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Effect of 4,4-Dideuteration of Reduced Nicotinamide–Adenine Dinucleotide Phosphate on the Mixed Function Oxidases of Hepatic Microsomes*

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ABSTRACT: It has been postulated for hepatic microsomal mixed-function oxidases that the electrons are transferred along an electron chain containing a flavoprotein, reduced nicotinamide–adenine dinucleotide phosphate–cytochrome *c* reductase, and the iron of cytochrome P-450. Sih, G. J., Tsong, Y. Y., and Stein, B. [(1968), *J. Amer. Chem. Soc. 90*, 5300] suggested, on the basis of kinetic evidence, that in the mitochondrial 11β-hydroxylase, reduced nicotinamide–adenine dinucleotide phosphate directly reduces the oxygen–oxygen bond. If such is the case, then the ratio of rates of hydroxylation for normal reduced nicotinamide–adenine dinucleotide phosphate to that of 4,4-dideuterio reduced nicotinamide–adenine dinucleotide phosphate (H:D) should be 2–3 due to the isotope effect of the deuterium. In hepatic microsomes the H:D is only 1.22 for ethylmorphine *N*-demethylase, 1.07 for aniline hydroxylase, and 1.12 for reduced nicotinamide–adenine dinucleotide phosphate–cytochrome P-450 reductase. The H:D of reduced nicotinamide–adenine dinucleotide phosphate–cytochrome *c* reductase is 2.00 when the deuterium is in the α position, but is 1.00 when it is in the β position. The H:D for endogenous and ethylmorphine-stimulated reduced nicotinamide–adenine dinucleotide phosphate oxidation were 0.98 and 1.18, respectively. Similarly for reduced nicotinamide–adenine dinucleotide phosphate stimulated oxygen uptake the H:D were 0.87 and 1.06, respectively. The failure to demonstrate a marked isotope effect clearly indicates that in the hepatic microsomes the proximal reducing agent of the oxygen–oxygen bond is an electron chain rather than a direct reduction by reduced nicotinamide–adenine dinucleotide phosphate.

In hepatic mixed function oxidase reactions, a molecule of oxygen, which is bound to both a substrate and cytochrome P-450, receives two electrons from a reducing agent, NADPH, to give a hydroxylated substrate and OH^-. At the present time the most widely accepted mechanism for this reaction postulates that the reduction occurs along an electron chain consisting of a flavoprotein, NADPH–cytochrome *c* reductase, and the cytochrome P-450 (Figure 1). This scheme for microsomes is supported by an extensive body of indirect evidence (Cooper *et al.*, 1965; Holtzman *et al.*, 1968). Further, Omura *et al.* (1966) have purified an analogous system from beef adrenal mitochondria which is capable of hydroxylating deoxycorticosterone in the 11β position but which differs from the microsomal system in requiring both a flavoprotein and a nonheme iron protein, in order to observe a NADPH–cytochrome *c* reductase activity.

Sih *et al.* (1968) have presented evidence for the 11β-hydroxylase system that the NADPH acts to donate a hydrogen to form a hydroperoxide rather than through an electron chain. This then rearranges to reduce the oxygen–oxygen bond. The modification of this proposal to the microsomal system is represented in Figure 2. Their arguments rest on three sets of observations. The first is that although it is possible to increase the concentration of the flavoprotein and adrenodoxin so that NADH will reduce the cytochrome P-450, it is not possible to observe hydroxylation with this cofactor. In order to achieve the same rate of reduction with NADH, they found it necessary to greatly increase the levels of the reductase and adrenodoxin. Yet, the limiting factor in the reduction is the low affinity of NADH for the flavoprotein, and if the concentration of NADH is increased, normal activity results. Secondly (D. Y. Cooper, personnel communication) they suggest that the scheme originally

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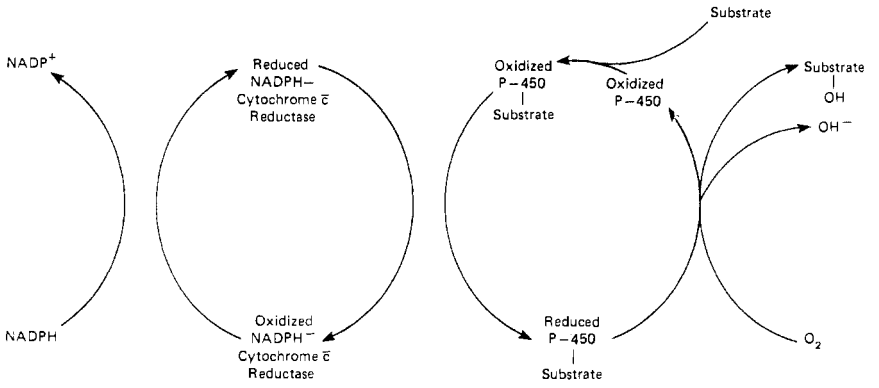

FIGURE 1: Scheme of hepatic mixed function oxidases in which electrons are transferred from NADPH through an electron chain consisting of NADPH–cytochrome *c* reductase and cytochrome P-450. The reduced cytochrome P-450 then serves as the proximal source of electrons for the reduction of the oxygen–oxygen bond.

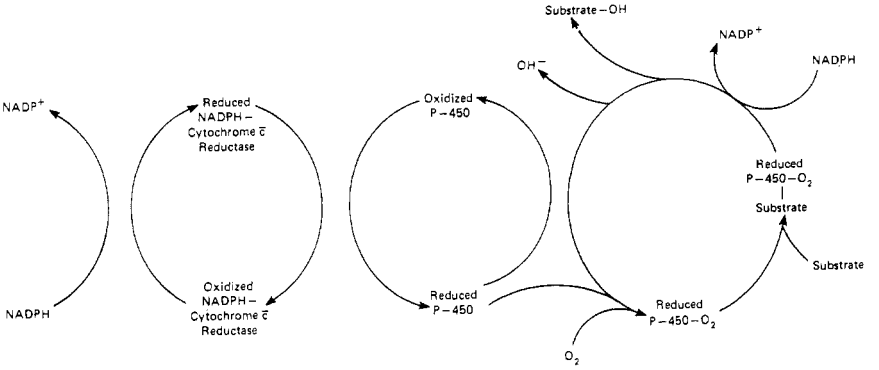

FIGURE 2: Scheme of hepatic mixed function oxidases in which the cytochrome P-450 is reduced by NADPH through NADPH–cytochrome *c* reductase. The oxygen–oxygen bond is directly reduced by a second molecule of NADPH.

proposed by Omura *et al.* (1966) should fit the "ping-pong bi–bi" kinetics of Cleland (1963). The binding of substrate to either oxidized or reduced microsomes (Remmer *et al.*, 1966) and enhancement of cytochrome P-450 reduction by substrate (Gigon *et al.*, 1968, 1969) suggest that the model should not follow these kinetics. Finally, Sih *et al.* (1968) showed that the stereochemistry of the NADPH for the reductase is different from the stereochemistry of the NADPH for the overall reaction. Das *et al.* (1968) have not observed a similar descrepancy for the microsomal system. The data from either set of workers may be misleading because the impurity of the systems could easily lead to extensive side reactions.

The question, therefore, still remains as to whether the basic mechanism is a transport of electrons from NADPH through an electron chain or whether there is a direct transfer of hydrogen from the NADPH to reduce the oxygen–oxygen bond. The ideal method for examining which of these two alternatives is correct would be to purify the product after using labeled hydrogen in the cofactor and determining whether the label was transferred from the cofactor to the product. Unfortunately, because of the rapid exchange of the hydroxyl hydrogen with the solvent, this method is not applicable (Holtzman *et al.*, 1967). An alternative approach is to determine the effect of 4,4-dideuteration of the NADPH on the rate of reactions. If the reaction is actually a direct reduction by NADPH, then it would be expected that the rate would be reduced at least two-threefold if this highly energetic step required the scission of a carbon–deuterium rather than a carbon–hydrogen bond (Melander, 1960). In the work presented below, I have found that the relative rates with the natural and deuterated cofactors for hydroxylations by hepatic microsomes is consistent with the proximal reducing agent being an electron chain and not the NADPH itself.

Methods

All hepatic microsomal preparations were obtained from 200–300 g, fed, untreated, male rats of either the Sprague-Dawley or Osborn-Mendel strains obtained from the National Institutes of Health colony.

The animals were sacrificed by cervical fracture. The livers were removed, chilled on ice, and homogenized with a glass teflon homogenizer with a 0.1–0.15 mm clearance in 3 ml of KCl–Tris (150 mM–20 mM, pH 7.4) per g of wet weight of liver. The homogenate was centrifuged at 9000*g* for 15 min in a Sorval RC2-B centrifuge and the supernatant centrifuged at 164,000*g* (average) for 38 min in a Spinco Model L centrifuge. The resulting pellet was suspended in KCl–Tris to give the equivalent of 2 g of liver/ml of suspension.

Protein was determined by the method of Sutherland *et al.*

(1949). NADP-4-d_1 was prepared by the hydrolysis of the CN^- complex of NADP in D_2O as described by San Pietro (1955). The crude mixture was purified on an AG-1-X8-formate column as described by Kornberg and Horecker (1953). The per cent of deuteration was determined by nuclear magnetic resonance on a Varian HA-100[1] spectrometer using trimethylsilylpropanesulfonate as reference. The doublet assigned to the 4-proton by Jardetzky and Wade-Jardetzky (1966) (δ 8.98 and 9.08 ppm) disappeared completely while the doublets at δ 8.32 and 8.40 ppm and δ 9.31 and 9.38 ppm assigned to the 5- and 6-protons, respectively, became simplified and broadened. This latter broadening may represent effects of the deuterium on the conformation and interaction of the nicotinamide and adenine rings (Jardetzky and Wade-Jardetzky, 1966; Cross and Fisher, 1969). These data indicate that there was greater than 90% replacement of the 4-proton by deuterium.

D-Glucose-1-d_1 was obtained from Isotope Inc. (Westwood, N. J.) (lot. 1318). The nuclear magnetic resonance spectrum of D-glucose had doublet peaks at δ 5.08 and 5.15 ppm which were not present in the D-glucose-1-d_1, confirming the nearly total substitution of deuterium in the glucose. The D-glucose-1-d_1 was phosphorylated to glucose 6-phosphate with hexokinase in the following manner: ATP (3.33 mmoles) and glucose (1.67 mmoles) were dissolved in Tris buffer (15 ml, 0.3 M, pH 7.4) containing $MgCl_2$ (0.1 mmole) and the pH was adjusted to 8.2 with 5 N NaOH (about 1.4 ml). The solution was placed in a pH-Stat, 500 units[2] of yeast hexokinase added, and the pH maintained at 8.2. At the end of the reaction (yield 95%) the reaction mixture was placed on an AG-1-X8-chloride column and the glucose 6-phosphate eluded with water. The eluate was acidified to pH 1, five volumes of acetone were added, and the mixture was centrifuged. Five volumes of ethanol were added to the clear supernatant and the mixture was centrifuged. The clear supernatant was made basic with 5 N NaOH and the glucose 6-phosphate collected by centrifugation (yield, 30%, contamination by adenine, 0.5 mole%).[3]

The effect of 1-deuteration of glucose and 4-deuteration of NADP on the reduction of NADP by glucose-6-phosphate dehydrogenase was determined as follows: ATP (15 μmoles in 2 M Tris, pH 7.4), glucose (20 μmoles), and yeast hexokinase (2 units) were added to 3 ml of KCl–Tris–$MgCl_2$ (150 mM–20 mM–5 mM) buffer at 37° in a Gilford 2400 recording spectrophotometer. Torula yeast glucose-6-phosphate dehydrogenase (0.03 unit)[3] was added and the reaction begun by the addition of 1 mg of NADP in water. The rate was determined by the initial change in absorbancy at 340 nm. The effect of deuteration of glucose and NADP on the rate of formation of NADPH through hexokinase was determined as above except 2 μ of glucose-6-phosphate dehydrogenase and 0.03 unit of hexokinase were used.

The mixed function oxidase activity was determined for ethylmorphine *N*-demethylase[4] and aniline hydroxylase as follows: to a 20-ml serum vial was added 1 ml of a solution of NADP (1.5 μmoles), glucose 6-phosphate (20 μmoles), $MgCl_2$ (15 μmoles), and glucose-6-phosphate dehydrogenase (2 units) dissolved in Tris buffer (0.15 M, pH 7.4) followed by 1 ml of substrate (6 mM) and 1 ml of a microsomal suspension (3 mg/ml of KCl–Tris). The vials were incubated at 37° for 10 min. When ethylmorphine was used as substrate, the reaction was terminated by the addition of 1 ml of 5% $ZnSO_4$ followed by 1.5 ml of a saturated $Ba(OH)_2$ and 0.5 ml of saturated $Na_2B_4O_7$ solution. The formaldehyde was determined in the clear supernatant by the addition of a half volume of a solution of 0.4 ml of acetylacetone/100 ml of 4 M $NH_4C_2H_3O_2$ and measuring of the absorbancy at 410 nm (Nash, 1953). When aniline was used as the substrate the reaction was terminated by putting the vessels in an ice bath. A 2.6-ml aliquot of incubate was mixed with solid NaCl and extracted into ethyl ether, and the *p*-aminophenol determined in aqueous alkaline phenol by the absorbancy at 650 nm (Holtzman and Gillette, 1968). Both reaction mixtures were saturated with substrate and cofactors. The reactions were linear with respect to time and protein concentration.

The initial rate of NADPH–cytochrome *c* reductase was determined by the method of Phillips and Langdon (1962) as previously described (Holtzman *et al.*, 1968). In this and subsequent assays the NADPH-generating system was modified to consist of NADP (2 μmoles), glucose 6-phosphate (10 μmoles), glucose-6-phosphate dehydrogenase (2 units), and sufficient 2 M Tris base to bring the pH to 7.4, all dissolved in 35 μl of KCl–Tris–$MgCl_2$. The concentration of NADPH was determined by measuring the absorbancy at 340 nm and the volumes were adjusted to give equal quantities of NADPH for all assays

NADPH–cytochrome P-450 reductase was determined by bubbling CO through the suspension of microsomes (3 mg/ml of KCl–Tris)in an Aminco anaerobic cuvet, warming to 37°, and measuring the initial change in absorbancy at 450 nm after the addition of NADPH (Holtzman *et al.*, 1968).

NADPH oxidase activity was determined by adding 3 mg of microsomal protein in 70 μl of KCl–Tris to a cuvet containing 3 ml of KCl–Tris–$MgCl_2$ at 37° followed by the addition of NADPH, and determining the initial rate of change in absorbancy at 340 nm.

Oxygen uptake was determined with a Clark electrode (YSI 5331) (Yellow Springs Instrument; Yellow Springs, Ohio). All assays were run in triplicate.

Results

As indicated in Table I both hexokinase and glucose-6-phosphate dehydrogenase are inhibited by the substitution of deuterium for hydrogen in the 1-position of glucose.

[1] These determinations were kindly performed by Mr. Edward Sokolski of the Section of Chemistry, Laboratory of Metabolism, National Heart Institute, Bethesda, Md.

[2] One unit of glucose-6-phosphate dehydrogenase or hexokinase activity leads to the reduction of 1 μmole of NADP/min at 25°.

[3] It is necessary to obtain a relatively pure deuterated glucose 6-phosphate instead of using the hexokinase reaction *in situ* since ADP has a marked effect on the rate of oxygen uptake (Hochstein *et al.*, 1964; Gotto *et al.*, 1965). Further, a small effect (30% increase) was observed on this parameter when isocitrate dehydrogenase was used to give β-deuterated NADPH. This effect was not due to the presence of glycerol in this enzyme preparation.

[4] Ethylmorphine was taken as a convenient example of hydroxylation since the N-demethylation was presumed to go through an intermediate hydroxylation

$$>NCH_3 \xrightarrow[\text{mixed function oxidase, } O_2, \text{ NADPH}]{} >NCH_2OH \xrightarrow{\text{spontaneously}} >NH + HCHO$$

TABLE I: The Effect of 1-Deuteration of Glucose and 4-Deuteration of NADP on Hexokinase and Glucose-6-Phosphate Dehydrogenase Activity.[a]

Enzyme Activity	NADP	Glucose	NADP Reduction (mmoles/min per mg of protein)
Hexokinase	4-H	1-H	12.5
	4-D	1-H	12.8
	4-H	1-D	9.5
	4-D	1-D	9.5
Glucose-6-phosphate dehydrogenase	4-H	1-H	40
	4-D	1-H	38
	4-H	1-D	15
	4-D	1-D	14

[a] Preparations and assays are described in the text.

TABLE II: The Effect of 4,4-Dideuteration of NADPH on the Activity of Ethylmorphine *N*-Demethylase and Aniline Hydroxylase in Rat Hepatic Microsomes.[a]

Enzyme	NADPH	Product	Rate of Product Formation (nmoles/min per mg of protein)	H:D[b]
Ethylmorphine *N*-demethylase	4,4-H_2	HCHO	4.05 ± 0.03[c]	
	4,4-D_2		3.29 ± 0.02	1.23
Aniline hydroxylase	4,4-H_2	*p*-Aminophenol	0.49 ± 0.01	
	4,4-D_2		0.44 ± 0.01	1.11

[a] Preparations and assays are described in the text. [b] H:D is the ratio of activity with the normal NADPH to that with 4,4-dideuterated NADPH. [c] Values are averages ± SEM.

For this reason all concentrations of NADPH were adjusted so that all assays in a given experiment were done with the same concentration of NADPH.[5]

The substitution of deuterium in the 4-position of NADPH led to a small decrease in the aniline hydroxylase and a slightly greater decrease in ethylmorphine *N*-demethylase activities (Table II). The ratio of activity with 4,4-H_2 to 4,4-d_2 NADPH (H:D) was only 1.11 and 1.23, respectively, rather than the 2–3 which would be expected if the rate limiting step were the rupture of a C–H bond.

[5] The concentration of NADPH was determined by measuring the absorbancy at 340 nm. The extinction coefficient appeared to be uneffected by deuteration, since the concentration of NADPH as determined by the absorbancy at 340 and 260 nm was in agreement.

TABLE III: The Effect of Deuteration of NADPH on NADPH–Cytochrome *c* Reductase Activity of Rat Hepatic Microsomes.[a]

NADP	Glucose 6-Phosphate	Cytochrome *c* Reduced (nmoles/min per mg of protein)
4-H	1-H	71.3 ± 2.3[b]
4-D	1-H	76.0 ± 1.3
4-H	1-D	35.7 ± 1.7
4-D	1-D	41.0 ± 1.3

[a] Preparations and assays are described in the text. [b] Values are averages ± SEM.

TABLE IV: The Effect of Ethylmorphine and 4,4-Dideuteration of NADPH on the NADPH–Cytochrome P-450 Reductase Activity of Rat Hepatic Microsomes.[a]

Additions	NADPH	Cytochrome P-450 Reduced[b] (nmoles/min per mg of protein)	H:D[c]
None	4,4-H_2	9.24 ± 0.00[d]	
	4,4-D_2	9.24 ± 0.00	1.00
Ethylmorphine (2 mM)	4,4-H_2	15.18 ± 0.66	
	4,4-D_2	13.84 ± 0.66	1.10
Difference	4,4-H_2	5.94	
	4,4-D_2	4.60	1.28

[a] Preparations and assays are described in the text. [b] ϵ_{450} of reduced cytochrome P-450 in CO *vs.* reduced cytochrome P-450 was taken as 91,000 (Omura and Sato, 1964). [c] H:D is the ratio of activity with the normal NADPH to that with 4,4-dideuterated NADPH. [d] Values are averages ± SEM.

The activity of NADPH–cytochrome *c* reductase is markedly affected by the stereochemistry of the deuteration of the NADPH (Table III). Since glucose-6-phosphate dehydrogenase inserts a hydrogen in the β configuration, these data would suggest that the hydrogen is removed from the α-hydrogen, in agreement with the results of Das *et al.* (1968). On the other hand, these authors did not observe a significant isotope effect, although with ^3H a five–tenfold effect would have been expected.

The addition of ethylmorphine greatly enhanced the rate of reduction of cytochrome P-450 in agreement with the results of Gigon *et al.* (1968, 1969) (Table IV). Further the rate of reduction with NADPH was only slightly affected by 4,4-dideuteration of NADPH. The similar effect of deuteration of NADPH on ethylmorphine *N*-demethylase activity and the stimulation of NADPH–cytochrome P-450 reductase activity by ethylmorphine lend further support to the concept that this reduction is the rate limiting step in the overall reaction.

TABLE V: The Effect of CO and Ethylmorphine on the NADPH Oxidase Activity of Rat Hepatic Microsomes.[a]

Addition	Atmosphere (%)	NADPH Oxidized (nmoles/min per mg of protein)	Inhibition (%)
None	80 N_2–20 O_2	14.2 ± 0.5[b]	
	80 CO–20 O_2	9.9 ± 0.3	31
Ethylmorphine (2 mM)	80 N_2–20 O_2	24.4 ± 0.9	
	80 CO–20 O_2	11.3 ± 0.3	54
Difference	80 N_2–20 O_2	10.2	
	80 CO–20 O_2	1.4	86

[a] Preparations and assays are described in the text. [b] Values are averages ± SEM.

TABLE VI: The Effect of Ethylmorphine and 4,4-Dideuteration of NADPH on the NADPH Oxidase Activity of Rat Hepatic Microsomes.[a]

Additions	NADPH	NADPH Oxidized (nmoles/min per mg of protein)	H:D[b]
None	4,4-H_2	28.3 ± 1.2[c]	
	4,4-D_2	28.6 ± 1.9	0.990
Ethylmorphine (2 mM)	4,4-H_2	56.5 ± 5.5	
	4,4-D_2	52.7 ± 1.9	1.066
Difference	4,4-H_2	28.2	
	4,4-D_2	24.1	1.170

[a] Preparations and assays are described in the text. [b] H:D is the ratio of activity with the normal NADPH to that with 4,4-dideuterated NADPH. [c] Values are averages ± SEM.

TABLE VII: The Comparison of Oxygen Uptake and Formaldehyde Formation for NADPH and Ethylmorphine in NADPH-Dependent Uptake of Oxygen by Rat Hepatic Microsomes.[a]

Additions	Oxygen Uptake (nmoles/min per mg of protein)	HCHO Formed (nmoles/min per mg of protein)
None	7.6 ± 0.1[b]	2.6 ± 0.05
Ethylmorphine (2 mM)	15.4 ± 0.3	10.4 ± 0.2
Difference	7.8	7.8

[a] Preparations and assays are described in the text. [b] Values are averages ± SEM.

TABLE VIII: The Effect of CO and Ethylmorphine on the NADPH-Dependent Uptake of Oxygen by Rat Hepatic Microsomes.[a]

Additions	Atmosphere (%)	Oxygen Uptake (nmoles/min per mg of protein)	Inhibition (%)
None	80 N_2–20 O_2	11.16 ± 0.21[b]	
	80 CO–20 O_2	5.60 ± 0.34	50
Ethylmorphine (2 mM)	80 N_2–20 O_2	25.51 ± 1.16	
	80 CO–20 O_2	8.83 ± 0.42	65
Difference	80 N_2–20 O_2	14.35	
	80 CO–20 O_2	3.23	77

[a] Preparations and assays are described in the text. [b] Values are averages ± SEM.

In agreement with the results of Orrenius (1965) there is a significant stimulation of the NADPH oxidase on the addition of ethylmorphine to the incubate (Table V). Further it is clear that while CO only partially inhibits the endogenous oxidation (30% of air value), it almost totally inhibits the substrate-stimulated oxidation. Further deuteration of NADPH has no effect on the endogenous oxidation, and only a small effect on the stimulated oxidase activity (Table VI).

As with the oxidation of NADPH, the NADPH-dependent uptake of oxygen is stimulated by the addition of ethylmorphine, in agreement with the report of Orrenius (1965). This increased uptake of oxygen with the addition of substrate is stoichiometric with the amount of product formed (Table VII). The metabolism of aniline is so slow in comparison to the endogenous uptake that the stoichiometry could not be established. As with NADPH oxidation there is also only a partial inhibition of the endogenous uptake of oxygen by CO, but a very substantial inhibition of the ethylmorphine-stimulated uptake (Table VIII). Again, as with product formation and NADPH oxidation, deuteration of the NADPH had little effect on the endogenous or substrate-stimulated uptake of oxygen (Table IX). It is unclear why the H:D for the endogenous uptake is less than 1. It could be due to some residual ATP or some other minor contaminant such as ethanol in the glucose 6-phosphate.

Discussion

It is clear from the failure to observe a marked isotope effect with deuterated NADPH on the formation of product, oxidation of NADPH, or uptake of oxygen that in the hepatic microsomal mixed function oxidases the rate limiting step is not the fission of the C–H bond of NADPH. Therefore, the data presented above are incompatible with the rate limiting step being the final transfer of a hydrogen from NADPH to form a hydroperoxide as suggested by Sih *et al.* (1968). Yet it is possible that some other step in the scheme they present is rate limiting. The penetration and binding of the substrate and oxygen are not likely candidates for the rate limiting steps as these rates are extremely rapid. Further,

TABLE IX: The Effect of Ethylmorphine and 4,4-Dideuteration of NADPH on the NADPH-Dependent Uptake of Oxygen by Rat Hepatic Microsomes.[a]

Additions	NADPH	Oxygen Uptake (nmoles/min per mg of protein)	H:D[b]
None	4,4-H_2	17.8 ± 0.0[c]	
	4,4-D_2	20.5 ± 0.0	0.87
Ethylmorphine (2 mM)	4,4-H_2	34.5 ± 1.2	
	4,4-D_2	32.6 ± 0.8	1.06
Difference	4,4-H_2	16.7	
	4,4-D_2	12.1	1.37

[a] Preparations and assays are described in the text. [b] H:D is the ratio of activity with the normal NADPH to that with 4,4-dideuterated NADPH. [c] Values are averages ± SEM.

from the data I have presented here, which is in agreement with previous work (Gigon *et al.*, 1968, 1969), the reduction of the NADPH–cytochrome *c* reductase is at least one order of magnitude more rapid than the overall reaction, indicating that the penetration of microsomes by NADPH and the subsequent reduction of the flavoprotein are not rate limiting either.

At the present time all evidence would indicate that the rate limiting step is the reduction of the cytochrome P-450 (Holtzman *et al.*, 1968; Omura *et al.*, 1966; Gigon *et al.*, 1968, 1969). Since the cytochrome is readily autooxidizable (Mason *et al.*, 1965) and is probably mostly in the ferri state (Imai and Sato, 1966), the relative rates of hydroperoxide formation and the competing reoxidation of the reduced cytochrome would determine the rate of product formation. Therefore, since the deuteration of the NADPH would not affect the rate of autoxidation, but would reduce the rate of formation of hydroperoxide two–threefold, only one-third to one-half the product would be formed for each reduction of the cytochrome P-450. Coupling this with a 10–20% decrease in the rate of reduction, there should be up to a fourfold reduction in the rate of formation of product.

In view of these considerations, Sih *et al.* (1968) appear to be wrong in postulating the formation of an intermediate hydroperoxide. Yet it could well be that, once the cytochrome is reduced and can bind molecular oxygen, a second electron chain may be required to transport electrons to reduce the oxygen–oxygen bond. Since the presence of nonheme iron in the microsomes is at best uncertain (Miyake *et al.*, 1967) this is not a likely candidate for a second chain. A disulfide moiety is probably not involved since the use of nonmercurial sulfhydro inhibitors has no effect on cytochrome P-450 enzymes (May and McCay, 1968). Cytochrome b_5 may be important in this reaction, but this is unlikely as NADH, with low concentrations of NADPH to reduce the cytochrome P-450, would be as effective as NADPH alone, a point which has never been demonstrated. Finally, another flavoprotein cannot be ruled out as the second electron donor.

The existence of a second electron chain can only be reconciled with the observation that the increase in NADPH oxidation on the addition of substrate is stoichiometric with the formation of product (Orrenius, 1965) if a large number of product molecules are formed for each reduction of the cytochrome P-450. On the other hand, such a scheme is not consistent with the observations of Gigon *et al.* (1968, 1969) who found that the increase in NADPH–cytochrome P-450 reductase activity with the addition of substrate was commensurate with the rate of product formation. These data indicate that the activity of the reductase is sufficient to account for the transport of electrons for the reduction of the oxygen–oxygen bond and no second chain need be postulated.

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A Comparison of Thiol Peptides of Heart Mitochondrial Malate Dehydrogenases from Pig, Chicken, and Tuna*

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ABSTRACT: The thiol groups of mitochondrial malate dehydrogenases (EC 1.1.1.37) from pig, chicken, and tuna hearts were alkylated with 1-[^{14}C]iodoacetate in 8 M urea, and the alkylated enzymes digested with trypsin and with mixed trypsin–chymotrypsin. Peptide mapping of the digests revealed a carboxymethylcysteine-containing peptide which appeared to be the same in all three malate dehydrogenases in its position on the peptide maps, its migration during electrophoretic purification at pH 3.5, and its amino acid composition. This peptide common to the three malate dehydrogenases contained one residue each of lysine, [^{14}C]carboxymethylcysteine, threonine, serine, glutamic acid (or glutamine), proline, and isoleucine when isolated from tryptic peptide maps. When isolated from tryptic–chymotryptic maps it contained all the same residues except for the absence of threonine. A second and larger peptide containing carboxymethylcysteine also appeared to be common to the tryptic peptide maps of all three enzymes and contained the same residues as the first peptide plus one additional residue each of threonine or serine, glycine, alanine, isoleucine, leucine, and possibly proline. The first peptide may be a chymotryptic fragment of the second, and both appear to represent a thiol-containing region in mitochondrial malate dehydrogenases which is conserved during evolution. The effect of iodoacetate on native chicken heart mitochondrial malate dehydrogenase in the presence and absence of substrates and coenzymes has been studied. A tryptic digest of the enzyme after total thiol alkylation in 8 M urea has been fractionated on a cation-exchange column, and the carboxymethylcysteine-containing peptides purified by paper electrophoresis and anion-exchange column chromatography. The peptides so isolated have been compared to those obtained from the peptide maps. The use of comparative peptide mapping in the identification of functionally essential regions in proteins is discussed.

The involvement of thiol groups in the operation of NAD-linked dehydrogenases has been established for several enzymes including glyceraldehyde-3-P dehydrogenase (Harris *et al.*, 1963), lactate dehydrogenase (Gruber *et al.*, 1962; DiSabato and Kaplan, 1963; Dube *et al.*, 1963), and alcohol dehydrogenase (Li and Vallee, 1965). Complete amino acid sequences around essential thiol groups in these enzymes have also been determined (Harris *et al.*, 1963; Fondy *et al.*, 1965; Holbrook and Pfleiderer, 1965; Gold and Segal, 1965; Holbrook *et al.*, 1967; Mella *et al.*, 1968; Harris, 1964; Li and Vallee, 1964).

A number of studies have implicated thiol groups in the catalytic mechanism of malate dehydrogenases. Green (1936) found that pig heart malate dehydrogenase was inhibited by iodoacetamide and similar findings were reported by Grimm and Doherty (1962) who showed that iodoacetamide-treated pig heart mitochondrial malate dehydrogenase bound substantially less NADH than did the native enzyme. Other workers (Wolfe and Neilands, 1956; Siegel and Englard, 1962; Devenyi *et al.*, 1966b; Kitto and Kaplan 1966; Seguin and Kosicki, 1967; Thorne and Kaplan, 1966; Siegel, 1967) have shown the sensitivity of malate dehydrogenases to thiol reagents such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoate). In general, the reaction with sulfhydryl reagents is sluggish in the absence of denaturing agents such as urea, indicative of buried thiol groups, and it is apparent that not all of the sulfhydryl groups are essential for activity (Siegel and Englard, 1962; Seguin and Kosicki, 1967; Devenyi *et al.*, 1966b; Siegel, 1967; Guha *et al.*, 1968). However, it has not been possible so far to label specifically and to identify an essential thiol region in malate dehydrogenases.

The active-site thiol region in lactate dehydrogenase was identified by Fondy *et al.* (1965) and Fondy and Kaplan (1965) by a structural comparison of thiol peptides obtained from lactate dehydrogenases of several different species widely separated in the evolutionary time scale. The essential thiol region in lactate dehydrogenases from frog, dogfish, and chicken muscle and from bovine and chicken heart showed very similar or identical primary sequences. Mella

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