EFFECT OF DRUG SUBSTRATES ON THE REDUCTION OF HEPATIC MICROSOMAL CYTOCHROME P-450 BY NADPH

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Received April 10, 1968

There is considerable evidence that the oxidation of various drugs and hydroxylation of steroids by NADPH-dependent enzymes in liver microsomes are mediated by cytochrome P-450 (Cooper et al., 1965). However, the mechanism of these enzyme reactions remains obscure. It has been postulated that NADPH reduced cytochrome P-450, either directly or indirectly by NADPH-cytochrome c reductase, and that the reduced cytochrome P-450 reacted with oxygen to form an "active oxygen" complex which in turn oxidized the drug or steroid (Gillette, 1966). However, the finding that various substrates caused changes in the visible absorption spectrum of liver microsomes even in the absence of NADPH (Remmer et al., 1966) suggested that substrates formed complexes with the oxidized form of cytochrome P-450 and that the rate limiting step of the reaction was reduction of the substrate-cytochrome P-450 complex. Accordingly, the N-demethylation of ethylmorphine by liver microsomes is more closely related to NADPH cytochrome P-450 reductase activity than to the amount of cytochrome P-450, the magnitude of the spectral change, or the activity of NADPH-cytochrome c reductase (Holtzman et al., 1968; Davies, Gigon and Gillette, unpublished).

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*Sponsored by the Swiss Academy of Medical Sciences.
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Two types of change in the difference spectrum of liver microsomes are produced by various substrates and inhibitors: "Type I" is characterized by a trough at about 420 mμ and a peak at about 385 mμ; "Type II" is characterized by a peak at about 430 mμ and a trough at about 394 mμ (Remmer et al., 1966; Schenkman et al., 1967). The present paper shows that substances which cause Type I spectral changes accelerate cytochrome P-450 reduction, whereas those which cause Type II spectral changes decelerate the reduction of this cytochrome. Moreover, the data suggest that the sex difference in microsomal ethylmorphine N-demethylase activity in rats may be related to the magnitude of the substrate-induced enhancement of cytochrome P-450 reduction.

METHODS. Adult male (180-200 g) and female (160-170 g) Sprague-Dawley rats (NIH colony) were allowed free access to laboratory chow and tap water. Animals were sacrificed by decapitation and hepatic microsomes were prepared as described previously (Kato and Gillette, 1965). Microsomal protein was estimated as described by Lowry et al., (1951). The Vmax values for ethylmorphine N-demethylation were determined as described by Castro and Gillette (1967).

NADPH cytochrome P-450 reductase was assayed by a method devised in this laboratory by Davies, Gigon and Gillette (unpublished). In this method carbon monoxide (CO), which had been deoxygenated by passage through an alkaline dithionite solution, was bubbled for 5 min through 3 ml of a microsomal suspension (5 mg protein/ml in 1.15% KCl - 0.02 M Tris-HCl, pH 7.4) in an anaerobic Aminco spectrophotometric cell (A1-65085). The plunger assembly containing 50 μl of NADPH-generating system[1] was then fitted to the cuvette. This amount of NADPH-generating

[1] The NADPH generating system consisted of 3 μmoles Tris-HCl, pH 7.4, 10 μmoles $MgCl_2$, 24 μmoles Na isocitrate, 1.2 μmoles NADP and 0.54 units isocitrate dehydrogenase.

system gave maximal rates of cytochrome P-450 reduction. Carbon monoxide was then passed through the inlet of the cuvette for 3 additional minutes. The cuvette was then sealed and transferred to the sample chamber of a Gilford Model 2000 spectrophotometer and allowed to equilibrate for 5 min at 37°C. The plunger was then quickly depressed and the absorbancy of the CO-cytochrome P-450 complex at 450 mμ recorded on the chart paper (12 inches per min). All reaction velocities were determined from the initial linear phase of the curve.

RESULTS AND DISCUSSION. As shown in Table 1, ethylmorphine, hexobarbital, SKF 525-A[1], aminopyrine and imipramine, all of which cause Type I spectral changes (Schenkman et al., 1967), markedly enhanced the rate of absorbancy change at 450 mμ. In contrast, aniline, nicotinamide and DPEA[2], which cause Type II spectral changes (Schenkman et al., 1967), decreased the rate of absorbancy change (Table 1).

Table 1

Effect of Drug Substrates on Hepatic Microsomal Cytochrome P-450 Reduction by NADPH in Male Rats*

Drug Substrates	Cytochrome P-450 Reduction (ΔOD 450 mμ/sec/mg protein x 10^{-3}) Control	In the presence of substrate	Conc. mM	% of control
Type I Substrates and Inhibitors				
Ethylmorphine	16.2	28.7	.4	177
Hexobarbital	11.0	18.7	.6	170
SKF 525-A	12.4	27.4	.1	221
Aminopyrine	12.2	17.3	.5	142
Imipramine	11.7	19.1	.5	162
Type II Substrates and Inhibitors				
Aniline	15.3	6.9	1.5-2.0	45
Nicotinamide	11.5	6.8	10	59
DPEA	12.2	4.4	0.1	36

*All values represent means of 3 experiments except those for aminopyrine and imipramine which represent single experiments.

[1] 2-diethylaminoethyl 2,2-diphenylvalerate HCl.

[2] 2,4-dichloro-6-phenylphenoxyethylamine HBr.

After complete reduction of cytochrome P-450 by either NADPH or dithionite in the presence of these compounds, the absorbancy values differed only 20% at most from those obtained in the controls. Thus the accelerated rate of absorbancy at 450 mμ cannot be explained by substrate-induced alterations in the molar extinction coefficient of the reduced cytochrome P-450 · CO complex, but probably represents changes in the rate of cytochrome P-450 reduction.

These findings raised the possibility that the ethylmorphine induced enhancement of cytochrome P-450 reduction by NADPH might be related to the maximum velocity of ethylmorphine N-demethylation. To evaluate this possibility we studied the effects of ethylmorphine on cytochrome P-450 reduction in liver microsomes from male and female rats (Table 2).

Table 2

Sex Differences in Ethylmorphine N-Demethylation and the Effect of Ethylmorphine on the Rate of Cytochrome P-450 Reduction by Rat Liver Microsomes*

Sex	Cytochrome P-450 Reduction (ΔOD 450 mμ/sec/mg protein x 10^{-3})			Ethylmorphine N-demethylation (Vmax)***
	Control**	Ethylmorphine**	Difference	
Male	16.2	28.7	12.5	11.5
Female	12.2	17.0	4.8	3.7
Male/Female	1.33	1.69	2.60	3.14

* The results are the means of 3 experiments.
** Determined at Vmax concentrations (NADPH) and ethylmorphine (0.4 mM).
***Formaldehyde formed (mμmoles/min/mg protein).

In the absence of ethylmorphine there was only a slight though significant sex difference in the reduction of cytochrome P-450, but in the presence of the substrate, the rate of reduction was 69% greater in the liver microsomes from males than in those from females. Clearly the sex difference in the absolute rate of cytochrome P-450 reduction either in the presence or the absence of ethylmorphine could not account for the

three-fold sex difference in ethylmorphine demethylation. But the sex difference in the substrate-induced increase in the reduction rate of the cytochrome closely paralleled the sex difference in ethylmorphine metabolism. These findings thus suggest the possibility that ethylmorphine combines with the cytochrome P-450 in liver microsomes to form a complex which is more readily reduced by NADPH cytochrome P-450 reductase than is cytochrome P-450 in the absence of the substrate. Moreover, the finding that the sex difference in the N-demethylation of ethylmorphine parallels the substrate-enhanced rate of reduction of cytochrome P-450 and not the absolute rate of reduction suggests that only a part of the cytochrome P-450 in microsomes from female rats is enzymatically active.

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