Biochemical Pharmacology, Vol. 30, No. 20, pp. 2767–2772, 1981.
Printed in Great Britain.

0006-2952/81/202767-06 $02.00/0

METABOLISM OF NITROSOACETOXYMETHYLMETHYLAMINE IN LIVER MICROSOMES

KLAUS E. APPEL*, NORBERT FRANK† and MANFRED WIESSLER†

Institute of Biochemistry and †Institute of Toxicology and Chemotherapy, German Cancer Research Centre, Im Neuenheimer Feld 280, D-6900 Heidelberg, West Germany

(*Received* 23 *December* 1980; *accepted* 23 *April* 1981)

Abstract—The carcinogen nitrosoacetoxymethylmethylamine (NAMM)‡ was incubated with mouse liver microsomes. The decomposition rate of NAMM and the formation of methanol were determined. After addition of an NADPH-regenerating system, formaldehyde formation resulting from metabolic degradation of the methyl group of NAMM was measured. The results suggest that NAMM is rapidly hydrolyzed by unspecific esterases to nitrosohydroxymethylmethylamine (NHMM). NHMM decomposes according to the activation mechanism of nitrosodimethylamine (NDMA). The formation of methanol proceeds with an even lower velocity. This indicates that intermediate breakdown products—probably NHMM or methyldiazohydroxide (MDH)—possess a distinct stability. Oxidative demethylation of NHMM or MDH was not detectable. The methanol formed is oxidised to formaldehyde by microsomal enzymes.

INTRODUCTION

The metabolic activation of the secondary carcinogen NDMA is generally accepted to be initiated by a cytochrome (cyt.) P-450 dependent oxidative dealkylation. This should lead to equimolar yields of formaldehyde and alkylating species, provided no further intermediate metabolic step exists [1, 2].

It has been shown by earlier studies that after induction of the microsomal monooxygenase system with phenobarbital (PB), 3-methylcholanthrene, or polychlorinated biphenyls, a depressed alkylation rate of biopolymers was observed when [^{14}C]NDMA was applicated *in vivo* or incubated in microsomal systems [3, 4]. The interpretation of these results on the basis of the above-mentioned activation mechanism proved difficult to reconcile with the assumption that NDMA-demethylase activity is increased after induction as is normal for other cyt. P-450 dependent demethylation reactions.

The discussion of this problem led to the speculation that the cause of the lowered alkylation rate of biopolymers may be a further oxidative demethylation of the second methyl group of NDMA by the induced cyt. P-450 [3]. A subsequent oxidative dealkylation of both alkyl groups is well known from tertiary amines, e.g. SKF 525-A or aminopyrine [5, 6]. NHMM or MDH are possible substrates for this second hydroxylation step. In an earlier paper, Hoch-Ligeti *et al.* [7] discussed the possible demethylation of monomethylnitrosamine to non-carcinogenic breakdown products. However, this reaction seems quite unlikely, since Druckrey *et al.* [8] have reported that monomethylnitrosamine is stable only at −70°. Although precise half-life times of these unstable reactive intermediates are not known Bartsch (in [9]) reported that the half-life of methylating intermediates formed from NDMA by liver microsomal monooxygenases was measured to be in the order of seconds in aqueous media. However, the stability of these compounds in the aprotic environment of lipoid membranes has not yet been determined. A certain stability is however a prerequisite of any metabolic reaction. It is also important in reference to a sufficiently stable transport form which is able to penetrate the nuclear membrane for the alkylation of genetic material.

Although it seems to be clear now [10–12] that after pretreatment of the animals with microsomal inducers, NDMA is demethylated with lower activity—a phenomenon which is explained by the existence of different forms of cyt. P-450 or by an altered nature of the microsomal membrane—we have tried to reveal if the presumed oxidative demethylation of NHMM or MDH is demonstrable.

As NHMM could not be synthesized, we used its derivative NAMM which has the same oxidation state. NAMM was additionally labelled (^{14}C) at the methyl group, thus enabling us to distinguish between labelled $^{14}CH_2O$ formed from the methyl group and the unlabelled CH_2O from the methylene group.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: [^{14}C]NAMM and NAMM were synthesized as already described [13, 14]; [^{14}C]methylamine and $^{14}CH_3OH$ were obtained from Amersham Buchler (Braunschweig, West Germany); 5,5-dimethyl-1,3-cyclohexanedione (Dime-

* Address correspondence to: Klaus E. Appel, Department of Toxicology, Institute of Pharmacology, Obere Zahlbacher Str. 67, D-6500 Mainz, West Germany.

‡ Abbreviations: NAMM, nitrosoacetoxymethylmethylamine; NDMA, nitrosodimethylamine; NHMM, nitrosohydroxymethylmethylamine; MDH, methyldiazohydroxide; cyt., cytochrome; PB, phenobarbital.

done), Merck (Darmstadt, West Germany); isocitrate dehydrogenase EC 1.1.1.42 and disodium salt of NADP, Boehringer (Mannheim, West Germany); trisodium-D,L-isocitrate, Serva (Heidelberg, West Germany); all other chemicals were of reagent grade and were commercially available.

Preparation of microsomes. Female NMRI mice were used, weighing 22–25 g. For induction of cyt. P-450, mice were pretreated with 0.1% PB in the drinking water for 5 days.

Animals were killed by decapitation. After bleeding, the livers were quickly removed, placed in ice-cold buffer I (0.25 M sucrose, 0.02 M Tris–HCl, 5.5 mM EDTA, pH 7.5), cut into small pieces and homogenised in 4 vol. of buffer I. After removal of cell debris, nuclei and mitochondria by centrifugation for 10 min at 500, 1000 and 10,000 rpm (Sorvall centrifuge, HB-4 rotor), the microsomal fraction was pelleted by centrifugation at 100,000 *g* for 60 min. The microsomal pellets were washed once with buffer II (0.12 M KCl and 0.05 M Tris–HCl, pH 7.5), resedimented by centrifugation at 100,000 *g* for 60 min, and resuspended in buffer II to give a final protein concentration of 2 mg/ml. Protein was determined by the method of Lowry *et al.* [15]. The concentration of cyt. P-450 was determined from the CO-difference spectra of dithionite reduced samples using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm.

Microsomal incubations and determination of formaldehyde. Microsomal incubations were performed in Eppendorf incubation tubes. The total incubation volume was 1 ml. The complete incubation medium contained 5 mM $MgCl_2$, 8 mM D,L-isocitrate, 1 mM NADP and 5 μl isocitrate dehydrogenase (20 mg/ml) in buffer II. In general, the microsomal protein content was 2 mg/ml. After 2 min of preincubation, the reaction mixture was started by addition of substrate. The Eppendorf incubation tubes were incubated at 37° under air in an Eppendorf incubator. CH_2O production was determined by measuring $^{14}CH_2O$ which was precipitated as formaldemethone by the procedure of Frisell and McKenzie as modified by Paik and Kim [16], using 8 mg of CH_2O as carrier. The precipitated formaldemethone was sucked over GFF-filters, washed once with ice-cold water, dried for 15 min and put into scintillation liquid (4 g PPO, 50 mg POPOP in 1 l. of toluene).

Determination of the half-life of NAMM. One milligram of NAMM was dissolved in 5 ml of a microsomal suspension (2 mg protein/ml). The mixture was stirred slightly at 37°. Aliquots of 200 μl were taken at different time intervals and extracted with 2.0 ml dichloromethane. The identification and quantification of NAMM were performed by a gas chromatograph equipped with a Thermal Energy Analyser as described elsewhere [17].

Determination of the formation rate of methanol. 2.5 mg of [^{14}C]NAMM (219 μCi/mmole) was dissolved in 0.5 ml H_2O which was added to 10 ml of a suspension of microsomes (2 mg protein/ml). The mixture was stirred slightly at 37°. Aliquots of 1 ml were taken at different time intervals and extracted by 10 ml of dichloromethane. Under these conditions, NAMM does not remain in the water phase, while the labelled breakdown products NHMM, MDH and methanol are extracted according to their partition coefficients. The portion of extracted methanol was 24 ± 1% ($n = 4$), which was determined in separate experiments. The alkylation of microsomal proteins, which was determined after exhaustive solvent extractions, was less than 0.5% of the applied dose of NAMM and was therefore neglected for quantitative calculations. The portion of NHMM and MDH which was extracted by dichloromethane could not be determined. In a first approach it is assumed that NHMM and MDH are completely extracted by the organic solvent. The radioactivity of the organic phases was determined. From these data it is possible to estimate the half-life of the sum of all ^{14}C-labelled dichloromethane extractable compounds. As the portion of methanol is subtracted, the values represent the decomposition rate of the sum of the compounds NAMM, NHMM and MDH and are inversely proportional to the formation rate of methanol. For the calculation of the half-life, a linear regression line was fitted for logarithms of concentration vs time. A confidence interval for the half-life was derived through the usual confidence region around the fitted regression line. The half-life values are expressed as weighted mean with the 95% confidence region in parentheses. If NHMM and/or MDH are not completely extracted by dichloromethane, the half-life of the sum of labelled compounds has in reality a higher value than that calculated. Therefore, the calculated formation rate of methanol represents a minimal value. Liquid counting was performed on a Mark III counter [Nuclear (Chicago, IL)] with Packard Instagel® as scintillation cocktail.

RESULTS AND DISCUSSION

It is known from investigations of the chemical stability of NAMM that the ester group can be hydrolytically cleaved both by water and by unspecific esterases [17]. The half-life in water is about 35 hr at 37°, whereas the half-life in serum is much shorter (about 3.5 min) due to the esterases. In blood, the velocity of decomposition is even twice as fast than in serum. Roller *et al.* [18] determined a half life of 40.4 min at 21° for the degradation by hog liver esterase at pH 7.0.

The hydrolytic cleavage leads to NHMM, which according to the proposed activation mechanism of NDMA decomposes to CH_2O and methyldiazohydroxide (MDH), which is probably the proximate alkylating agent (see also Fig. 4).

The half-life of NAMM was determined in liver microsomes as described in Materials and Methods. It was found to be 1.2 min (0.9–1.3), which indicates a rather rapid breakdown corresponding with that in blood and demonstrates a relatively high esteratic activity in microsomes. (Fig. 1, linear regression line I).

The formation rate of methanol was slower compared with the degradation rate of NAMM (Fig. 1, linear regression line II). Methanol was formed with a half-life of 11.3 min (5.4–16.3). Obviously the formation rate of methanol does not proceed simultaneously with the degradation rate of NAMM. This implicates that intermediate breakdown

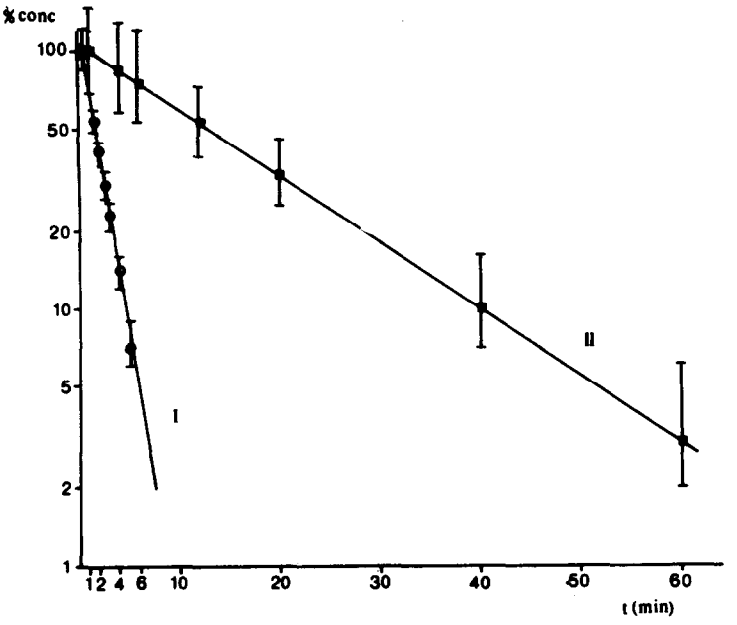

Fig. 1. Determinations of the half-lifes of NAMM (I) and of the sum of the compounds NAMM, NHMM and MDH (II) in mouse liver microsomes (2 mg protein/ml). I and II are linear regression lines of the logarithms of concentration on time. As described in Materials and Methods, II is inversely proportional to the formation rate of methanol.

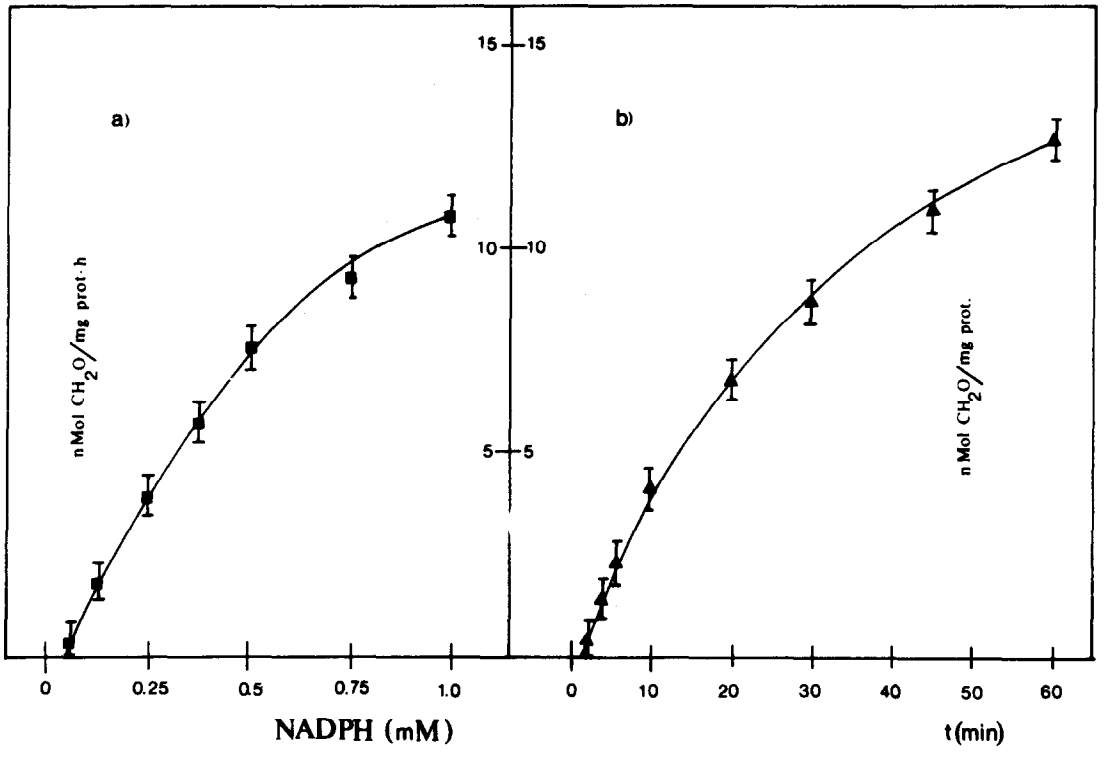

Fig. 2. (a) NADPH-dependent formaldehyde formation from the ^{14}C-labelled methyl group of NAMM in mouse liver microsomes. The concentration of [^{14}C]NAMM was 0.75 mM. (b) Time course of formaldehyde formation from the ^{14}C-labelled methyl group of NAMM. The incubation mixture contained 2 mg of protein/ml and an NADPH-regenerating system. The concentration of [^{14}C]NAMM was 0.75 mM.

Table 1. $^{14}CH_2O$ formation from both [^{14}C]NAMM and [^{14}C]methanol in liver microsomes from either control or PB-pretreated mice.

	nmole CH_2O/mg protein/hr ($n = 4$)					
	NAMM			Methanol		
Concentration (mM)	0.188	0.375	0.75	0.188	0.375	0.75
Control microsomes	7.01 ± 0.24	11.51 ± 0.48	17.11 ± 2.1	5.27 ± 0.63	9.13 ± 1.44	14.8 ± 2.0
PB-induced microsomes	3.33 ± 0.14	6.27 ± 0.29	10.07 ± 1.06	1.73 ± 0.2	3.80 ± 0.87	9.93 ± 0.19
Per cent inhibition	−52.5	−45.5	−41.1	−67.2	−58.3	−33.0

The concentrations of microsomal cytochromes were: 0.98 ± 0.14 nmole cyt. P-450/mg protein, 0.53 ± 0.11 nmole cyt. *b*5/mg protein for control microsomes; and 2.2 ± 0.2 nmole cyt. P-450/mg protein, 1.04 ± 0.09 nmole cyt. *b*5/mg protein for PB-induced microsomes.

products—probably NHMM or MDH—possess a certain stability in the order of some minutes in the microsomes. These results lead to the conclusion that NHMM or MDH is the transport form which is able to penetrate the nuclear membrane to alkylate DNA. Recently Gold and Linder [19] have also found out that enzymatically formed α-hydroxynitrosamines might be sufficiently stable to diffuse away from the site of their formation before further decomposition and therefore may be considered as "transportable" metabolites. This distinct but limited stability of NHMM or MDH is a prerequisite for any possible metabolic reaction. In order to ascertain if the methyl group of NHMM or MDH can be oxidatively demethylated by the microsomal monooxygenase system, [^{14}C]NAMM—labelled at the methyl group—was incubated with liver microsomes and increasing concentrations of NADPH. An oxidative dealkylation of NAMM itself seems unlikely in view of its rather high instability in the microsomes.

The dependence of $^{14}CH_2O$ formation on increasing concentrations of NADPH is shown in Fig. 2a. Figure 2b demonstrates the time dependent $^{14}CH_2O$ formation from [^{14}C]NAMM with liver microsomes and an NADPH-regenerating system. Thus we can conclude from these data that CH_2O is generated from the methyl group of NAMM. This metabolic process seems to be catalyzed by the microsomal mixed-function oxidase system.

To characterize further a possible cyt. P-450 dependent hydroxylation of the methyl group of possibly NHMM or MDH, an enhancement of the $^{14}CH_2O$ formation was attempted by using liver microsomes from PB-pretreated animals. Table 1 demonstrates that PB pretreatment inhibited $^{14}CH_2O$ formation instead of inducing it, as had been expected. This result was unusual in view of other cyt. P-450 dependent demethylation reactions which are enhanced by PB-induction, e.g. dealkylation of aminopyrine.

It was first shown by Orme-Johnson and Ziegler [20] that components of the microsomal fraction can oxidise methanol to formaldehyde in the presence of NADPH and O_2. Since methanol is a metabolic product of NAMM [18] and therefore CH_2O can arise from CH_3OH under these conditions of incubation, ^{14}C-labelled methanol was incubated in equivalent doses as [^{14}C]NAMM and $^{14}CH_2O$ was measured. The same result as obtained in the experiment with NAMM—an inhibiting effect after PB-induction—was observed (Table 1). Thus, this experiment suggests the possibility that the CH_2O may come from methanol. There have been conflicting opinions on the enzyme mechanism of alcohol oxidation. Initially, the group of Lieber has shown that hepatic microsomes oxidize various alcohols and that the microsomal ethanol-oxidizing system (MEOS) has characteristics (requirement for NADPH, dioxygen and partial inhibition by CO) which resemble those commonly found among microsomal monooxygenase systems [21, 22]. Although some authors attributed the MEOS-activity to catalase-contaminating microsomes [23], to catalase plus alcohol-dehydrogenase (ADH) [24], or to unidentified enzymes [25], others describe a major part of the MEOS-activity to a catalase—and ADH-independent pathway [22, 26]. Actually, a number of workers observed ethanol oxidation in a purified reconstitution monooxygenase system [27, 28], whereas others have failed to observe it [29, 30].

From our results in which PB-pretreatment does not enhance CH_2O formation we are unable to deduce which enzyme mechanism is involved in methanol oxidation. Further support for the assumption that CH_2O may be formed from methanol and not from oxidative demethylation of NHMM or MDH comes from the comparison of Lineweaver and Burk plots of CH_2O formation either from NAMM or methanol. Lineweaver and Burk plots of CH_2O formation either from NAMM or methanol are nearly identical (Fig. 3). The K_m values were 0.71 ± 0.21 mM ($n = 4$) for NAMM and 0.90 ± 0.35 mM ($n = 4$) for methanol. In addition the V_{max} values were of the same order of magnitude. The V_{max} values were 0.49 ± 0.07 nmole CH_2O/mg/min ($n = 4$) for NAMM and 0.5 ± 0.05 nmole CH_2O/mg/min ($n = 4$) for methanol. These results suggest that the measured CH_2O comes from methanol rather than from oxidative demethylation of NHMM or MDH.

Recent studies have shown that nitrite is a metabolic product when nitrosamines are incubated with liver microsomes and NADPH [31]. Incubation of NAMM under the same conditions did not produce any detectable amounts of nitrite over various periods of time. This result indicates that the intact

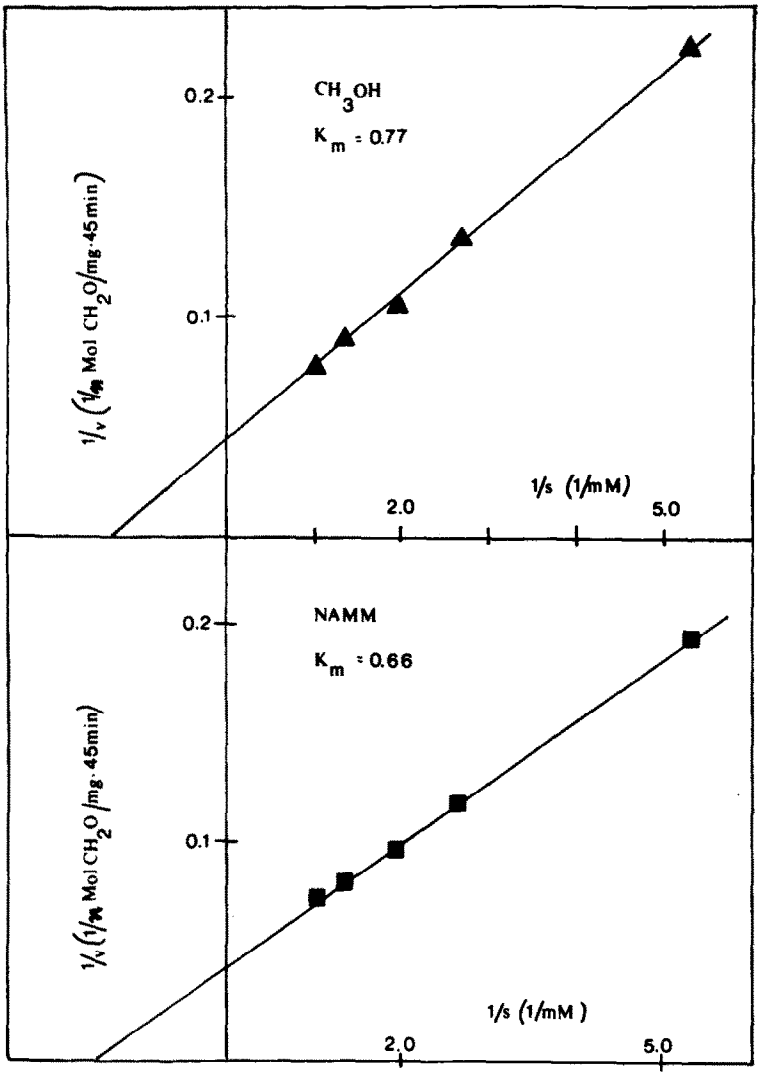

Fig. 3. Lineweaver and Burk plots for the determination of K_m and V_{max} values for both [^{14}C]NAMM and [^{14}C]methanol in mouse liver microsomes.

nitrosamine molecule is necessary for the metabolic formation of nitrite. Therefore we can exclude that nitrite is formed from the metabolites NHMM or MDH.

Finally, it should be mentioned that NAMM is probably not a metabolic product of NDMA. When ^{14}C-labelled NDMA was applied (10 mg/kg i.p.), NAMM was not found in the blood of rats after various periods of time (the detection limit for NAMM was 15 pg).

In summary, the data show that the hydrolytic degradation of NAMM by esterases is a rapid process, while the formation of methanol is slower. Formaldehyde is formed by oxidation of methanol in the presence of microsomal enzymes, NADPH and dioxygen (Fig. 4). There is experimental evidence

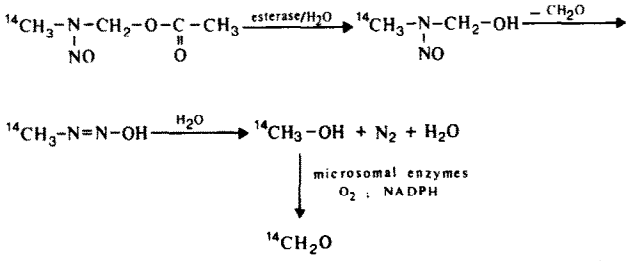

Fig. 4. Metabolic scheme of nitrosoacetoxymethylmethylamine.

for a distinct stability of NHMM or MDH or of both, which is in the order of a few minutes in the microsomes. This stability is probably sufficient for reaching the genetic material. It is probably not sufficient to make the experimental proof that NHMM or MDH can act as substrates of a cyt. P-450 dependent demethylation reaction.

Acknowledgements—The authors thank Dr. Hallier for helpful discussions and Mrs. Holzmann, Mainz, for her kindness in preparing the manuscript.

REFERENCES

1. P. N. Magee and J. M. Barnes, *Adv. Cancer Res.* **30**, 559 (1967).
2. P. Czygan, H. Greim, A. J. Garro, F. Hutterer, F. Schaffner, H. Popper, O. Rosenthal and D. Y. Cooper, *Cancer Res.* **33**, 2983 (1973).
3. K. E. Appel, M. Schwarz, R. Rickart and W. Kunz, *J. Cancer Res. Clin. Oncol.* **94**, 47 (1979).
4. W. Kunz, K. E. Appel, R. Rickart, M. Schwarz and G. Stöckle, in *Primary Liver Tumours* (Eds. H. Remmer, H. M. Bolt, P. Bannasch and H. Popper), p. 261. M.T.P. Press, Lancaster, (1978).
5. J. Gilette, H. Sasame and B. Stripp, *Drug Metab. Dispos.* **1**, 164 (1973).
6. M. R. Franklin, *Pharmac. Ther. Arch.* **2**, 227 (1977).
7. C. Hoch-Ligeti, M. F. Argus and J. C. Arcos, *J. Natn. Cancer Inst.* **40**, 535 (1968).
8. H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, *Z. Krebsforsch.* **69**, 103 (1967).
9. T. Kuroki and C. Drevon, *Nature, Lond.* **271**, 368 (1978).
10. J. C. Arcos, G. M. Bryant, N. Venkatesan and M. F. Argus, *Biochem. Pharmac.* **24**, 1544 (1975).
11. R. Kato, H. Shoiji and A. Takanaka, *Gann* **58**, 467 (1967).
12. M. Schwarz, K. E. Appel, D. Schrenck and W. Kunz, *J. Cancer Res. Clin. Oncol.* **97**, 233 (1980).
13. M. Wiessler, *Tetrahedron Lett.* **30**, 2575 (1975).
14. H. Braun and M. Wiessler, *J. labelled Compd.* **14**, 887 (1978).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. W. K. Paik and S. Kim, *Archs. Biochem. Biophys.* **165**, 369 (1974).
17. N. Frank, C. Janzowski and M. Wiessler, *Biochem. Pharmac.* **29**, 383 (1980).
18. R. P. Roller, D. R. Shrimp and L. K. Keefer, *Tetrahedron Lett.* **25**, 2065 (1975).
19. B. Gold and W. B. Linder, *J. Am. chem. Soc.* **101**, 6772 (1979).
20. W. H. Orme-Johnson and D. M. Ziegler, *Biochem. biophys. Res. Commun.* **21**, 78 (1965).
21. C. S. Lieber and L. M. DeCarli, *Science* **162**, 917 (1968).
22. C. S. Lieber and L. M. DeCarli, *J. biol. Chem.* **245**, 2505 (1970).
23. R. G. Thurman, H. G. Ley and R. Scholz, *Eur. J. Biochem.* **25**, 420 (1972).
24. K. J. Isselbacher and E. A. Carter, *Biochem. biophys. Res. Commun.* **39**, 530 (1970).
25. M. K. Roach, W. N. Rees and P. J. Creaven, *Biochem. biophys. Res. Commun.* **36**, 596 (1969).
26. A. G. Hildebrandt, M. Speck and L. Roots, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **281**, 371 (1974).
27. G. T. Miwa, W. Levin, P. E. Thomas and A. Y. H. Lu, *Archs. Biochem. Biophys.* **187**, 464 (1978).
28. K. Ohnishi and C. S. Lieber, *J. biol. Chem.* **252**, 7124 (1977).
29. R. G. Thurman, W. R. Mekemma, H. J. Brentzel and S. Hesse, *Fedn. Proc. Fedn. Am. Socs exp. Biol.* **34**, 2075 (1975).
30. K. P. Vatris and M. J. Coon, in *Alcohol and Aldehyde Metabolizing Systems*, Vol. II, p. 307. Academic Press, New York (1977).
31. K. E. Appel, D. Schrenck, M. Schwarz, B. Mahr and W. Kunz, *Cancer Lett.* **9**, 13 (1980).