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An Approach Measurement of the Stoichiometric Relationship between Hepatic Microsomal Drug Metabolism and the Oxidation of Reduced Nicotinamide Adenine Dinucleotide Phosphate

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SUMMARY

Various methods for estimating the stoichiometric relationship between drug oxidation and NADPH oxidation in hepatic microsomes are discussed. Of these, two methods give values approaching 1:1 for a number of type I substrates. One method relates the metabolism to the substrate-dependent NADPH oxidation corrected for CO-sensitive, endogenous NADPH oxidation. The correction was obtained by measuring the rate of NADPH oxidation in an atmosphere of 9:1 $CO–O_2$. The other method relates the CO-sensitive drug oxidation to CO-sensitive NADPH-oxidation.

INTRODUCTION

A large number of drugs are oxidized by hepatic microsomal enzymes that are usually referred to as mixed-function oxidases. As defined by Mason (1), NADPH-dependent, mixed-function oxidases should utilize equivalent amounts of NADPH, O_2, and substrate. Subtraction of the rate of endogenous from substrate-stimulated NADPH oxidation, however, has rarely provided this kind of stoichiometry. Among the exceptions are the *N*-oxidation of certain tertiary amines by intact hepatic microsomes (2), the *N*-demethylation of aminopyrine by hepatic microsomes from phenobarbital-treated rats (3), and the *N*-demethylation of benzphetamine by solubilized preparations (4).

This study was undertaken to develop methods of correction for the NADPH oxidation unrelated to drug oxidation, thereby permitting the measurement of NADPH oxidation solely related to substrate oxidation. With such methods, it should be possible to determine the stoichiometric relationships between the metabolism of type I substrates and NADPH oxidation.

THEORETICAL BASIS OF METHODS

Possible reasons for failing to obtain the characteristic stoichiometric relationships between the rates of oxidation of NADPH and substrate predicted by the mixed-function oxidase mechanism may be seen with

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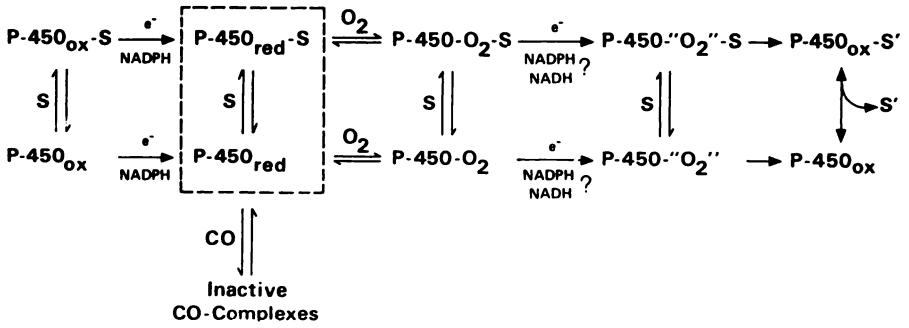

FIG. 1. *Theoretical relationships between substrate (S) and NADPH oxidation by the mixed-function oxidase system*

the aid of Fig. 1, which shows a simplified version of our current view of the mechanism of cytochrome P-450 enzyme systems. Let us assume for the moment that the only pathway of NADPH oxidation in liver microsomal preparations is through the cytochrome P-450 system. In absence of substrate, all oxidation of NADPH would occur along the lower pathway. In the presence of a substrate at concentrations sufficient to saturate the enzyme completely (V_{max} concentrations), however, all oxidation of NADPH would occur along the upper pathway and none would occur along the lower pathway. Under the assumed conditions, therefore, the total rate of NADPH oxidation in the presence of V_{max} concentrations of substrate should correspond to the V_{max} of substrate metabolism; i.e., index I (Table 1) should be 1.0. In fact, when appreciable amounts of NADPH are oxidized along the lower pathway, in the absence of substrate, it would be a mistake to subtract the rate of NADPH oxidation in the absence of substrate from that in the presence of substrate, i.e., index II. To illustrate this fact with the idealized system shown in Fig. 1, let us assume that the rate of NADPH oxidation in the absence of substrate is 6 nmoles/min/mg and, in the presence of V_{max} concentrations of substrate, 10 nmoles/min/mg. Since the V_{max} for the metabolism of the substrate would also be 10 nmoles/mg, index II would be 2.5 instead of 1.0. Indeed, the theoretical ratio of 1.0 would be obtained by index II only when the rate of NADPH oxidation along the lower pathway is zero in the absence as well as in the presence of substrate.

TABLE 1
Definition of indices I–IV

Index	Definition
I	$\frac{V_{max}\text{ (metabolism) in air}}{\text{Rate of NADPH oxidation in air with } V_{max} \text{ concentrations of substrate}}$
II	$\frac{V_{max}\text{ (metabolism) in air}}{\text{Rate of NADPH oxidation in air with } V_{max} \text{ concentrations of substrate, minus rate of NADPH oxidation in air without substrate}}$
III	$\frac{V_{max}\text{ (metabolism) in air}}{\text{Rate of NADPH oxidation in air with } V_{max} \text{ concentrations of substrate, minus rate of NADPH oxidation in CO–O}_2 \text{ without substrate}}$
IV	$\frac{V_{max}\text{ (metabolism) in air minus } V_{max}\text{ (metabolism) in CO–O}_2}{\text{Rate of NADPH oxidation in air with } V_{max} \text{ concentration of substrate, minus rate of NADPH oxidation in CO–O}_2 \text{ with } V_{max} \text{ concentrations of substrate}}$

Studies with liver microsomes, however, have invariably shown that the rate of NADPH oxidation in the presence of V_{max} concentrations of substrate is always greater than the rate of substrate oxidation; i.e., index I < 1. Although part of the excess NADPH oxidation undoubtedly occurs through pathways completely unrelated to the cytochrome P-450 system, which catalyzes the metabolism of the substrate, other reasons for a low index I value are also possible. For example, the substrate may undergo reactions which are not detected by the

assay system; indeed, most drugs are metabolized by several different reactions. Alternatively, the substrate may combine with cytochrome P-450, but for some reason may not be metabolized. Ullrich and Diehl (5) recently showed that perfluorohexane stimulated NADPH oxidation by liver microsomes but was not defluorinated. With such abortive complexes, the difference in rates of NADPH oxidation in air in the presence and absence of substrate may thus exceed the rate of product formation; i.e., index II also < 1.

In most instances, however, the rate of product formation is greater than the difference in the rates of NADPH oxidation in the presence and absence of substrate (index II > 1), suggesting that an appreciable portion of the endogenous rate of NADPH oxidation passes through the cytochrome P-450 system that catalyzes substrate oxidation. We might be able to decrease the portion of the endogenous rate of NADPH oxidation mediated by cytochrome P-450 by measuring the rate of NADPH oxidation in the absence of substrate but in the presence of an atmosphere containing a high concentration of CO, which inhibits electron flux through cytochrome P-450 (index III, Table 1). Of course we could not expect to inhibit all the electron flux through the system, because even in an atmosphere containing 90% CO and 10% O_2 the electron flux could still be appreciable. Indeed, the inhibitory effect of CO on NADPH oxidation through the system would probably be slightly less in the absence of substrate than in its presence, since the degree of inhibition by CO depends not only on the relative affinities of the reduced form of cytochrome P-450 for oxygen and CO but also on the relative rates at which the reduced form is formed and oxidized (6). On the other hand, liver microsomal preparations can contain other cytochrome P-450 systems that are not involved in the metabolism of the substrate, as well as other CO-sensitive, NADPH-dependent enzyme systems, such as cytochrome oxidase in mitochondria, which usually contaminate liver microsomal preparations to a minor extent. For these reasons, the endogenous rate of NADPH oxidation in the presence of $CO–O_2$ can provide a value for NADPH oxidation by enzyme systems independent of the cytochrome P-450 system. This value will be overestimated because of incomplete blockade of the cytochrome P-450 system and underestimated because of the inhibitory effect of CO on other CO-sensitive systems. When these factors are about equal to or smaller than the rate of substrate-dependent NADPH oxidation in air (i.e., the upper pathway of Fig. 1), index III (Table 1) should approach 1.0 when the 1:1 stoichiometry is valid.[4]

In some situations index IV may provide a better estimate of the stoichiometry of the reaction than does index III. For example, CO may inhibit the electron flux through cytochrome P-450 more effectively in the presence of substrates than in their absence. Substrates may decrease other NADPH-dependent systems, such as lipid peroxidation. Moreover, substrates may be metabolized by CO-insensitive reactions, such as the conversion of dimethylaniline to dimethylaniline *N*-oxide. As with index III, however, index IV will still be invalid when the substrate forms abortive complexes or when liver microsomes contain CO-sensitive enzyme systems that are not involved in metabolism of the substrate.

METHODS

Male Sprague-Dawley rats from Hormone Assay, Chicago (180–200 g), were decapitated at 8:00 a.m., and hepatic microsomes were prepared as described previously (7). Protein was determined according to the biuret method (8). Metabolism of substrates was accomplished in a 3.0-ml incubation mixture containing 5 mM $MgCl_2$, 12 mM

[4] When liver microsomal preparations contain relatively large activities of CO-sensitive enzymes that are not involved in the metabolism of drugs, this method will give invalid results. Since many of these extraneous CO-sensitive enzyme systems are also sensitive to cyanide, their contribution to NADPH oxidation can be diminished by the addition of 1.0 mM cyanide, which has little effect on cytochrome P-450. When this technique is used, however, it should be realized that cyanide can react with aldehydes such as formaldehyde to form cyanohydrins, and thereby cause an apparent decrease in aldehyde formation, unless appropriate control experiments are also carried out.

glucose 6-phosphate, 1.0 unit of glucose 6-phosphate dehydrogenase, 0.33 mM NADP, 50 mM Tris buffer (pH 7.4), 5–10 mg of microsomal protein, and various amounts of substrate. The mixtures were incubated for 10 min in air or in an atmosphere of 9:1 CO–O_2.[5]

The *N*-demethylation of aminopyrine, benzphetamine, ethylmorphine, and *N*-methyl-*p*-chloroaniline was estimated by measuring the amount of formaldehyde formed (9). The metabolism of imipramine was estimated by measuring both the amount of formaldehyde formed and the amount of drug disappearing as described by Dingell *et al.* (10). Hexbarbital metabolism was estimated by measuring the remaining substrate according to Cooper and Brodie (11), as modified by Bush.[6] Isoamyl alcohol was omitted from the heptane phase to decrease the extraction of norhexobarbital. V_{max} values were calculated as described by Davies *et al.* (12). NADPH oxidation was measured as described by Gigon *et al.* (13); the microsomal suspension was gassed with 90% CO–O_2 for 5 min at 37°, the plunger assembly, containing 50 µl of an NADPH solution (0.5 µmole), was inserted, and the gassing was continued for 3 min. The cuvette was then sealed and transferred to a Gilford model 2000 spectrophotometer, and the disappearance of absorbance at 340 nm was recorded at 37°. Studies comparing NADPH oxidation in an atmosphere of 1:9 O_2–N_2 with that in air revealed that O_2 in concentrations as low as 10% was not rate-limiting. Thereafter NADPH oxidation in air was compared with that in the CO–O_2 atmosphere. With different concentrations of the substrates, an estimation of V_{max} for NADPH oxidation was obtained and was found to be close to the value obtained with high concentrations of substrate (approximately 10 × K_m). The latter value was therefore used to represent substrate-stimulated NADPH oxidation. For reasons discussed in the following section, the V_{max} values for substrate oxidation were usually estimated from Lineweaver-Burk plots.

[5] For hexobarbital and imipramine (substrate disappearance), it was not possible to obtain a good estimate of V_{max} in CO–O_2 atmospheres because of very low activity. Two of the highest substrate concentrations were then chosen, and the velocities obtained were compared with the corresponding ones in air. The percentage inhibition was the same for both substrate concentrations and therefore was used to calculate the V_{max} in CO–O_2.

[6] M. T. Bush, personal communication.

RESULTS AND DISCUSSION

As expected, Table 2 shows that the index I values for *N*-demethylation of the various substrates by liver microsomes of male rats were less than 1.0, indicating that NADPH is oxidized along pathways which do not lead to formaldehyde formation. On the other hand, the endogenous NADPH oxidation is apparently not affected by benzphetamine, since index II for this reaction approaches 1.0. However, the index II values for *N*-demethylation of the other substrates considerably exceeded 1.0, suggesting either that most of the substrates appreciably inhibit the endogenous rate of NADPH oxidation or that a significant portion of the endogenous NADPH oxidation proceeds through the cytochrome P-450 system that catalyzes the *N*-demethylation of these substrates.

By contrast, the values of indices III and IV for the *N*-demethylation of most of the substrates approached 1.0, indicating that the assumptions made in the derivations of these indices are reasonably valid. However, the index for the *N*-demethylation of imipramine was still considerably less than 1.0.

A lack of correlation between NADPH oxidation and formaldehyde formation would be expected when the drugs are metabolized along pathways other than *N*-demethylation. For example, the V_{max} for *N*-demethylation of imipramine represented only about 50% of the V_{max} for total metabolism of the drug as measured by the disappearance of the substrate (Table 3). Thus, measurement of total microsomal metabolism either by determining all the metabolites or by determining the disappearance of substrate, suggests that relation of the maximal velocity to NADPH oxidation according to indices III and IV would give a stoichiometry of 1:1. In accordance with this view, Table 2 shows that the values of index III approached 1.0 when the V_{max} values for the

TABLE 2

Calculation of indices I–IV

Values are the means of three or four animals ± standard errors.

Substrate	$B - A$	$B - C$	$B - D$	Index I (V_{max} in air), B	Index II (V_{max} in air), $B - A$	Index III (V_{max} in air), $B - C$	Index IV (V_{max} in air − V_{max} in CO–O_2), $B - D$
Ethylmorphine	6.65 ± 0.35	9.65 ± 1.08	7.84 ± 1.32	0.84 ± 0.37	2.00 ± 0.15	1.1 ± 0.10	1.2 ± 0.16
Aminopyrine	2.67 ± 0.61	9.40 ± 1.05	8.20 ± 0.59	0.81 ± 0.005	4.26 ± 1.10	1.04 ± 0.12	0.88 ± 0.07
Benzphetamine	7.91 ± 0.41	10.9 ± 1.3	10.7 ± 1.3	0.55 ± 0.041	1.10 ± 0.073	0.81 ± 0.03	0.75 ± 0.03
p-Chloro-*N*-methyl-aniline	3.93 ± 0.59	7.62 ± 0.75	4.84 ± 0.23	0.90 ± 0.055	2.60 ± 0.11	1.3 ± 0.10	1.1 ± 0.10
Imipramine	2.92 ± 0.77	8.02 ± 0.51	7.60 ± 0.46	0.40 ± 0.46	1.97 ± 0.81	0.55 ± 0.06	0.40 ± 0.05
Hexobarbital[a]	5.48 ± 0.60	9.60 ± 0.86	8.03 ± 0.66	0.60 ± 0.49	1.60 ± 0.20	0.91 ± 0.12	0.82 ± 0.07
Imipramine[a, b]	5.60	9.50	7.90	0.85	1.52	1.1	

[a] Hexobarbital plus imipramine V_{max} determined by substrate disappearance.

[b] Determined in a pooled sample from two animals.

TABLE 3

Metabolism of type I substrates and oxidation of NADPH in the absence and presence of carbon monoxide
Values are the means ± standard errors of three or four animals, except as noted below.

Substrate	Type I substrate metabolism		NADPH oxidation			
	V_{max} in air	V_{max} in CO–O_2	*A* (endogenous in air)	*B* (substrate in air)	*C* (endogenous in CO–O_2)	*D* (substrate in CO–O_2)
	nmoles/mg protein/min		*nmoles/mg protein/min*			
Catabolism[a]						
Ethylmorphine	11.2 ± 0.36	2.27 ± 0.10	7.72 ± 1.12	13.4 ± 0.82	3.68 ± 0.30	5.53 ± 0.51
Aminopyrine	9.43 ± 0.40	2.30 ± 0.14	9.02 ± 0.85	11.7 ± 0.77	2.35 ± 0.35	3.52 ± 0.20
Benzphetamine	8.75 ± 0.80	0.91 ± 0.032	8.16 ± 0.79	16.8 ± 0.48	5.15 ± 0.89	6.16 ± 0.88
p-Chloro-*N*-methylaniline	10.1 ± 1.0	5.39 ± 0.57	7.29 ± 0.40	11.2 ± 0.72	3.57 ± 0.30	6.41 ± 0.91
Imipramine	4.48 ± 0.27	1.35 ± 0.32	8.51 ± 0.31	11.4 ± 0.56	3.23 ± 0.23	3.50 ± 0.33
Anabolism[b]						
Hexobarbital	8.3 ± 0.20	1.66 ± 0.045	8.45 ± 0.50	13.9 ± 0.66	4.36 ± 0.20	5.89 ± 0.38
Imipramine[c]	8.5		6.39	10.0	2.51	

[a] Nanomoles of formaldehyde formed per milligram of protein per minute.
[b] Nanomoles of substrate disappearing per milligram of protein per minute.
[c] Determined in a pooled sample of two animals.

total metabolism of imipramine and hexobarbital were estimated by extrapolation of the Lineweaver-Burk plots.

High substrate concentrations, however, inhibited the metabolism of *N*-methyl-*p*-chloroaniline, imipramine, and hexobarbital, but did not inhibit the substrate-dependent NADPH oxidation (Table 4). Indeed, the velocities measured at the highest substrate concentrations were only about 40–50% of the calculated V_{max} values (cf Tables 3 and 4), because of substrate inhibition. Thus, at high concentrations of these substrates, there is an uncoupling mechanism between NADPH oxidation and drug metabolism. Whether the substrate inhibition represents an allosteric change of the reduced form of cytochrome P-450 or another alteration is not understood.

Nevertheless, substrate inhibition cannot account for all unusually low index III and IV values. In other studies we found that the values for index II as well as indices III and IV were less than 1.0 when we studied the metabolism of hexobarbital by liver microsomes from female rats or from male rats receiving spironolactone (14). The reason for these unusually low values is not clear at present, but high substrate concentrations or treatment of animals with spironolactone might decrease the affinity of substrate for the reduced and oxygenated forms of cytochrome P-450 without affecting its affinity for the oxidized form. In this case, the rate of substrate metabolism would be less than the difference between the rates of NADPH oxidation in the presence and absence of substrate, and thus give an index II value less than 1.0.

In any event, the use of indices III and IV reported in the present paper provides a better understanding of the stoichiometric relationships between metabolism of a number of type I substrates and NADPH oxidation than can be obtained solely by the methods used in the past.

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TABLE 4

Effect of substrate inhibition on indices III and IV

Values are the means ± standard errors of three animals, except as indicated. The substrate concentrations used were those above which metabolism is not increased. The substrate concentrations employed for stimulating NADPH oxidation were the same as those used in the incubation mixtures described in the text, except for the presence of of 1 mM hexobarbital.

Substrate	Concentration	Metabolism		NADPH oxidation				Index III	Index IV
		Air	$CO–O_2$	*A* (endogenous in air)	*B* (substrate in air)	*C* (endogenous in $CO–O_2$)	*D* (substrate in $CO–O_2$)		
	mM	*nmoles/mg protein/min*		*nmoles/mg protein/min*					
Catabolism[a]									
Ethylmorphine	2.5	9.60 ± 0.32	1.84 ± 0.10	7.72 ± 1.12	13.4 ± 0.82	3.68 ± 0.30	5.53 ± 0.51	0.98	0.99
p-Chloro-*N*-methylaniline	1.0	5.39 ± 0.57	3.00 ± 0.31	7.29 ± 0.40	11.2 ± 0.72	3.57 ± 0.30	6.41 ± 0.91	0.70	0.50
Anabolism[b]									
Hexobarbital	0.4	3.76 ± 0.14	0.82 ± 0.11	8.89 ± 0.58	13.1 ± 0.57	4.66 ± 0.22	6.55 ± 0.31	0.44	0.45
Imipramine[c]	0.4	4.68		6.39	9.5	2.51	—	0.67	—

[a] Nanomoles of formaldehyde formed per milligram of protein per minute.

[b] Nanomoles of substrate disappearing per milligram of protein per minute.

[c] Determined in a pooled sample from two animals.

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