Biochemical Pharmacology, Vol. 29, pp. 383–387.

0006-2952/80/0201-0383 $02.00/0

STABILITY OF NITROSOACETOXYMETHYLMETHYLAMINE IN *IN VITRO* SYSTEMS AND *IN VIVO* AND ITS EXCRETION BY THE RAT ORGANISM

NORBERT FRANK, CHRISTINE JANZOWSKI and MANFRED WIESSLER
Institute of Toxicology and Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, G.F.R.

(*Received* 18 *April* 1979; *accepted* 27 *August* 1979)

Abstract—The stability of the carcinogen nitrosoacetoxymethylmethylamine (NAMM)* was determined in water, serum and blood as well as in an *in vivo* assay. The half lifes of NAMM in the different media show that the dominant enzymatic degradation occurs within a few seconds after intravenous application. Nevertheless NAMM is stable enough for a passage through the body, which is also indicated by the determination of traces of NAMM in urine and exhalation air. This could explain the carcinogenesis of NAMM away from the application site.

Nitrosodimethylamine—a potent carcinogen [1, 2]—exerts its carcinogenic effects after activation by the liver monooxygenase system [3, 4]. The resulting, highly reactive nitrosohydroxymethylmethylamine (III) itself, a degradation product or a conjugate, is responsible for the methylation of genetic material. The stable model compound nitrosoacetoxymethylmethylamine (NAMM) (II) delivers nitrosohydroxymethylmethylamine (III) after hydrolysis or attack of esterases without any further enzymatic activation by liver oxygenase system (see Fig. 1)

The results of the experiments on the carcinogenesis of NAMM in the rat are summarized in Table 1. After intravenous, oral, subcutaneous and intrarectal application, local effects were observed. This could be explained by generation of the ultimate carcinogen from NAMM at the site of application. In addition to these local effects, systemic effects are also seen after intraperitoneal and subcutaneous application of the compound. Tumors were also found in fore-stomach, hematopoietic system, prostata and kidney after subcutaneous application.†‡ This systemic effect must be explained by a transport mechanism either of the intact NAMM or of a metabolite or its conjugate. It is not yet known whether the stability of NAMM is sufficient for transport of the intact substance to the target organ.

We therefore studied the kinetics of the decomposition of NAMM in *in vitro* systems after chemical hydrolysis or enzymatic cleavage in serum and blood and compared these results to those of the corresponding *in vivo* assay. The urine excretion and exhalation rate of intact NAMM were also measured to complete the study.

* Abbreviations: NAMM—Nitrosoacetoxymethylmethylamine, GC—gas chromatography, MS—mass-spectrometry, TEA—Thermal Energy Analyzer.

† M. Habs, personal communication.

‡ H. R. Scherf, personal communication.

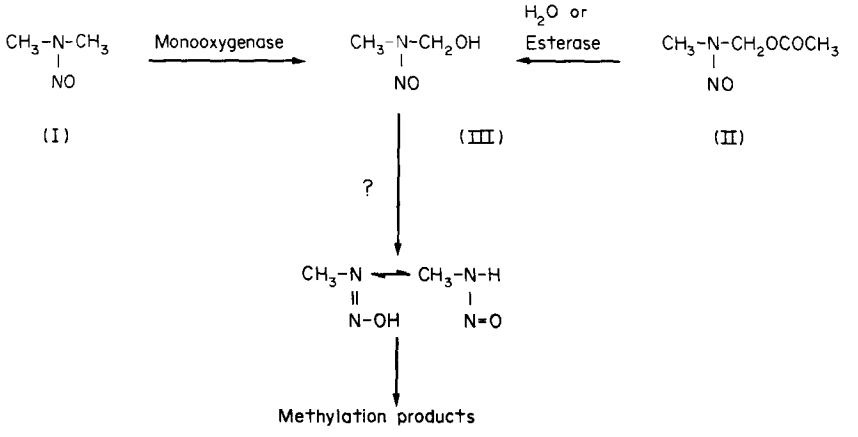

Fig. 1. Comparative decomposition scheme of nitrosodimethylamine (I) and nitrosoacetoxymethylmethylamine (II).

Table 1. Biological effects in rats after application of NAMM

Mode of application	Single dose (mg/kg/week)	Total dose (mg/kg)	Effective no. of animals	Tumor bearing animals	Rate (%)	Survival time (days)	Animal with tumors of (%)		References
p.o.	2 × 3.5	140	16	13	81	170 ± 13	Fore-stomach*	100	[5]
							Others	0	
p.o.	2 × 1.75	70	20	18	90	184 ± 22	Fore-stomach*	100	[5]
							Others	0	
i.v.	1 × 5.0	125	25	23	92	189	Lung*†	76	
							Heart‡	60	[6]
							Others	12	
i.v.	1 × 2.5	90	37	36	97	258	Lung*†	82	
							Heart‡	41	[6]
							Others	11	
s.c.	2 × 2.5	152.5	35	35	100	229	Subcutis§	69	
							Lung*†	69	[6]
							Others	9	
i.p.	1 × 13.2	13.2	30	30	100	126–483	Small intestine†	83	[7]
							Others	50	
i.r.¶	2 × 3.5	112.0	109	107	98	230	Colon†‖	98	
							Others	1	[6]

* Squamous cell carcinomas.
† Adenocarcinomas.
‡ Rhabdomyosarcomas.
§ Sarcomas.
‖ Undifferentiated sarcomas.
¶ Intrarectal.

MATERIALS AND METHODS

NAMM and ^{14}C-labelled NAMM were synthesized as described previously [8, 9]. Solvents were of analytical grade. Extrelut®was purchased by Fa. Merck, Darmstadt, G.F.R. Heparin-Riker 5000®was used as anticoagulate. For gas chromatographic analysis, a Pye Unicam 104 gas chromatograph (Philips GmbH, Kassel, G.F.R.) equipped with a flame ionization detector and a Thermal Energy Analyzer (TEA, Thermo Electron Corp., Waltham, U.S.A. were used. GC/MS analysis was performed on a LKB 9000 mass spectrometer (Bromma, Sweden), coupled to a Pye Unicam 104 gas chromatograph. The radioscans were recorded with a Berthold scanner LB 242 k (Berthold, Wildbad, G.F.R.)

Determination of the half life of NAMM in water. From a solution of about 1 mg NAMM in 1.0 ml water maintained at 37°, aliquots of 1 μl were injected in the GC at different times compared to an external NAMM standard. Separations were performed on a glass column (2.2 m × 0.4 mm i.d.) packed with 3% Carbowax 20 M TPA on Gaschrom Q (80–100 mesh). Injector temperature was 200°, the detector temperature 300° and the oven temperature 160°. The carrier gas was nitrogen, at a flow rate of 30 ml/min. The amounts of NAMM were plotted against the time, and the half life was calculated as shown in Calculations.

Determination of half life of NAMM in serum and in blood in vitro. Male SD rats weighing about 250 g, fasted overnight, were injected with 1000 I.E. heparin and exsanguinated. Suitable amounts of NAMM were added to 5–10 ml of blood (serum), stirred at 37°, in order to obtain an inital concentration of 0.2 mg NAMM/ml in the reaction mixture. Aliquots of 100 μl were taken at different time intervals and pipetted into 2 ml of dichloromethane in a separating funnel.

Water (900 μl) was added to each separating funnel, containing 100 μl of the reaction mixture and 2 ml of dichloromethane. After shaking, the organic phase was separated and filtered over a cotton wad. Extraction of the aqueous phase was repeated with 2 ml of dichloromethane. The extracts were combined and concentrated to a volume of 0.2 ml in a graduated vial under a stream of nitrogen. The amounts of NAMM in the samples were determined gas chromatographically with a TEA detector. For confirmation of the results, two samples were examined by GC/MS; the mass spectra of the eluting peak proved that the original NAMM was detected by the TEA detector (Fig. 2).

In separate recovery experiments, defined specific amounts of NAMM (20 μg/20 μl H_2O) were added to a mixture of 100 μl of blood, 900 μl of distilled water and 2 ml of dichloromethane. After isolation and gas chromatographic determination, the median recovery of NAMM was found to be 64% (from 9 observations, ranging from 61 to 67%). This value was taken to correct for losses of NAMM during clean up of the samples. The half life was calculated as shown in Calculations.

Determination of half life of NAMM in vivo. Male SD rats, weighing about 200 g, were injected with 1000 I.E. heparin and anesthetized with diethylether. A cannule was introduced into the plexus orbitalis, while they were simultaneously injected with a solution of NAMM (5 mg/0.5 ml water) into the tail vein. The end of the injection procedure was taken as t_0. Periodically 4 drops of blood (approximately 120 μl) were collected in a separating funnel containing 2 ml of dichloromethane. The clean up of the samples was performed according to the methods described for the *in vitro* experiments. The amount of NAMM in the samples was determined gas chromatographically with the TEA detector. The half life was calculated as shown in Calculations.

Gas chromatographic determination of NAMM with the TEA-detector. Gas chromatographic separations were carried out on a glass column (1.2 m × 2.0 mm i.d.) packed with 10% Carbowax 20 M – Therephthalic acid on Gas Chrom Q (80–100 mesh). The temperature of the column oven was

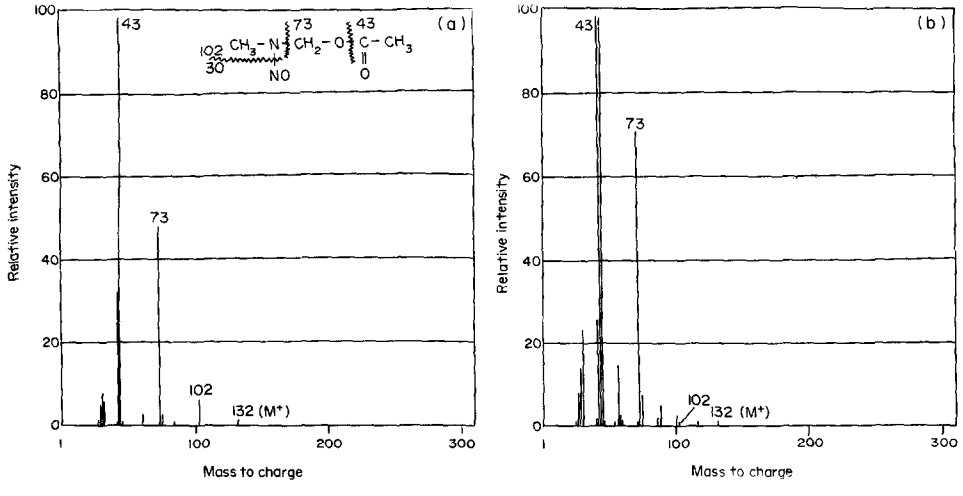

Fig. 2. Mass spectra of NAMM. (a) shows the standard spectrum, and (b) the spectrum after isolation from blood and GC separation.

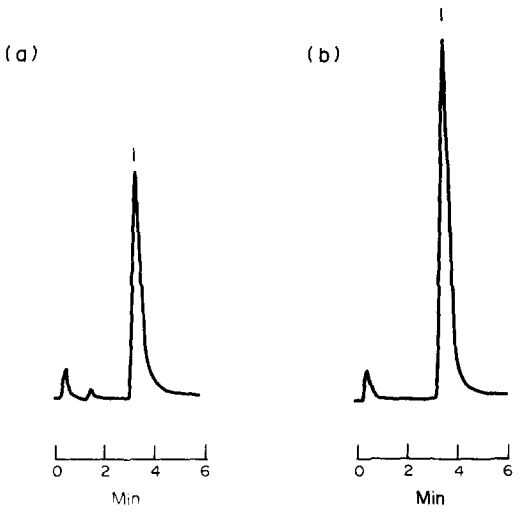

Fig. 3. Typical GC-TEA-chromatogram. a = standard: 4 μl containing 20 ng of NAMM (1). b = sample: 4 μl of a blood extract containing 31 ng of NAMM (1).

150° for 10 sec and was then programmed to rise by 10°/min to 200°. The injection port temperature was 200° and the carrier gas was helium at a flow rate of 30 ml/min. Aliquots of 4 μl were injected into the gaschromatograph. A typical chromatogram is shown in Fig. 3.

Determination of the amount of NAMM exhaled. Two male SD rats of about 150 g fasted overnight were injected i.v. with 5 mg/kg NAMM in 0.9% NaCl. They were placed in a desiccator. The expired air was sucked for 7 hr through a trap with 100 ml dichloromethane and a trap with conc. H_2SO_4. (Control experiments confirmed that NAMM completely remains in dichloromethane under the conditions applied.) After evaporation under a nitrogen stream to a volume of 1.0 ml, the residue was analyzed by GC/TEA and GC/MS.

Determination of ^{14}C-NAMM in urine. Sixteen male SD rats with an average weight of 130 g were injected i.v. with a solution of 10.4 mg ^{14}C-NAMM (5 mg/693 μCi/kg) in 0.9% NaCl. The urine was collected in a vessel cooled with a dry ice/methanol mixture for a period of 30 hr. The yield of 55 ml was diluted with ethanol (500 ml), filtered and evaporated to a volume of 21.1 ml. This concentrate was extracted three times with dichloromethane (total 75 ml) dried over Na_2SO_4 and evaporated. A thin-layer chromatogram on silica gel (solvent: dichloromethane 100; diethylether 70; *n*-hexane 50) shows seven points in the u.v.-light (254 nm) and two peaks in the radioscan. The peak with an r_f-value of 0.89 corresponds to the peak of the origin compounds on the same plate.

* In a control experiment, the half life of NAMM was measured in serum deactivated through heat pretreatment (100°, 5 min). The resulting value of 26 hr compares more to the half life in water (35 hr) than to the decomposition value of NAMM in serum (3.5 min).

Table 2. Half life of NAMM in different media

Medium	Half life	$\bar{M}$*
H_2O	32 hr; 40 hr	35 hr
Serum	3.8 min; 3.0 min; 4.6 min	3.5 min
Blood *in vitro*	0.8 min; 0.7 min; 2.7 min	1.6 min
In vivo	12 sec; 19 sec	16 sec

* $\bar{M}$ = weighted mean.

Determination of NAMM in urine. 15 male SD rats with an average weight of 110 g were injected i.v. with a solution of 8.25 mg NAMM (5 mg/kg) in 0.9% NaCl. The urine was collected in a vessel cooled with a dry ice/methanol mixture for a period of 24 hr. The yield of 90 ml was divided in 5 portions and poured over columns (120 × 25 mm) filled with Extrelut—an inorganic matrix for liquid/liquid partition chromatography—and extracted 5 times with 30 ml dichloromethane. The combined organic phases were evaporated to 1.0 ml and analyzed for NAMM with GC/TEA.

CALCULATIONS

Assuming that the decomposition of NAMM *in vitro* follows an exponential law, we fitted a linear regression line for logarithms of concentration on time. From this the half life was estimated to be that time at which a reduction of concentration to the half had taken places. A confidence interval for the half life was derived through the usual confidence region around the fitted regression line. The *in vivo* assay needs a separate mode of calculation of the half life. As explained later, an increase and a decrease of the NAMM concentration in the blood can be observed simultaneously. This situation is usually described by a so called 'Bateman' function [10]. Thus the half life was calculated from the estimated parameters of a Bateman function. These estimates were derived by non-linear regression methods.

RESULTS AND DISCUSSION

The results on the half life of NAMM in various systems are shown in Table 2. The decomposition of NAMM in water due to spontaneous hydrolysis is relatively slow at pH 7.4. In contrast to the results in water, the half life in serum is much shorter. Therefore the spontaneous hydrolysis of NAMM can be neglected in the following discussion.

It is probable that the accelerated cleavage in the serum is caused by esterases.* This correlates with the findings from Roller *et al.* [11], who describes the decomposition by hog liver esterase in phosphate buffer at pH 7.0 with a half life of 40.4 min at 21°.

In blood the velocity of decomposition is twice as fast as in serum. This may be caused by a loss in activity during the preparation of the serum, which could be explained by the removal of the erythrocytes and the enzyme rich granulocytes, and also by deactivation during the preparation.

In the *in vivo* experiment, the decomposition is overlapped by a distribution effect. We therefore

detect increasing amounts of NAMM within the first seconds after application. After 11 sec the maximal value was achieved (23% NAMM of theorie). Therefore the half life had to be calculated according to the Bateman function, as shown in Calculations. Since the calculated hydrolysis of NAMM *in vivo* is much faster than *in vitro* ($t_{1/2}$ = 16 sec vs 96 sec), it is unlikely that the non-specific esterases in the blood are solely responsible for this acceleration.

Another effect which must also be considered is the possibility of uptake of NAMM through organs and/or extracellular fluids other than blood. If the distribution of NAMM is similar to that of nitrosodimethylamine [12, 13], a rapid uptake can be expected. Therefore the value of 16 sec indicates the maximal decomposition velocity. The actual velocity could be slower and lie between 16 and 96 sec, as was found for the *in vitro* decay in blood.

The amounts of recovered NAMM in urine and exhalation air are extremely small (<0.01%) and NAMM was not found in all experiments. Nevertheless, it is clear that NAMM is stable enough for a passage through the whole organism even in traces.

The values obtained for the stability of NAMM demonstrate that the decomposition rate is slow enough to enable the distribution of small amounts of unchanged NAMM within the whole organism after an intravenous application.* This is supported by the fact that NAMM was found in the exhalation air and in the urine. The results could mean that the carcinogenic effects of a cleavage of NAMM do not only take place at the application site. It is therefore not cogent to require the transport of intermediates or conjugates. On the other hand we cannot exclude the transport of a stable intermediate or a conjugate to explain the organotropy after subcutaneous and intraperitoneal application.

* The circulation time of blood in the rat is about 5 sec (Frank, Ivankovic, unpublished results).

Acknowledgements—The authors thank Dr. J. Wahrendorf for his help in calculations and statistics, Dr. B. L. Pool for her assistance in preparing the English manuscript, Mrs. A. Reinemut and Mrs. G. Kudlek for her excellent technical work and the Deutsche Forschungsgemeinschaft for the financial support of the project.

REFERENCES

1. P. N. Magee and J. M. Barnes, *Br. J. Cancer* **10**, 114 (1956).
2. H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, *Z. Krebsforsch.* **69**, 103 (1967).
3. P. Czygan, H. Greim, A. J. Garro, F. Hutterer, F. Schaffner, H. Popper, O. Rosenthal and D. Y. Cooper, *Cancer Res.* **33**, 2983 (1973).
4. R. C. Cotrell, B. G. Lake, J. C. Phillips and S. D. Gangolli, *Biochem. Soc. Trans.* **5**, 311 (1977).
5. M. Wiessler and D. Schmähl, *Z. Krebsforsch.* **85**, 47 (1976).
6. M. Habs, D. Schmähl and M. Wiessler, *Z. Krebsforsch.* **91**, 217 (1978).
7. J. M. Ward, J. M. Rice, P. P. Roller and M. L. Wenk, *Cancer Res.* **37**, 3046 (1977).
8. M. Wiessler, *Tetrahedron Lett.* **30**, 2575 (1975).
9. H. Braun and M. Wiessler, *J. labelled Comp.* **14**, 887 (1978).
10. W. H. Wagner, in *Pharmakokinetik* (Eds. E. Gladtke and H. M. v. Hattingberg), p. 137. Springer, Berlin, (1977).
11. P. P. Roller, D. R. Shrimp and L. K. Keefer, *Tetrahedron Lett.* **25**, 2065 (1975).
12. P. N. Magee, *Biochem J.* **64**, 676 (1956).
13. D. F. Heath, *Biochem. J.* **85**, 72 (1962).