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METABOLISM OF FORMALDEHYDE DURING *IN VITRO* DRUG DEMETHYLATION

EFFECTIVENESS OF PROTECTION BY SEMICARBAZIDE

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Abstract—Formaldehyde formation is usually determined as a measure of the metabolism of drug substrates *in vitro*. We have studied the fate of formaldehyde added to incubates of rat liver microsomes or 9000 *g* supernatant fraction as well as the effectiveness of semicarbazide in protecting formaldehyde from metabolic degradation. Formaldehyde is known to be oxidized by a cytosolic NAD- and GSH-dependent (form)aldehyde dehydrogenase. We found that, in addition, some other NAD-independent reactions take place in the cytosol. We observed, moreover, that formaldehyde is also metabolized by the 9000 *g* supernatant fraction fortified with cofactors for hepatic monooxygenase in the absence of NAD. This finding could be attributed to a hitherto unknown, cytosolic NADP-dependent, GSH-requiring dehydrogenase. The microsomal fraction metabolized formaldehyde only to a small extent. Therefore, in order to use formaldehyde formation as a parameter of drug metabolism, semicarbazide is necessary to protect formaldehyde from further metabolism in the 9000 *g* supernatant fraction and microsomes. By determining amounts of both formaldehyde and *p*-chlor-aniline during *p*-chlor-*N*-methylaniline demethylation, it was shown that semicarbazide (4 mM) only partially protected formaldehyde from further metabolism in the 9000 *g* supernatant, although semicarbazone formation from the added formaldehyde and semicarbazide progressed more rapidly than formaldehyde metabolism. As higher semicarbazide concentrations inhibit microsomal demethylations, it is concluded that determination of formaldehyde is not a suitable method for determining drug demethylation by the 9000 *g* liver supernatant. In microsomal incubates, only a low semicarbazide concentration (1.0 mM) was necessary to protect formaldehyde from further metabolism.

During demethylation reactions catalysed by hepatic microsomal cytochrome P-450, formaldehyde is formed. The amounts of this product are usually taken as a measure of the velocity of the reaction. *In vivo*, formaldehyde is further metabolized to formic acid and ultimately to carbon dioxide. Exhaled carbon dioxide, in fact, is used as an estimate of the capacity for hepatic demethylation *in vivo* [1–3]. However, *in vitro* oxidation of formaldehyde may also occur [4–8]. Since the earliest publications in the field, therefore, 1–10 mM semicarbazide has been used by most workers [9–17] to protect formaldehyde by forming a semicarbazone. Recently, however, Matsubara *et al.* [18] reported a strong inhibitory effect of semicarbazide on aminopyrine demethylation by rat liver microsomes, whereas, at the same time, it seemed that semicarbazide addition was not necessary because formaldehyde apparently remained stable in microsomal incubates. The authors concluded that semicarbazide should be omitted from such incubates. Dewaide and Henderson [19] observed that high concentrations of semicarbazide interfered with the reaction of formaldehyde with Nash reagent, a finding which might explain the apparent inhibitory effect of semicarbazide on demethylation reactions.

The present study was undertaken in order to determine whether or not formaldehyde is metabolized during incubations with liver microsomes or with the 9000 *g* supernatant fraction under conditions used to determine demethylation reactions and to decide which cofactors are necessary. In addition, the protection of formaldehyde by semicarbazide from further metabolic conversion was investigated in both the microsomal suspensions and the 9000 *g* liver supernatant fraction. Different substrates for demethylation were investigated, including *p*-chlor-*N*-methylaniline, the demethylation of which was determined by two methods. In this way, the effect of semicarbazide could be investigated on hepatic microsomal demethylation reactions as well as on formaldehyde recovery.

MATERIALS AND METHODS

Male Wistar (WU) rats, weighing about 200 g, were obtained from the central Animal Breeding Institute TNO, Zeist, The Netherlands. All animals were kept under similar conditions in Makrolon cages, with a bedding of wood shavings, and received standard food pellets (Hope Farms) and water *ad lib*.

Chemicals. Ethylmorphine, aminopyrine and cocaine (Pharm.Ned.) were obtained from Brocacef; analytical grade *N*-methylaniline and *p*-chlor-aniline (PCA) from Fluka; *p*-chlor-*N*-methylaniline

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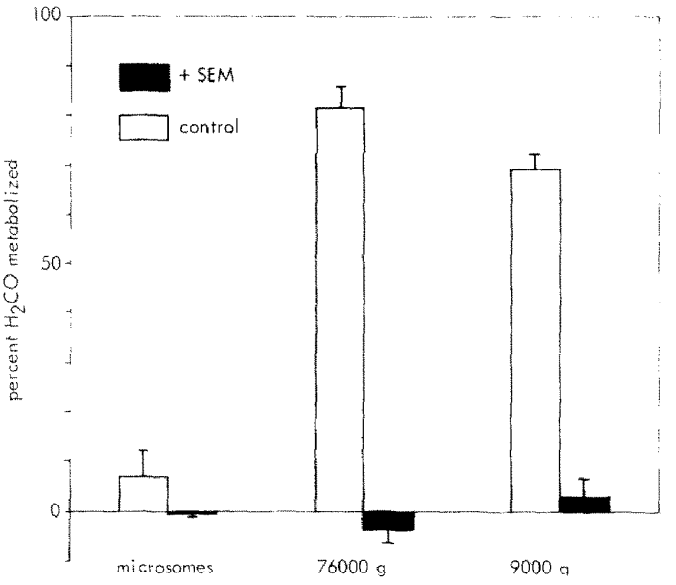

Fig. 1. Effect of semicarbazide (4.17 mM) on the metabolism of formaldehyde (0.167 mM) in incubation mixtures containing rat liver microsomes, 76,000 *g* supernatant fraction or 9000 *g* supernatant fraction. Values represent means ± S.E.M. (N = 6).

(PCNMA) from CalBiochem. Monomethylaminopyrine was a gift from Hoechst. NADP (grade I), NAD (grade I), NADH (grade I), glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase (grade I) were purchased from Boehringer. Diethylmaleate was obtained from Fluka. All other chemicals used were of at least reagent grade purity.

Enzyme determinations. Ethylmorphine, aminopyrine, cocaine, *N*-methylaniline and monomethylaminopyrine demethylations were assayed by measuring the formation of formaldehyde. *p*-Chlor-*N*-methylaniline demethylation was determined by measuring the formation of both formaldehyde and *p*-chlor-aniline.

The 9000 *g* supernatant fraction and the microsomes, prepared as described by van den Berg *et al.* [20], were incubated at 37°, with shaking, air being freely admitted. The incubation mixture contained NADP (1.5 μmole), glucose-6-phosphate (12.5 μmoles), glucose-6-phosphate dehydrogenase (1 I.U.) (for microsomal incubates), $MgCl_2$ (12.5 μmoles), substrate, semicarbazide as indicated in the tables and figures, and 9000 *g* supernatant or microsomes, derived from 1/6 g liver, in a total volume of 3 ml. The incubation mixtures used for formaldehyde metabolism are given in the figures. The reaction was initiated by the addition of the enzyme and stopped after 10 min by mixing 1 ml of the 9000 *g* supernatant incubates or 3 ml of the microsomal incubates with 0.5 ml $ZnSO_4$ (40%) and 1.0 ml $Ba(OH)_2$ (saturated). After centrifugation, formaldehyde was determined using the Nash reagent [21], as described by van den Berg *et al.* [20]. Demethylation of *p*-chlor-*N*-methylaniline was also determined by the formation of *p*-chlor-aniline, as described by Kupfer and Bruggeman [22].

The rate of formation of a semicarbazone from formaldehyde and semicarbazide was determined by recording the absorbance at 232 nm using an Aminco DW 2A recording spectrophotometer in the split beam mode. The reaction rate constant k was calculated using the equation $\Delta A = \Delta A_{max}(1 - e^{-kt})$.

RESULTS

Effect of semicarbazide on the determination of formaldehyde with Nash reagent. It has been reported by Dewaide and Henderson [19] that concentrations of semicarbazide exceeding 10 mM interfere with the reaction of formaldehyde with Nash reagent. Semicarbazide has been used in concentrations of 1–10 mM in measurements of hepatic microsomal demethylations. However, using the highest of these semicarbazide concentrations (10 mM), we failed to demonstrate interference with formaldehyde determinations carried out with Nash reagent, at formaldehyde concentrations ranging from 0.03 to 0.167 mM. This is in agreement with the results of Dewaide and Henderson [19] who also found no important inhibitory effect with up to 8 mM semicarbazide.

Metabolism of formaldehyde by different liver cell fractions. Figure 1 shows the percentage disappearance of added formaldehyde in different liver cell fractions, using the same incubation conditions as those used for demethylation reactions. In microsomal incubations there was very little disappearance (± 6 per cent) of formaldehyde in the absence of semicarbazide and none in the presence of 4.17 mM of this compound. In the 9000 *g* and the 76,000 *g* supernatant incubation mixtures without semicarbazide, 70 and 80 per cent, respectively, of the formaldehyde disappeared. In the presence of 4.17 mM semicarbazide, metabolism of formaldehyde was reduced to about 4 per cent.

We investigated the cofactors necessary for formaldehyde metabolism in the 9000 *g* liver superna-

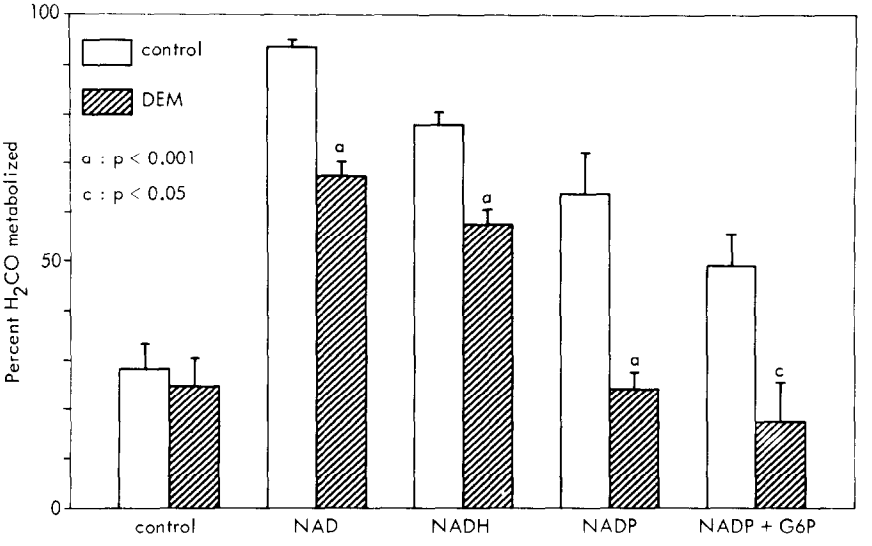

Fig. 2. Effect of GSH depletion by diethylmaleate on the metabolism of formaldehyde (0.167 mM) in different incubation mixtures containing the 9000 *g* supernatant fraction. Cofactor concentrations were 0.5 mM NAD, 0.5 mM NADH, 0.5 mM NADP and 4.17 mM G-6-P. Values represent means ± S.E.M. (N = 3).

tant fraction (Fig. 2). All incubation mixtures contained Mg^{2+}. In the absence of NAD or NADP some formaldehyde metabolism still took place (Fig. 2), presumably performed by peroxisomal catalase [8, 23] which does not require these cofactors. It was found that NADP significantly increased formaldehyde metabolism. The presence of G-6-P did not alter this effect of NADP, indicating that no reductive metabolism takes place. NAD had a strong stimulatory effect on the disappearance rate of formaldehyde which must have been due to a specific NAD formaldehyde dehydrogenase [4, 6, 7, 23]. NADH exerted a similar effect. This could have been due to formation of NAD which is formed from NADH by the enzyme NADH oxidase [24, 25]. NAD formaldehyde dehydrogenase is known to be GSH dependent [5, 7, 26]. To investigate whether GSH is also a necessary cofactor for the NADP-dependent dehydrogenase, the effect of GSH depletion was studied. GSH was depleted to 5–10 per cent of control livers, which contained 4.2 ± 0.2 μmoles/g, by diethylmaleate, administered i.p. 1 hr prior to liver removal, at a dose of 0.7 ml/kg. This treatment had no effect on formaldehyde metabolism in the 9000 *g* control incubates (Fig. 2). The metabolism of formaldehyde was significantly lowered, however, in incubates containing NAD or NADH, as cofactors. In the presence of NADP, GSH depletion completely inhibited the NADP-induced stimulation of formaldehyde metabolism. GSH addition completely restored the enzyme activity (unpublished results).

Effect of semicarbazide on demethylation of different substrates. Using aminopyrine and ethylmorphine at low concentrations, relative to the apparent K_m of the demethylation reaction, we studied the effect of semicarbazide, in concentrations from 1 to 10 mM, on the rate of demethylation in both hepatic microsomes and the 9000 *g* supernatant fraction (Fig. 3). Using hepatic microsomes and aminopyrine as substrate, a favourable effect on the amount of formaldehyde determined was observed at low semicarbazide concentrations, whereas higher concentrations did not significantly inhibit aminopyrine demethylation (Fig. 3). A similar effect of low semicarbazide concentrations was observed using ethylmorphine as a substrate (Fig. 3). High semicarbazide concentrations, however, inhibited ethylmorphine demethylation.

Using the 9000 *g* liver supernatant fraction, completely different results were obtained, in comparison to those obtained with microsomes. In the absence of semicarbazide, considerably less formaldehyde could be detected during ethylmorphine demethylation (Fig. 3). By increasing semicarbazide concentrations formaldehyde recovery was clearly improved, but the amounts of formaldehyde measured at low semicarbazide concentrations were significantly lower than the amounts measured when using microsomes (Fig. 3). An optimum was found at 4.17 mM semicarbazide; higher concentrations did not improve formaldehyde recovery and inhibited ethylmorphine demethylation. This was also found for aminopyrine demethylation, but, using this substrate, there was no difference between the amount of formaldehyde formed by microsomes and the 9000 *g* supernatant fraction at low concentrations of semicarbazide. At high semicarbazide concentrations we even observed a somewhat faster demethylation using the 9000 *g* supernatant fraction (Fig. 3). The effect of semicarbazide on the demethylation reaction was also investigated for other substrates. We found no influence of semicarbazide, in concentrations of 1.0 and 4.17 mM, on the production of formaldehyde during microsomal demethylation of *N*-methylaniline or cocaine at either low or high substrate concentrations (Table 1). A significant protecting effect on formaldehyde recovery was only

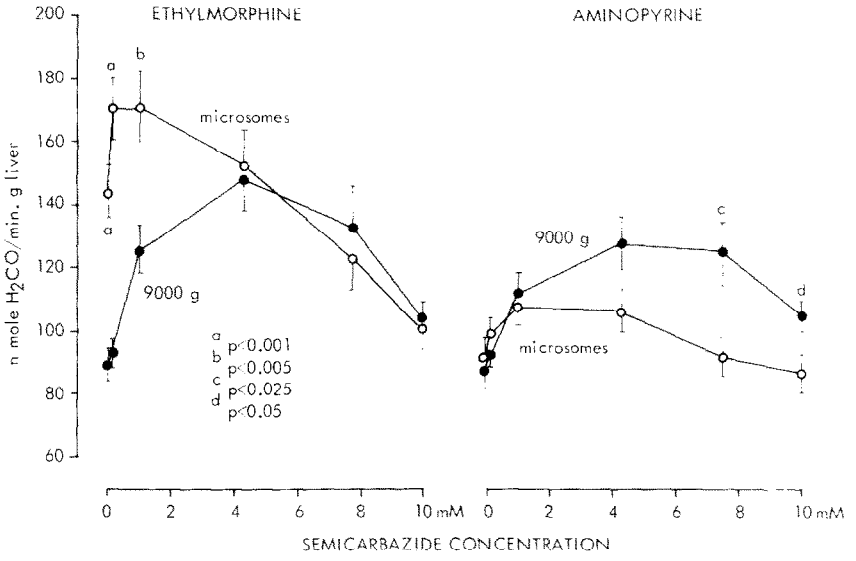

Fig. 3. Effect of different concentrations of semicarbazide on the recovery of formaldehyde produced as a result of demethylation of ethylmorphine (0.13 mM) and aminopyrine (0.13 mM) by rat liver 9000 *g* supernatant fraction or microsomes. Values represent means ± S.E.M. (N = 10).

observed with the high (1.3 mM) concentration of monomethylaminopyrine, as also was observed using ethylmorphine as substrate.

Effect of semicarbazide on p-*chlor*-N-*methylaniline demethylation determined using both products of microsomal and 9000* g *incubations.* In order to clarify whether the formaldehyde formed is completely protected by semicarbazide, we determined both PCA and formaldehyde formed in the same incubations during PCNMA demethylation in 9000 *g* supernatant fraction and microsomal incubates. Semicarbazide did not significantly lower PCA production in microsomal incubates (Table 2). Using the 9000 *g* supernatant fraction similar observations were made. The demethylation reaction itself therefore was not inhibited by semicarbazide at concentrations up to 4.17 mM.

In microsomal incubates formaldehyde formation equalled PCA production in the presence of semicarbazide, using 0.13 mM PCMNA. At a higher substrate concentration, formaldehyde formation was slightly but significantly lower than PCA production, even when semicarbazide was present. However, using hepatic 9000 *g* supernatant fraction as the enzyme source, the amount of formaldehyde determined was much lower than the PCA concentration. Even 4.17 mM semicarbazide could only partially abolish this difference. It seems, therefore, that protection of formaldehyde by semicarbazide is incomplete, especially when the rate of formaldehyde formation is high due to high substrate concentrations.

Rate of formaldehyde–semicarbazone formation. Formaldehyde has no absorption spectrum in the ultraviolet region above 200 nm, while semicarbazide has an absorption peak at 204 nm. When combined, however, a large semicarbazone peak appears at 232 nm, whereas the smaller 204 nm semicarbazide peak disappears. The formation of semicarbazone was followed at 232 nm for different formaldehyde concentrations and with both 1.0 and 4.17 mM semicarbazide in 0.1 M phosphate buffer, pH 7.4 (Fig. 4). The reaction rate constant for complex

Table 1. The effect of semicarbazide on the N-demethylation of various substrates in liver microsomal incubates (nmoles formaldehyde/min/g liver)*

	Substrate concentration (mM)	Semicarbazide (mM)		
		0	1.0	4.17
N-Methylaniline	0.13	7.9 ± 1.7	11.2 ± 2.6	11.2 ± 1.8
	1.3	34.6 ± 4.3	39.7 ± 3.2	35.8 ± 5.0
Cocaine	0.13	31.7 ± 4.6	31.3 ± 3.8	23.3 ± 2.7
	1.3	103.0 ± 6.2	105.8 ± 6.1	94.8 ± 6.1
Monomethylaminopyrine	0.13	50.9 ± 4.7	50.0 ± 2.3	48.5 ± 2.3
	1.3	115.8 ± 3.8	150.7 ± 7.7†	155.6 ± 4.7‡

* Results are means ± S.E.M. for six determinations.
† $P < 0.01$ for difference from 0 mM semicarbazide.
‡ $P < 0.001$ for difference from 0 mM semicarbazide.

Table 2. The effect of semicarbazide on the N-demethylation of PCNMA and formaldehyde recovery from hepatic microsomes and the 9000 *g* supernatant fraction (nmoles/min/g liver)*

Cell fraction	PCNMA concentrations (mM)	Product measured	Semicarbazide (mM) 0	1.0	4.17
Microsomes	0.13	HCHO	39.6 ± 5.2	50.7 ± 4.4	49.2 ± 4.4
	0.13	PCA	40.0 ± 5.4	49.9 ± 2.0	46.4 ± 3.0
			n.s	n.s	n.s
	1.3	HCHO	87.6 ± 10.9	109.8 ± 5.8	99.6 ± 6.3
	1.3	PCA	136.7 ± 4.0	135.3 ± 4.7	124.9 ± 5.3
			$P < 0.01$	$P < 0.01$	$P < 0.01$
Supernatant fraction (9000 *g*)	0.13	HCHO	17.1 ± 4.1	25.3 ± 3.4	24.7 ± 3.4
	0.13	PCA	52.3 ± 8.8	52.0 ± 7.7	50.3 ± 9.4
			$P < 0.01$	$P < 0.01$	$P < 0.05$
	1.3	HCHO	54.3 ± 3.8	80.3 ± 4.9†	109.6 ± 6.4‡
	1.3	PCA	199.2 ± 17.7	180.0 ± 12.0	173.4 ± 13.9
			$P < 0.001$	$P < 0.001$	$P < 0.001$

* Results are means ± S.E.M. for six determinations.
† $P < 0.01$ for difference from 0 mM semicarbazide.
‡ $P < 0.001$ for difference from 0 mM semicarbazide.

formation using 4.17 mM semicarbazide was 0.86 ± 0.02 per min. At 1 mM, the reaction rate constant was 0.21 ± 0.01 per min. In both cases the reaction rates were independent of the initial concentrations of formaldehyde.

DISCUSSION

Hepatic microsomal demethylations are usually determined by measuring formaldehyde formation. This method may lead to artificially low results, because formaldehyde may be further metabolized in the incubate. Formaldehyde metabolism in rat liver has been studied by several investigators. It was found that formaldehyde is mainly dehydrogenated by a NAD- and GSH-dependent formaldehyde dehydrogenase [4–8, 25]. The importance of GSH in formaldehyde metabolism was also demonstrated by Jones *et al.* [27] using isolated hepatocytes. The subcellular localization of this enzyme was investigated by Koivula *et al.* [4] who found two NAD-dependent formaldehyde dehydrogenases in the cytoplasmic fraction and almost no dehydrogenase activity towards formaldehyde in the microsomal fraction. In addition, some dehydrogenation of formaldehyde by nonspecific aldehyde dehydrogenases may take place, these enzymes also requiring NAD as a cofactor [4, 5, 25]. As yet, no formaldehyde dehydrogenase activity has been found in rat liver which requires NADP as a cofactor [4, 7, 25]. In microsomal or 9000 *g* supernatant-containing incubates NAD is not usually added, but NADP is, because this is a cofactor for microsomal monooxygenase. Therefore, we were interested to know (i) whether or not formaldehyde metabolism takes place under these circumstances, (ii) the subcellular localization of the enzyme system responsible for formaldehyde metabolism and (iii) the cofactors required for the reaction.

The present data show that, in addition to the

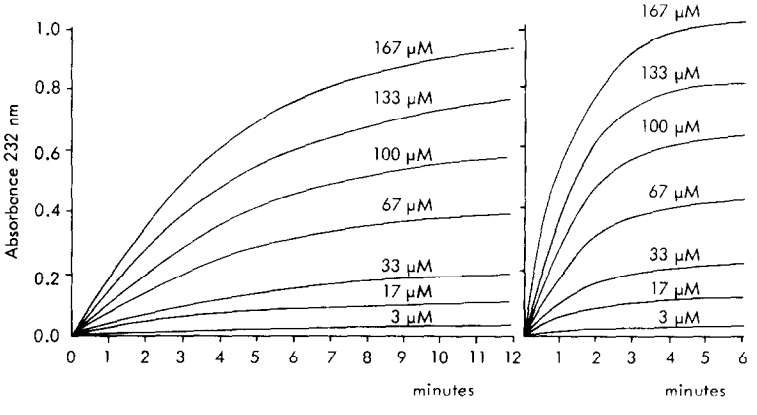

Fig. 4. Rate of semicarbazone formation from formaldehyde (3–167 μM) and semicarbazide 1 mM (left) and 4.17 (right) at 37°.

known NAD-dependency of formaldehyde dehydrogenase, there is also NADP dependency. NAD and NADP added together had no additional effect (unpublished results). The enzymes are localized in the cytosol and have either a complete (NADP) or partial (NAD) requirement for GSH. Formaldehyde dehydrogenase, which is both NADP-and GSH-dependent, has as yet not been described in rats. In human liver, on the other hand, Uotilla and Koivusalu [28] detected an NADP- and GSH-dependent formaldehyde dehydrogenase. Formaldehyde metabolism, therefore, is likely to occur in incubates containing the 9000 *g* liver supernatant fraction, but some metabolism also occurs in microsomal incubates, as shown in the present work.

Semicarbazide is usually added to protect formaldehyde by forming a semicarbazone. This may lead to inhibition of drug demethylation reactions, as recently shown by Matsubara [18]. The present work shows that addition of semicarbazide has sometimes a small inhibitory effect on hepatic microsomal demethylation reactions. This effect, however, is much smaller than reported by Matsubara. The apparent inhibitory effect of semicarbazide is not due to interference with the Nash reaction, as was also shown in the present work. The effect of semicarbazide increased with increasing concentration and was also dependent on the concentration and the nature of the substrate. The protection of added formaldehyde by semicarbazide seemed to be complete when 1 mM semicarbazide was present in the microsomal incubates. Addition of 4 mM semicarbazide to the 9000 *g* supernatant-containing incubates led to maximal recovery of the formaldehyde produced by demethylation of ethylmorphine and aminopyrine. Higher concentrations clearly inhibited these demethylation reactions. Even 4 mM semicarbazide inhibits some demethylation reactions, especially at low substrate concentrations. Through studying PCNMA demethylation, by measuring both the formation of formaldehyde and PCA, it was demonstrated that 4.17 mM semicarbazide did not completely protect formaldehyde. This could have been due to a relatively slow formation of the semicarbazone, since the rate constant for the complex formation using 4.17 mM semicarbazide with added formaldehyde was found to be 0.86 per min. The amount of added formaldehyde metabolized by the 9000 *g* liver supernatant fraction in the absence of semicarbazide is 70 per cent, from which a reaction rate constant of 0.12 per min can be calculated. This proves that the complex formation takes place much faster than the metabolism of formaldehyde. Nevertheless, the amount of formaldehyde recovered is lower than the amount of PCA determined when 4.17 mM semicarbazide is present in the 9000 *g* supernatant-containing reaction mixtures used for PCNMA demethylations. These apparently contradictory results may be due to the fact that formaldehyde is formed during demethylation reactions within the microsomal membrane and is not added to the reaction mixture as in our experiments. If the formaldehyde dehydrogenase is somehow concentrated near the microsomal membrane, it might oxidize the formaldehyde before it comes into contact with the semicarbazide. An analogous explanation was provided by Denk *et al.* [29] for their finding that formaldehyde generated during microsomal aminopyrine demethylation is metabolized more efficiently by mitochondria than added formaldehyde. This was attributed to juxtaposition of mitochondria and endoplasmic reticulum fragments. Absorption of formaldehyde dehydrogenase onto the microsomal membrane might also explain the residual enzyme activity observed when microsomes are used. The recovery of formaldehyde is somewhat lower than found by Kupfer and Bruggeman [22] and Fuller *et al.* [30] for 1.5 mM PCNMA. At lower substrate concentrations the recovery is even lower, which may lead to error in the determination of kinetic constants for hepatic oxidative demethylation reactions.

It appears from the foregoing that 4.17 mM semicarbazide is not sufficient to protect formaldehyde from further metabolism if it is formed by oxidative demethylation using 9000 *g* supernatant fraction as the enzyme source. Because higher semicarbazide concentrations, and even 4.17 mM, might inhibit the demethylation reactions, it should be regarded as impossible to determine accurately hepatic *N*-demethylation by estimation of formaldehyde formation using 9000 *g* supernatant fraction as the enzyme source. When microsomes are used, addition of 1 mM semicarbazide is required to protect formaldehyde for further metabolism.

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