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INTRAHEPATIC MUTAGENESIS ASSAY: A SENSITIVE METHOD FOR DETECTING *N*-NITROSOMORPHOLINE AND IN VIVO NITROSATION OF MORPHOLINE

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Summary

An intrahepatic host-mediated mutagenicity assay capable of detecting low levels of *N*-nitrosomorpholine (NMOR) is described. The indicator organism was *Salmonella typhimurium* TA1530 which had been injected intravenously 10 min prior to the administration of the test compound. The bacteria were subsequently recovered from the liver and scored for revertants by standard methods. The lower limit of detectibility of this system for intubated NMOR was 0.2 μg/g body weight.

This assay was then used to study the formation of NMOR in vivo from morpholine and nitrite which had been sequentially gavaged to mice. Under acidic conditions (pH 3.4) 12—19% of the administered morpholine was converted to NMOR in the presence of excess nitrite. This nitrosation, and the subsequent uptake and activation of the NMOR, took place so rapidly that most of the total mutagenic response was complete within 15 min. This response was inhibited by prior intubation of ascorbic acid, a known inhibitor of nitrosation, and enhanced by sodium thiocyanate, a nitrosation catalyst.

For many classes of carcinogenic compounds there is a rough correlation between their mutagenicity in in vitro microbial mutagenicity assays and their carcinogenic potency [14]. A notable exception to this observation has been the *N*-nitroso compounds [14,15]. Thus a strongly carcinogenic *N*-nitrosamine is frequently a weak mutagen in such an assay.

We therefore have been actively seeking a microbial mutagenicity test which is more sensitive for low levels of the carcinogenic *N*-nitrosamines than previous tests and which does not substantially increase the complexity of the assay or the time required to complete it. We now report the results of our first efforts in this direction.

The assay chosen employs an intrahepatic host-mediated bacterial mutagenicity test not unlike those which have been reported previously using other detector organisms for other compounds [8,17,20]. As the test compound we chose *N*-nitrosomorpholine (NMOR), both because it is a well-studied member of the *N*-nitrosamine group, and because its precursor, morpholine (MOR), is readily nitrosated by nitrite or airborne NO_x [5]. Considering the widespread use of MOR in industry and its presence as a contaminant in certain foods [22], it seemed that a mutagenicity test which would also measure the extent of such nitrosation in vivo would be extremely valuable. This has been attempted previously using an intraperitoneal host-mediated mutagenicity assay to detect nitrosation of dimethylamine [7] or MOR [4]. However, in neither case was detection of nitrosation of the amine at low levels attempted. The present report describes an intrahepatic host-mediated assay which, when combined with certain other optimization procedures, is capable of detecting in vivo nitrosation of small doses of MOR. This sort of sensitivity increase may ultimately help investigators to determine whether the ingestion of low levels of amines such as MOR may represent a potential health hazard.

Materials and methods

Bacterial strain

A *his*$^-$ auxotroph (TA1530) of *S. typhimurium*, kindly provided by Dr. B.N. Ames (University of California, Berkeley), was used as the mutational indicator. Reversions from histidine dependence to histidine independence were scored as described previously [2]. The genetic markers of this strain were described by Ames et al. [2]. 4 other *S. typhimurium* strains (G46, TA1535, TA92 and TA100A) were found to be less sensitive to NMOR in this assay.

Animals

Female CD-1 mice (Charles River Laboratories, Wilmington, MA) of 6—7 weeks old (weighing between 22 and 28 g) were used throughout this investigation. The animals were starved overnight prior to each experiment.

Chemicals and media

Sodium nitrite and sodium thiocyanate were purchased from Fisher Scientific Co., Fairlawn, NJ. MOR, ascorbic acid and NMOR were obtained from Eastman Chemicals (Rochester, NY), Aldrich (Milwaukee, WI) and Thermo Electron (Waltham, MA), resp.

The culture medium for overnight culturing and the complete and minimal media for measuring survival and reversions, respectively, have been described previously [1,2].

Mutagenicity test

An intrahepatic host-mediated assay was employed throughout this study. Bacterial cells (*S. typhimurium* TA1530) from overnight culture were washed twice by centrifugation in 0.9% saline. The washed cells were resuspended in

saline and adjusted to 2.5×10^{10} cells/ml. 0.1 ml of this suspension was injected into the tail vein of each mouse. 10 min later each animal in the experimental groups received 0.2 ml MOR and 0.2 ml of sodium nitrite sequentially by gavage. Both MOR and nitrite were dissolved in 0.5 M citrate buffer and the pH was adjusted to pH 3.4. Control animals were intubated with nitrite alone, MOR alone, or only the buffer solution. At the indicated times the animals were sacrificed by cervical dislocation. The liver of each animal was immediately removed and homogenized in 2 ml of saline with a motor-driven Teflon homogenizer. The homogenates were filtered through a cotton plug in a Pasteur pipette and, following appropriate dilution, were then plated in triplicate on media designed to determine either bacterial survival or *his*$^+$ reversions [1]. The survival and revertant plates were scored for colonies after incubating at 37°C for 18 and 40 h resp. Results are reported as revertants/10^7 surviving bacteria. Each data point is the average of 6 determinations (duplicate animals run on 3 separate days).

Results

The dose mutagenic response curve for *S. typhimurium* TA1530 cells located in the livers of mice treated with NMOR is shown in Fig. 1. There is a linear log

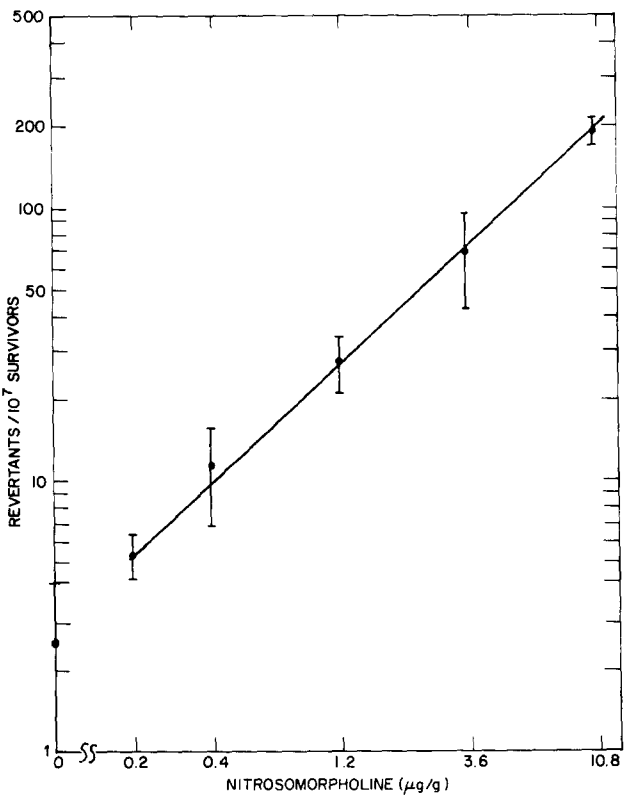
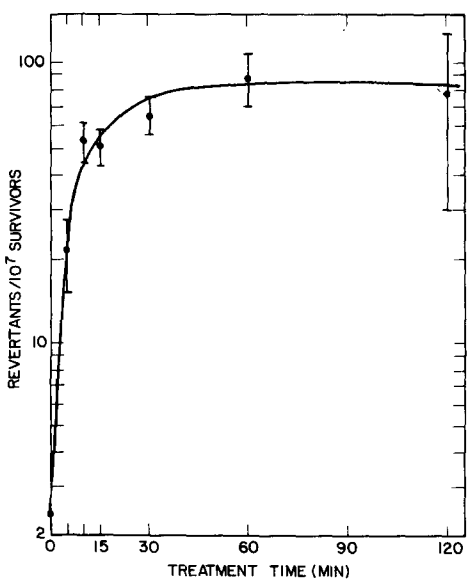


Fig. 1. *his*$^+$ reversion induction of *S. typhimurium* TA1530 located in the livers of mice intubated 2 h previously with *N*-nitrosomorpholine. Each point is the mean ± SD of at least 6 mice.

dose—log response relationship from the lowest dose causing detectable mutagenicity (0.2 μg/g body weight) to at least 10.8 μg/g. At this time (2 h after treatment), the bacterial titre in the livers of untreated animals was 43 ± 12% of the administered dose. There was no significant difference between treated and control animals in terms of bacterial survival. Virtually all these bacteria were trapped within the liver, as the bacterial concentration in heart blood taken from several animals was always less than 1% of the liver concentration.

When animals are injected with sodium nitrite and MOR, there is a well documented non-enzymatic formation of NMOR in the stomach [12,13,19,21]. This nitrosated product is quickly absorbed and converted to a mutagenic metabolite(s), as shown by the rapid increase in reversion frequency during the first 10 min of the experiment (Fig. 2). Nitrosation, absorption and activation are essentially complete within 30 min.

The dose dependence of this reaction is evident from the increase in the *S. typhimurium* TA1530 reversion level in animals dosed with a fixed amount of sodium nitrite (120 μg/g) and variable amounts of MOR (Fig. 3). No mutagenic activity above the background level was observed when MOR or nitrite was intubated alone. However, when dosed in combination with nitrite, MOR caused an increase in reversion frequency which was 58 times the background level at the highest dose tested (40 μg/g). Even the lowest MOR level tested (4 μg/g) caused a 9-fold increase in *his*$^+$ reversions over the background level. By comparing the reversion frequencies following the direct administration of various amounts of NMOR (Fig. 1) with those in Fig. 3, it is possible to estimate the extent of conversion of MOR to NMOR under these conditions,



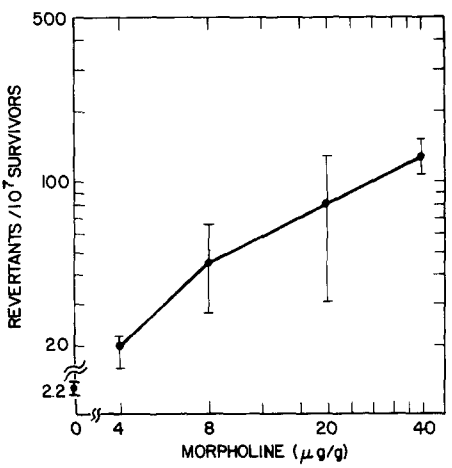


Fig. 2. Time course of *his*$^+$ reversion induction of *S. typhimurium* TA1530 located in the livers of mice previously intubated with 20 μg/g MOR and 120 μg/g sodium nitrite (bars = SD).

Fig. 3. *his*$^+$ reversion induction of *S. typhimurium* TA1530 located in the livers of mice 2 h after intubation of sodium nitrite (120 μg/g) and morpholine (bars = SD).

TABLE 1

ESTIMATION OF IN VIVO NITROSATION OF MOR BY NITRITE VIA THE INTRAHEPATIC HOST-MEDIATED ASSAY

Precursor		Amount of MOR nitrosated (μg/g)	% MOR nitrosated
MOR (μg/g)	Sodium nitrite (μg/g)		
4	120	0.62	15.5
8	120	1.59	19.8
20	120	3.29	16.4
40	120	4.94	12.3

which were designed to maximize yields. As shown in Table 1 between 12 and 19% of the MOR is nitrosated under these conditions. When the pH is not controlled the amount of MOR nitrosated is much more variable and generally lower (data not shown), probably due to the fact that both the MOR and $NaNO_2$ solutions can significantly raise the stomach pH (I.S. Krull and K. Mills, personal communication).

To further demonstrate the relationship between the extent of MOR nitrosation and bacterial reversion frequency, animals were gavaged with a known catalyst of nitrosation (sodium thiocyanate) or an inhibitor of nitrosation (ascorbic acid). As shown in Table 2, a 120 μg/g dose of sodium thiocyanate caused a significant increase in the number of intrahepatic revertants over controls dosed with nitrite and MOR alone. Lower doses (4 or 20 μg/g) of thiocyanate did not cause a significantly increased revertant response, possibly because the molar ratio of thiocyanate to nitrite (resp., 1 : 40 or 1 : 8) may have been insufficient to form enough of the nitrosating intermediate, NOSCN [9], to cause such an effect.

Gavage of ascorbic acid caused a dose-related inhibition of reversions, as shown in Fig. 4. This is not unexpected in view of the well-documented inhibitory effects of ascorbic acid on nitrosation due to its affinity for nitrite [3]. At

TABLE 2

EFFECT OF THIOCYANATE ON THE MUTAGENICITY OF MOR PLUS NITRITE IN *S. TYPHIMURIUM* TA1530 WITH THE INTRAHEPATIC HOST-MEDIATED ASSAY [a]

Concentration of MOR (μg/g)	Concentration of sodium nitrite (μg/g)	Concentration of sodium thiocyanate (μg/g)	Revertants/10^7 survivors (mean ± S.E.)
0	0	0	2.3 ± 0.07
4	0	120	2.9 ± 1.2
0	120	120	2.3 ± 0.8
4	120	0	10.0 ± 3.0
4	120	4	17.9 ± 4.6
4	120	20	18.4 ± 5.6
4	120	120	43.9 ± 11.2

[a] MOR, nitrite and thiocyanate were prepared in citrate buffer, pH 2.6.

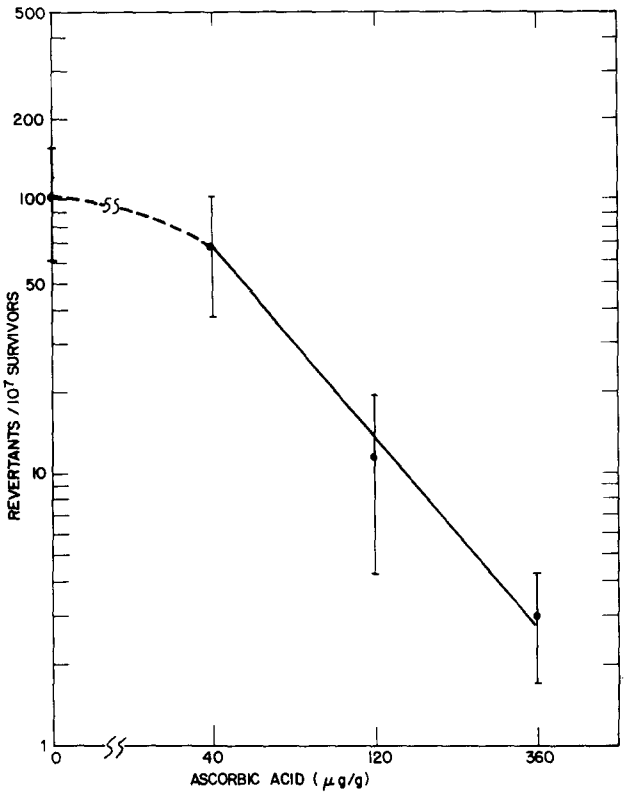


Fig. 4. Effect of ascorbic acid on the induction of *his*$^+$ reversions of *S. typhimurium* TA1530 in the intrahepatic host-mediated assay. The ascorbic acid was administered by gavage immediately after morpholine (40 μg/g) and before sodium nitrite (120 μg/g). The bacterial cells were recovered 2 h after dosing (bars = SD).

a dose (360 μg/g) slightly more than equimolar to nitrite (1.08 : 1), the ascorbic acid completely inhibited in vivo nitrosation, as the reversion frequency (3.0 ± 1.3) was insignificantly above the rate in control groups given only MOR plus ascorbic acid (2.0 ± 0.9 revertants/10^7 survivors) or nitrite plus ascorbic acid (2.5 ± 1.0).

Discussion

In this report we have described a sensitive host-mediated system for detecting NMOR and in vivo nitrosation of MOR. We believe this sensitivity increase is the result of a number of factors. The NMOR-detecting organisms are located inside the liver rather than in the intraperitoneal cavity, as was the case in most previously reported studies [4,26]. Thus the bacteria are closer to the source of the "activated" (mutagenic) metabolite(s) of NMOR, which is thought to be made most actively in the hepatocytes [16]. To further test this hypothesis, we compared mutagenicity levels in bacteria located intraperitoneally vs. intrahepatic bacteria in the same animals. The latter indicator cells exhibited a 10-fold higher reversion frequency than the former (n = 3).

A second possible source of increased sensitivity could be the fact that the MOR and nitrite were gavaged in buffered acidic (pH 3.4) solutions. Nitrosation is known to proceed most rapidly at this pH [9]. We also used a large excess of nitrite compared to MOR. This was done because nitrite is rapidly absorbed from the stomach [10,18] and because the rate of nitrosation is related to the square of the nitrite concentration [9].

These optimization procedures have enabled us to detect in vivo nitrosation of MOR at lower doses and in higher yield than in previous studies using mutagenicity assays. However, the yield figures (12—19%) are only estimates because the mutagenic effects of directly gavaged NMOR were used to calculate the extent of nitrosation of MOR. While the time course of mutagenic response to MOR + NO_2^- and NMOR are very similar (data not shown) there is no assurance that the physiological response to the two is sufficiently similar to be able to directly relate them. For example, there may be an impaired response to NMOR formed from the precursors, as nitrite has been reported to have a direct microsomal enzyme inhibitory effect which suppresses mutagenicity following administration of nitrosodimethylamine [6].

In a recent study using a chemical method for detecting nitrosation, Krull and co-workers [13] found that intragastric nitrosation of MOR in the presence of excess nitrite was very rapid, with maximum levels of NMOR being present 9 min after intubation. The rapid mutagenic response which we have described here tends to corroborate their work and demonstrate that the subsequent uptake and enzymatic activation of NMOR is also very rapid. The mutagenic response was essentially complete within 30 min of administration of the precursors.

To further test that the in vivo mutagenicity assay was indeed measuring the nitrosation product of MOR, we examined the response in the presence of well-known modifiers of nitrosation. Ascorbic acid is known to compete with MOR for the available nitrite, thus inhibiting nitrosation [3,19]. As predicted, we found a dose—response relationship for this inhibitor such that the mutagenicity decreased to background levels when the ascorbic acid level was increased to be approx. equimolar with nitrite. A number of anions have been shown to enhance the rate of MOR nitrosation in vitro and in vivo, with thiocyanate being among the most effective [9,13]. We have also corroborated this observation by showing that thiocyanate does, indeed, increase the mutagenic response to gavaged nitrite plus MOR.

In summary, this report describes in vivo nitrosation of MOR at relatively low levels and the detection of a resultant mutagenic metabolite(s) in the liver. The significance of these findings for humans is uncertain, as it would be unusual to find simultaneous ingestion of MOR with an excess of nitrite. However several investigators [11,23—25] have reported that nitrate ingestion by humans (usually in the form of certain vegetables) resulted in salivary nitrite levels that exceed 100 μg/ml. Thus there is a distinct possibility that simultaneous ingestion of MOR with a source of nitrate could result in nitrosation occurring in the acidic conditions of the stomach.

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