aniline hydroxylase, and 3,4-benzo[*a*]pyrene hydroxylase of hepatic microsomes were inhibited 22–32% by substitution of D_2O for H_2O in the incubation medium. The effect on the first two enzymes was not related to the pH or the time course of incubation. The inhibition was linear with D_2O concentration, occurred immediately, and was rapidly reversible. The inhibition was purely noncompetitive. NADPH–cytochrome *c* reductase in the intact microsomes was inhibited 15%, while in the solubilized form it was inhibited 51%. Basal NADPH oxidase and NADPH–cytochrome P-450 reductase activities were not greatly affected, but were significantly inhibited in the presence of 2 mM ethylmorphine. In both cases the difference between the stimulated and basal activities was stoichiometric with ethylmorphine *N*-demethylase and exhibited the same degree of inhibition. These findings suggest that, in spite of the lipid nature of the microsomal membrane, changes in hydration of the lipoprotein play a significant role in the mixed-function oxidases.

INTRODUCTION

Mixed-function oxidases are an integral part of the liver microsomal membrane and exhibit maximal activity only when bound to the intact membrane. The removal of phospholipids by lipases (1–3) leads to profound changes in spectral properties and a loss of activity, suggesting an intimate relationship between the enzymatic protein and the membrane lipids. Furthermore, the higher activity found with very lipid-soluble substrates and the inactivity of polar substrates (4) suggest that these enzymes not only are associated with the membrane but are actually buried in it. Yet, in spite of the hydrophobic character of this complex, we have found that deuterated water has a significant and reproducible inhibitory effect on their activity (5).

Deuterated water may inhibit enzymatic reactions either as a reactant or through its hydration of the enzymatic protein. In the latter case, the enzyme hydrated with deuterated rather than normal water may less readily undergo the tertiary structural changes necessary for activity. As with mitochondrial oxidative phosphorylation, the mixed-function oxidases have no formal requirement for water. To explain the rather diffuse inhibitory effect of deuterated water on mitochondrial oxidative phosphorylation, which they and others have observed, Tyler and Estabrook (6) have postulated the existence of "cryptic protolysis" in the mito-

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chondrial electron chain (7–10). Although this inhibition of oxidative phosphorylation might fit into the mechanism postulated by Mitchell (11), in which increased chemical activity of the free proton is the driving force for phosphorylation, no such scheme seems applicable to the mixed-function oxidases.

More recently, Ahmed *et al.* (12) have demonstrated inhibition of the ($Na^+ + K^+$)-activated ATPase of brain microsomes, which is also a membrane-bound lipoprotein complex. Unlike the generalized inhibition of mitochondrial oxygen uptake, D_2O competitively inhibited the Na^+-activated step and appeared actually to stimulate the K^+-activated step (12). These authors suggested that the D_2O acted by altering the structure of the lipoprotein rather than participating in the enzymatic reaction.

Similarly, our results indicate that the primary effect of deuterated water is to alter the hydration of the microsomal membrane proteins, with a resultant inhibition of the conformational changes necessary for activity. This effect is most pronounced in the inhibition of the coupling of electron transport to the presence of type I substrates (13–17).[2] The correlation of the inhibition of this process with the inhibition of the overall mixed-function oxidase activity further supports the suggestion of Gigon, Gram, and Gillette (14, 15) that the rate-determining step of the mixed-function oxidases is stimulation of the transport of electrons down the electron chain by type I substrates.

METHODS

All animals used in these experiments were fed, untreated, 200–300-g male Sprague-Dawley rats obtained from Charles Riber Laboratories, Inc.

The animals were killed by cervical fracture. The livers were removed, chilled on ice, and homogenized in 0.15 M KCl–0.02 M Tris, pH 7.4 (3 ml/g of liver), with a Teflon-glass homogenizer (0.10–0.15-mm clearance). The homogenate was centrifuged at 9000 × *g* for 15 min in a Sorvall RC-2B centrifuge with an SS-34 rotor. The supernatant solution was centrifuged at 165,000 × *g* (avg) for 38 min in a Beckman L-3-50 centrifuge with a 50 Ti rotor. The microsomal pellet was resuspended in KCl-Tris (the equivalent of 2 g of liver per milliliter of suspension), and protein was determined by the method of Sutherland *et al.* (18).

Deuterated water (99.5% deuterium) was obtained from Mallinckrodt (lots TTS and XBJ) and redistilled in glass. The pH and pD were determined with an Ag–AgCl–Ag combination electrode (Metrohm EU147X). In spite of the reports of an apparent discrepancy between the pH and pD (pD = reading +0.4) (19) when estimated with a calomel reference electrode, the results obtained below indicated that the nominal pD values obtained with this electrode without adding 0.4 were optimal. Except as noted below, all incubations were conducted in KCl–Tris–$MgCl_2$ (0.15 M–0.02 M–0.005 M; pH or nominal pD = 7.4).

In the incubations for the determination of ethylmorphine *N*-demethylase, aniline hydroxylase, and 3,4-benzo[*a*]pyrene hydroxylase activities, an NADPH-generating system was prepared by adding NADP (0.33 mM), glucose 6-phosphate (5 mM), and glucose 6-phosphate dehydrogenase (0.67 unit/ml)[3] to the appropriate buffer. Solutions of ethylmorphine and aniline hydrochloride (neutralized with NaOH) (0.15 M, 40 μl) were added to 3 ml of KCl–Tris–$MgCl_2$–NADPH solution to give a final concentration of 2 mM. The 3,4-benzo[*a*]-pyrene was added as an acetone solution to the empty vials, the solvent was blown off, and the incubation solution was added. At the end of the incubation, crystals of this substrate remained on the sides of the vessels. The vessels were warmed for 2 min with shaking, and the microsomal suspension was added to give a final concentration of 1 mg of microsomal protein per milliliter (50–70 μl of 60–45 mg of protein per milliliter). In these incubations all substrate concentrations were at least 8 times higher than the apparent K_m (greater than 90% of the V_{max} value for ethylmorphine) and were

[2] A type I substrate, when added to hepatic microsomes, gives a difference spectrum with a peak at 385 nm and a trough at 423 nm.

[3] One unit of glucose 6-phosphate dehydrogenase activity will reduce 1 μmole of NADPH per minute at 25°.

linear with respect to time and protein concentration.

The mixtures were incubated for 10 min at 37° in a shaking water bath under air. The ethylmorphine *N*-demethylase reaction was terminated by the addition of 5% $ZnSO_4$ (1 ml), followed by saturated $Na_2B_4O_7 \cdot 10H_2O$ (0.5 ml) and saturated $Ba(OH)_2$ (1.5 ml). Formaldehyde was determined in the clear supernatant solution with acetylacetone in 4 M ammonium acetate (0.5 volume) (20). The aniline hydroxylase reaction was terminated by placing the vessels in ice, extracting the products with ether from a saturated NaCl solution, and determining the formation of *p*-aminophenol by the yield of indophenol (21). The reaction of 3,4-benzo[*a*]pyrene hydroxylase was also terminated by placing the mixture on ice. The hydroxylated derivatives were extracted into hexane (10 ml) and back-extracted into 1 M NaOH, and the products as determined fluorometrically (activating wavelength, 395 nm; emission, 509 nm) were compared with 8-hydroxy-3,4-benzo[*a*]-pyrene[4] (22).

For determination of the effect of pH or pD on ethylmorphine *N*-demethylase and aniline hydroxylase, the Tris was omitted and a buffer of the appropriate pH (1 M PO_4^- or Tris, 0.15 ml) was added to each vessel. Kinetic constants for ethylmorphine *N*-demethylase and aniline hydroxylase were determined after the substrate solution (ethylmorphine, 75 mM; aniline, 30 mM) had been added with a Hamilton dispenser (PB-600) to the incubation medium.

NADPH–cytochrome *c* reductase activity was determined in an Aminco-Chance split-beam, dual-wavelength spectrophotometer. The KCl–Tris–$MgCl_2$ buffer was warmed to 37° for 5 min, and horse heart cytochrome *c* (Sigma) (50 mg/ml, 40 μl) and freshly dissolved NADPH (Sigma) (100 mg/ml, 10 μl) were added. The reaction was begun by adding 10 μg of microsomal protein per milliliter. The rate was determined from the initial change in the absorbance at 554–540 nm, assuming a millimolar extinction coefficient of 18.7.

NADPH oxidase activity was assayed after warming the buffer at 37° for 5 min in the Aminco-Chance instrument, and adding NADPH (100 mg/ml, 10 μl) and finally the microsomal suspension to give a concentration of 1 mg/ml. The rate was determined by the initial change in absorbance at 340–390 nm, using a millimolar extinction coefficient of 6.2.

Dichlorophenolindophenol reductase activity was determined in the same fashion as NADPH–cytochrome *c* reductase, except that the Aminco-Chance apparatus was used in the split-beam mode with the light at 605 nm and a millimolar extinction coefficient of 20.

NADPH–cytochrome P-450 reductase activity was assayed after the addition of 2.8 ml of KCl–Tris–$MgCl_2$ buffer to an Aminco anaerobic cuvette, followed by the microsomal suspension (9 mg of protein) and ethylmorphine (0.15 M, 40 μl) as indicated. The mixture was gassed for 5 min with carbon monoxide which had been deoxygenated by passage through a solution of 5% sodium dithionite–0.05% anthroquinone-2-sulfonic acid in 0.1 M NaOH. The cuvette was capped after NADPH (100 mg/ml, 10 μl) had been placed in the plunger and the gas phase flushed with carbon monoxide for 1 min. The cuvette was warmed in the Aminco-Chance apparatus for 7 min at 37°, and the reductase activity was determined by the initial change in absorbance 450–490 nm. A millimolar extinction coefficient of 91 was used (2).

All assays were done in triplicate. The kinetic constants for ethylmorphine *N*-demethylase and aniline hydroxylase were estimated by inspection from a double-reciprocal plot and confirmed by computer analysis (23). For all studies on the components of the mixed-function oxidases, an assay of ethylmorphine *N*-demethylase was run on the same day with the same batch of buffer and microsomes and was used for the comparisons made.

RESULTS

As can be seen in Table 1, significant inhibition of mixed-function oxidase activity

[4] The 8-hydroxy-3,4-benzo[*a*]pyrene was kindly supplied by Dr. Harry Gelboin of the National Cancer Institute.

TABLE 1

Effect of deuterated water on ethylmorphine N-demethylase, aniline hydroxylase, and 3,4-benzo[a]pyrene hydroxylase activities of male rat hepatic microsomes

Preparations and assays are described in the text.

Substrate	Product	Buffer	Rate	$D_2O:H_2O$[a]
			nmoles product/min/mg protein	
Ethylmorphine	Formaldehyde	H_2O	9.79 ± 0.04[b]	
		D_2O	7.66 ± 0.36	0.783
Aniline	*p*-Aminophenol	H_2O	0.355 ± 0.017	
		D_2O	0.233 ± 0.011	0.657
3,4-Benzo[a]pyrene	8-Hydroxy-3,4-benzo[a]pyrene	H_2O	0.0197 ± 0.0013	
		D_2O	0.0136 ± 0.0006	0.691

[a] Ratio of activity in buffer containing 99% deuterated water to that with standard water.

[b] Averages ± standard errors of triplicate incubations.

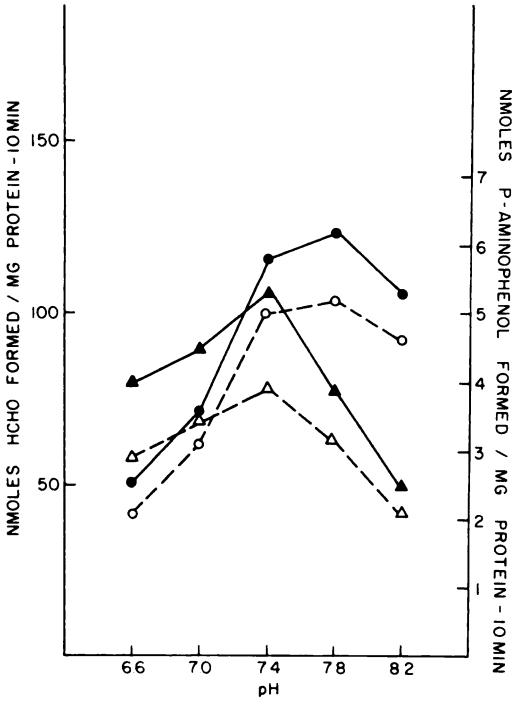

FIG. 1. *Effect of pH on ethylmorphine N-demethylase in H_2O buffer (●——●) and D_2O buffer (○- - -○) and on aniline hydroxylase in H_2O buffer (▲——▲) and D_2O buffer (△- - -△)*

Preparations and assays are described in the text.

was demonstrated for each of the three substrates examined. The similar degree of inhibition for each of the substrates might suggest that they possess the same rate-limiting steps, presumably the reduction of cytochrome P-450 (24, 25). This inhibition could have been an artifact of change in the extinction coefficient of the colorimetric products in the deuterated water. This seems unlikely, since the aniline and 3,4-benzo[a]-pyrene products had been extracted, so that no effect on color development should have occurred. In the case of formaldehyde, there was no change in the absorbance whether determined in water or in deuterated water [ϵ_{H_2O} = 7720, ϵ_{D_2O} = 7820]. Furthermore, there was no effect on the extinction coefficient of NADPH [ϵ_{H_2O} = 6620, ϵ_{D_2O} = 6360] or in the concentration of NADPH in the incubation mixture when a generating system was used.

Because of the question of the relationship of pD to pH in the incubation mixture, a curve of activity with respect to both pH and pD was determined for ethylmorphine *N*-demethylase and aniline hydroxylase. As can be seen in Fig. 1, the ethylmorphine *N*-demethylase activity reached a peak at a pH or nominal pD of 7.8, and that of aniline hydroxylase, at 7.4. Clearly there was no shift of the deuterated water curve toward the acid, as would be expected if the pD were really 0.4 unit higher than the reading.

Another possibility is that the initial rates were the same but that the deuterated water denatured the enzymes so that the yield of product was continued in normal deuterated not radioactive but not in deuterated water. As Fig. 2 shows, however,

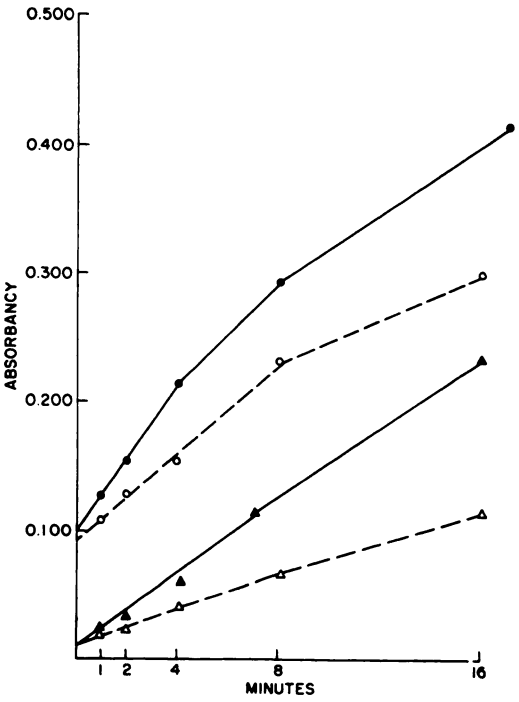

FIG. 2. *Time course of ethylmorphine N-demethylase in H_2O buffer* (●——●) *and D_2O buffer* (○- - -○) *(absorbance at 412 nm) and of aniline hydroxylase in H_2O buffer* (▲——▲) *and D_2O* Buffer (△- - -△) *(Absorbance at 650 nm)*

Preparations and assays are described in the text.

both enzymes in both solvents displayed essentially linear reactions for at least 10 min.

It is of some interest that the inhibition was linear with the concentration of deuterium (Fig. 3). Furthermore, when microsomes suspended and incubated in deuterated water were compared with those suspended and incubated in standard water, the inhibition appeared rapidly and did not progress with time (Fig. 4). At the end of 90 min, when microsomes suspended in deuterated water were incubated in buffer containing standard water, there was a total reversal of inhibition, indicating that irreversible denaturation of the enzyme had not occurred.

Deuterated water had no effect on the apparent K_m of either ethylmorphine *N*-demethylase (K_m in H_2O = 0.20 mM; K_m in D_2O = 0.20 mM) or aniline hydroxylase (K_m in H_2O = 0.053 mM; K_m in D_2O = 0.048 mM), but exhibited purely noncompetitive inhibition of the reaction, with a decrease in the V_{max} of ethylmorphine *N*-demethylase from 9.17 to 6.45 nmoles of formaldehyde per minute per milligram of protein, and in the V_{max} of aniline hydroxylase, from 0.625 to 0.444.

An Arrhenius plot of ethylmorphine *N*-demethylase and aniline hydroxylase in standard and deuterated water (Fig. 5) shows only small differences in the activation energies in either solvent.

As can be seen in Table 2, the basal level of NADPH oxidase was essentially unaffected by the presence of deuterated water, but the stimulated values were significantly

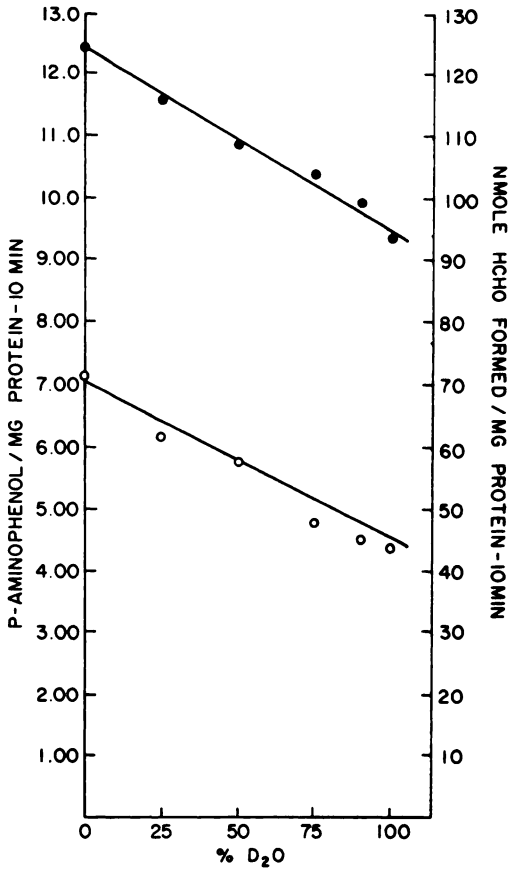

FIG. 3. *Effect of varying concentrations of D_2O on activity of ethylmorphine N-demethylase* (●——●) *and aniline hydroxylase* (○——○).

Preparations and assays are described in the text.

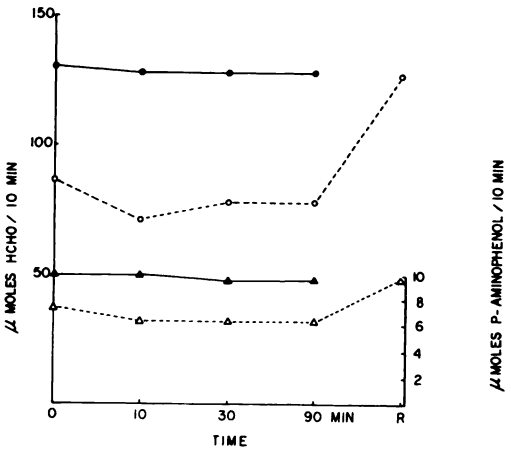

FIG. 4. *Effect of storage of microsomes on ethylmorphine N-demethylase activity in H_2O buffer* (●– –●) *and D_2O buffer* (○- - -○) *and on aniline hydroxylase in H_2O buffer* (▲– –▲) *and D_2O buffer* (△- - -△).

The enzymatic activity of microsomes stored in H_2O buffer was determined in H_2O buffer, while those stored in D_2O buffer were assayed in D_2O buffer except for point *R*, where the activity of the enzymes was determined in H_2O buffer for microsomes after storage in D_2O buffer for 90 min. Preparations and assays are described in the text.

inhibited. Moreover, for aminopyrine *N*-demethylase, in agreement with the results of Orrenius (26), the difference between the basal rate and the rate in the presence of ethylmorphine was stoichiometric with the rate of formation of formaldehyde in both the presence and absence of deuterated water. Clearly the stimulation of the NADPH oxidase by ethylmorphine was inhibited to the same extent as that of the *N*-demethylase.

From Table 3 it is readily apparent that microsomal NADPH-cytochrome *c* reductase was inhibited far less than ethylmorphine *N*-demethylase by deuterated water. It is not surprising that the inhibition of these two reactions was not the same, since the reductase does not appear to be involved in the rate-limiting step in the overall mixed-function oxidase activity. What is surprising is that the solubilized and partially purified preparation of Lu, Junk, and Coon (27) was much more sensitive to deuterated water than were the other reactions. Furthermore, the reduction of 2,6-dichlorophenolindophenol also showed a greater sensitivity to the presence of deuterated water, even in intact microsomes.

Like NADPH oxidase, the basal levels of NADPH-cytochrome P-450 reductase were relatively unaffected by the substitution of deuterated water for standard water (Table 4). The difference (Δ) between the rates in the presence and absence of 2 mM ethylmorphine was also stoichiometric with the formation of formaldehyde. It is this difference in reductase activity which is inhibited by the presence of deuterated water, and this inhibition is the same as that for the *N*-demethylase.

DISCUSSION

In spite of the highly lipid nature of the microsomal membrane, significant and reproducible inhibition of the mixed-function oxidase activity of hepatic microsomes occurred when deuterated water was substituted for ordinary water. This inhibition was both rapid and readily reversible. A number of sites in the enzyme complex may be affected by this substitution.

The first enzyme in the electron chain is a flavoprotein which presumably is related to NADPH-cytochrome *c* reductase, originally separated from the membrane and purified by Strittmatter (28, 29). In this preparation the membrane is incubated with a proteolytic enzyme, with the loss of NADPH-

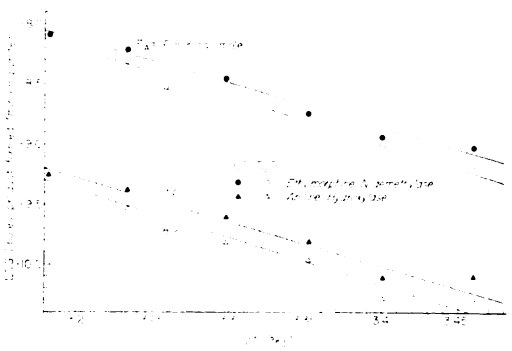

FIG. 5. *Arrhenius plot of ethylmorphine N-demethylase in H_2O buffer* (●– –●) *and D_2O buffer* (○– –○) *and of aniline hydroxylase in H_2O buffer* (▲- - -▲) and *D_2O buffer* (△- - -△)

Preparations and assays are described in the text.

TABLE 2

Effect of deuterated water on NADPH oxidase and ethylmorphine N-demethylase activities of male rat hepatic microsomes

Preparations and assays are described in the text.

Assay	Buffer	2 mM ethylmorphine	Rate	Δ[a]
			nmoles/min/mg protein	
NADPH oxidase	H_2O	−	4.32 ± 0.12[b]	
	H_2O	+	10.72 ± 0.33	6.40
	D_2O	−	4.45 ± 0.08	
	D_2O	+	8.85 ± 0.20	4.40
Ethylmorphine *N*-demethylase	H_2O	+	6.00 ± 0.12	
	D_2O	+	3.99 ± 0.08	

[a] Δ is the difference between NADPH oxidase activities in the presence and absence of 2 mM ethylmorphine.

[b] Averages ± standard errors of triplicate incubations.

TABLE 3

Effect of deuterated water on NADPH-cytochrome c reductase, NADPH diaphorase, and ethylmorphine N-demethylase

Preparations and assays are described in the text.

Assay	Buffer	Rate	D_2O:H_2O[a]
		nmoles/min/mg protein	
NADPH-cytochrome *c* reductase	H_2O	48.7 ± 1.8[b]	
	D_2O	41.6 ± 1.2	0.854
NADPH-cytochrome *c* reductase, solubilized[c]	H_2O	757 ± 13	
	D_2O	370 ± 8	0.489
2,6-Dichloroindophenol reductase	H_2O	183 ± 5	
	D_2O	108 ± 4	0.590
Ethylmorphine *N*-demethylase	H_2O	5.42 ± 0.08	
	D_2O	3.80 ± 0.12	0.701

[a] Activity in deuterated water divided by activity in standard water.

[b] Averages ± standard errors of triplicate incubations.

[c] Preparation of Lu, Junk, and Coon (27).

cytochrome P-450 reductase activity. The rate-limiting step for cytochrome *c* reductase activity for both the purified enzyme and intact microsomes is reduction of the flavin (21, 30). In the reduction of cytochrome P-450, however, the rate-limiting step is the transfer of electrons from the flavin to the cytochrome (24, 25). It is possible that if reduction of the flavin were sufficiently inhibited, the over-all reaction would also be inhibited. For example, the hydrogens of the reduced flavin readily exchange with the medium, so that there could be an isotope effect on the transport of electrons to the cytochrome. In view of the relatively slight inhibition of NADPH-cytochrome *c* reductase activity in intact microsomes, this does not seem likely. Furthermore, the failure of deuterated water to inhibit either the basal NADPH oxidase or NADPH-cytochrome P-450 reductase activities, both of which are presumed to be mediated by the same flavoprotein, would seem to exclude this as the site of inhibition.

It is possible that the deuterated water could exert its inhibitory effect on the catalytic site of the enzyme. Yet the apparent Michaelis-Menten constants for both ethylmorphine *N*-demethylase and aniline hydroxylase were not affected. The mode of

TABLE 4

Effect of deuterated water on NADPH-cytochrome P-450 reductase and ethylmorphine N-demethylase activities of male rat hepatic microsomes

Preparations and assays are described in the text.

Assay	Buffer	2 mM ethylmorphine	Rate	Δ[a]
			nmoles/min/mg protein	
NADPH-cytochrome P-450 reductase	H_2O	−	21.6 ± 0.3[b]	
	H_2O	+	33.5 ± 0.8	11.9
	D_2O	−	19.9 ± 0.2	
	D_2O	+	27.5 ± 0.5	7.6
Ethylmorphine *N*-demethylase	H_2O	+	11.0 ± 0.10	
	D_2O	+	7.5 ± 0.05	

[a] Δ is the rate of NADPH-cytochrome P-450 reductase in the presence minus that in the absence of 2 mM ethylmorphine.

[b] Averages ± standard errors of triplicate incubations.

inhibition appears to be purely noncompetitive, with a decrease only in maximal velocity. Alternatively, the deuterated water could bind to the enzymatic protein and "temporarily denature" the enzyme, with a loss of sites. Such a mechanism of inhibition would be difficult to test.

Our observations suggest that this decrease in maximal mixed-function oxidase velocity is due to a decrease in the ethylmorphine-stimulated NADPH-cytochrome P-450 reductase activity, so that less reduced cytochrome P-450 is present. Of all the results, the failure of deuterated water to inhibit the basal NADPH oxidase and NADPH-cytochrome P-450 reductase, while showing significant inhibition of the stimulation by ethylmorphine (Δ value), strongly supports the suggestion of Gigon, Gram, and Gillette (14, 15) that it is this Δ value for NADPH-cytochrome P-450 reductase which determines the rate of the over-all mixed-function oxidase reaction. The stoichiometry of the Δ value for 1-electron transfer with the formation of formaldehyde tends to confirm this assertion.

The most likely mechanism for this inhibition would therefore appear to be changes in hydration of the membrane proteins, with concomitant changes in their conformation to make them less active in stimulating the NADPH-cytochrome P-450 reductase. We have too little information at this time to speculate on the precise alterations which might be involved in this process.

A further interesting aspect of this work is the observation that deuterated water inhibited the metabolism of all the substrates to approximately the same extent. If, as the data suggest, this inhibition resulted from a direct action of the water on the substrate stimulation of NADPH-cytochrome P-450 reductase, all the substrates studied should be able to bind and activate the stimulatory site for the reductase, and this activation may be an important controlling methanism in their metabolism. In the case of aniline, the stimulatory effect is masked by the binding of the substrate directly to the heme (30). This binding inhibits the apparent increase in reductase activity.[5]

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[5] The decrease in activity of the reductase may be more apparent than real, since the determination is dependent on the assumption that the rate-limiting process is the reduction of the heme and not the binding of CO to the reduced heme. The competition between the binding of aniline and CO may increase the apparent slowing of the reductive process.

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