

# Some Factors Affecting the Host-Mediated Assay Response

by Errol Zeiger\*

## Introduction

The host-mediated assay was initially conceived as a system for determining the mutagenic potential of mammalian metabolites of foreign compounds for bacteria (1). In practice, a genetically defined indicator microorganism is injected into the peritoneal cavity of mice which are then administered the substance under test, either orally or parenterally. After a specified time—usually 3 hr—the mouse is sacrificed and the organisms are recovered; they are then handled by standard bacteriological techniques for determination of the mutant frequency. An increase in the mutation frequency above the solvent control indicates an induction of mutation.

In order to further understand the relationship between the host animal's metabolic state and the mutagenic response of the indicator microorganisms, the following study was undertaken.

Three compounds were studied: two carcinogenic nitrosamines, dimethylnitrosamine (DMNA) and *N*-nitrosomorpholine (NM), which need metabolic activation for their mutagenic and carcinogenic activities (1-7) and the nitrosamide *N*-nitroso-*N*-methylurea (NMU), which does not require metabolic

activation for expression of its mutagenic and carcinogenic properties (8-12).

The mice used as the host animals were subjected to a number of nutritional variables, such as 24 hr starvation, an all-casein diet, and a protein-free diet, which have documented effects on DMNA metabolism. In addition, the potent inhibitor of DMNA metabolism, aminoacetonitrile (AAN), was also employed, and the subsequent effects on DMNA, NM, and NMU mutagenicity were measured.

Reconstruction studies with tester strains of the indicator microorganisms, in the presence and absence of DMNA and AAN, were performed to determine whether the appearance of mutants in the host-mediated assay was the result of a true mutational phenomenon or whether it was simply a process of selection for the preexisting mutants in the population.

## Materials and Methods

### Strains

*Salmonella typhimurium* his G-46, a mis-sense mutant, was obtained from Dr. Bruce Ames, University of California, Berkeley. *S. typhimurium* Z-34 (his<sup>+</sup> met<sup>-</sup>) was derived from *S. typhimurium* G-46 by spontaneous mutation.

### Host-Mediated Assay

The host-mediated assay procedures employed were an adaptation of those previous-

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ly published (1,7) and are presented in more detail elsewhere (manuscript in preparation).

The mice were injected IP with 2.0 ml of a suspension of *S. typhimurium* G-46. Where AAN was employed, it was injected subcutaneously (SC) at this time at a dose level of 200.0, 100.0, or 20.0 mg/kg. One hour after the bacterial injection, the mice were injected intramuscularly (IM) with DMNA (20.0 mg/kg), NM (100.0 mg/kg), or NMU (10.0 mg/kg) in saline. The mice were then sacrificed at intervals of 5, 10, 15, 20, 30, 60, and 120 min following mutagen injection, and the bacteriological procedures were performed as previously described (7). The spontaneous reversion frequency was determined at 120 min.

## Results

### Effects of Dietary Variables on the Mutagenic Response

The mice maintained on a complete semi-synthetic diet (SSD) for 6-8 days yielded

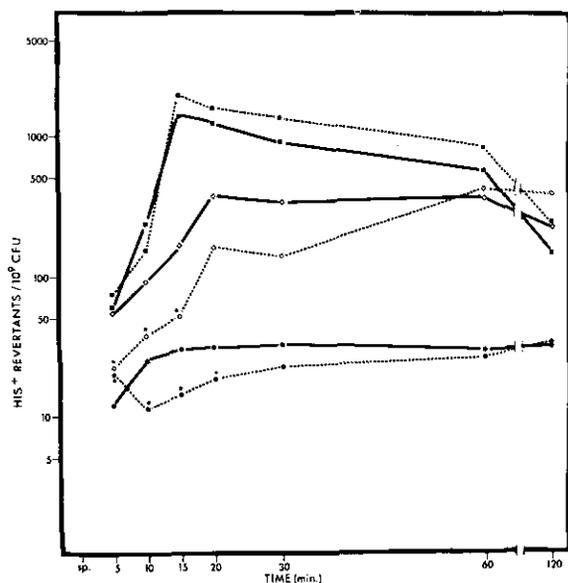


FIGURE 1. Comparison of the mutagenicity of (◇) 20.0 mg/kg DMNA, (●) 100.0 mg/kg NM, and (■) 10.0 mg/kg NMU, in chow (—) and semi-synthetic diet-fed (-) mice. Asterisks (\*) denote  $p < 0.05$  when compared to the corresponding chow diet.

a depressed mutagenic response to both DMNA and NM when compared to chow-fed mice (Fig. 1). The response after DMNA injection was depressed between 5 and 30 min and attained a maximum at 60 min as opposed to the chow-fed maximum response at 20 min. With the exception of the 5 min value, the NM response, like the DMNA response, suggests a depressed exposure of the indicator organisms to the mutagenic compound.

Overall, the effects observed with NMU were contrary to the DMNA and NM results; the mean reversion frequencies at most time intervals were higher in the SSD-fed mice than in the chow-fed mice (Fig. 1), but the differences between the means were not significant ( $p > 0.05$ ).

Maintenance of the mice on a protein-free diet for 8 days resulted in a dramatic depression in the mutagenic response to DMNA when compared to mice maintained on SSD (20% casein) (Fig. 2). NM, however, yielded an initial enhancement of mutagenicity followed by a depression (Fig. 3). Starvation for 24 hr following SSD main-

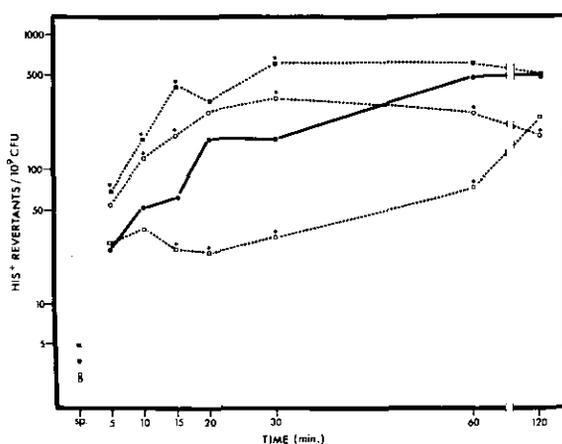


FIGURE 2. Effects of (●) complete semisynthetic diet, (□) protein-free diet (○) complete semisynthetic diet followed by 24-hr starvation and (■) complete semisynthetic diet followed by 24-hr casein diets on the mutagenicity of 20.0 mg/kg DMNA for *S. typhimurium* G-46 in the mouse. sp. denotes spontaneous reversion frequency; the asterisks (\*) indicate  $p < 0.05$  when compared to complete semisynthetic diet.

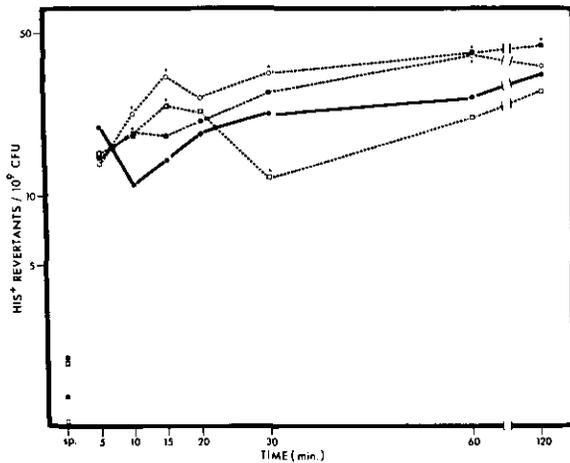


FIGURE 3. Effects of (●) complete semisynthetic diet, (□) protein-free diet, (○) complete semisynthetic diet followed by 24-hr starvation, and (■) complete semisynthetic diet followed by 24-hr casein on the mutagenicity of 100.0 mg/kg NM for *S. typhimurium* G-46 in the mouse. Asterisks (\*), sp. as in Fig. 2.

tenance produced an enhanced mutagenic response to both DMNA (Fig. 2) and NM (Fig. 3). The DMNA response, however, following the initial enhancement, was depressed at 60 and 120 min (Fig. 2). When pure casein, alone, was fed to the mice for 24 hr following SSD feeding, both DMNA (Fig. 2) and NM (Fig. 3) mutagenicity were enhanced; DMNA to a greater extent than NM.

When NMU was administered to mice maintained on a protein-free diet, an erratic response was obtained (Fig. 4). Feeding pure casein alone for 24 hr following SSD maintenance resulted in a dramatic depression in the mutagenic response at all time intervals (Fig. 4). The significantly depressed response is strong evidence for the involvement of a metabolic scheme which has the capability of "detoxifying" NMU.

#### Effects of Aminoacetonitrile Pretreatment on the Mutagenic Response

AAN, administered subcutaneously to the mice 1 hr prior to the injection of DMNA or NM depressed the mutagenic response at all dose levels. The responses obtained with

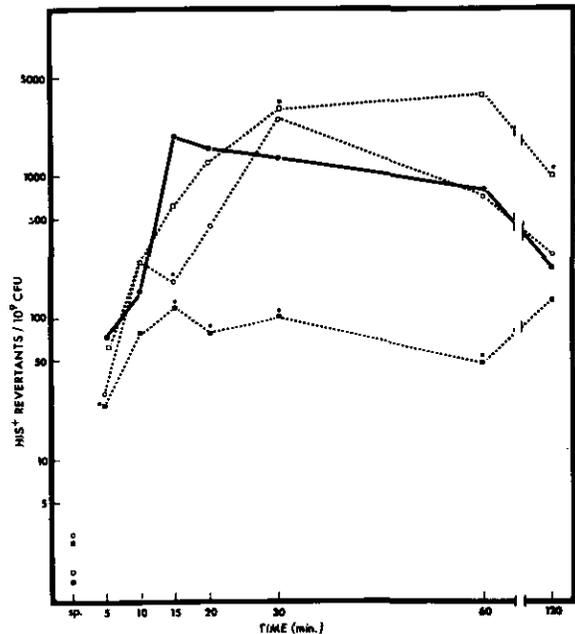


FIGURE 4. Effects of (●) complete semisynthetic diet, (□) protein-free diet, (○) complete semisynthetic diet followed by 24-hr starvation, and (■) complete semisynthetic diet followed by 24-hr casein on the mutagenicity of 10.0 mg/kg NMU for *S. typhimurium* G-46 in the mouse. Asterisks (\*), sp. as in Fig. 2.

DMNA appear dose-related (Fig. 5). The gradual increases in mutation frequency exhibited by 20.0 and 100.0 mg/kg indicate that active mutagen is being released to the cells in the peritoneal cavity at a depressed rate as compared to the untreated mice; the constant, low-level, mutagenic response seen after 200.0 mg/kg AAN may be the result not of liver metabolism, but of non-hepatic activation of DMNA.

The absence of a dose-relationship in the mice challenged with NM (Fig. 6) may be related to the comparatively low mutagenic response obtained in mice not treated with AAN. Therefore, there may not be sufficient latitude between the control NM mutation level and the inhibited mutation levels to effect a dose-related depression.

The level of AAN (200.0 mg/kg) that caused the greatest inhibition of mutagenic response with DMNA and NM yielded the greatest enhancement of NMU mutagenicity (Fig. 7). The expected corresponding dose

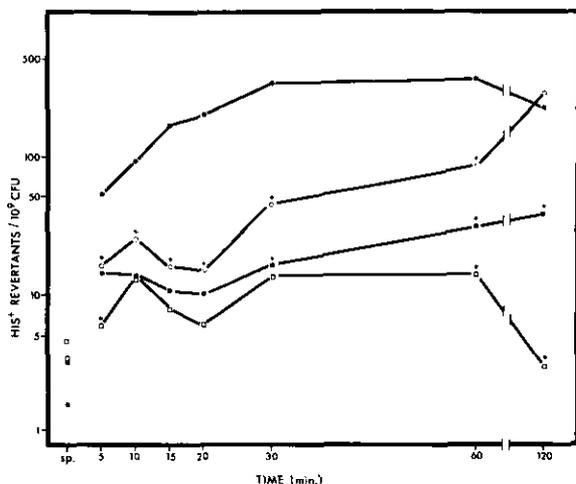


FIGURE 5. Effects of AAN pretreatment on the mutagenicity of 20.0 mg/kg DMNA for *S. typhimurium* G-46 in chow-fed mice: (□) AAN=200.0 mg/kg; (■) 100.0 mg/kg; (○) 20.0 mg/kg; (●) 0 mg/kg. Asterisks (\*) denote  $p < 0.05$  when compared to the next lowest AAN concentration.

effect with 100.0 and 20.0 mg/kg was not seen; both doses yielded peak reversion frequencies at 30 min. The higher mutant frequency was elicited by 20.0 mg/kg; the maximum mutant frequency seen with 100.0

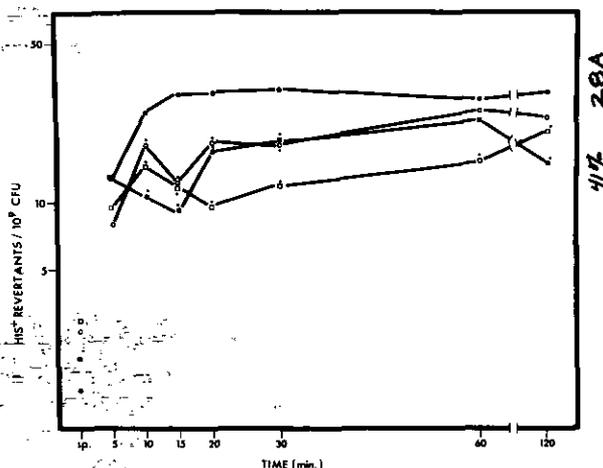


FIGURE 6. Effects of AAN pretreatment on the mutagenicity of 100.0 mg/kg NM for *S. typhimurium* G-46 in chow-fed mice: (□) AAN=200.0 mg/kg; (■) 100.0 mg/kg; (○) 20.0 mg/kg; and (●) 0 mg/kg. Asterisks (\*) denote  $p < 0.05$  when compared to 0 mg/kg AAN.

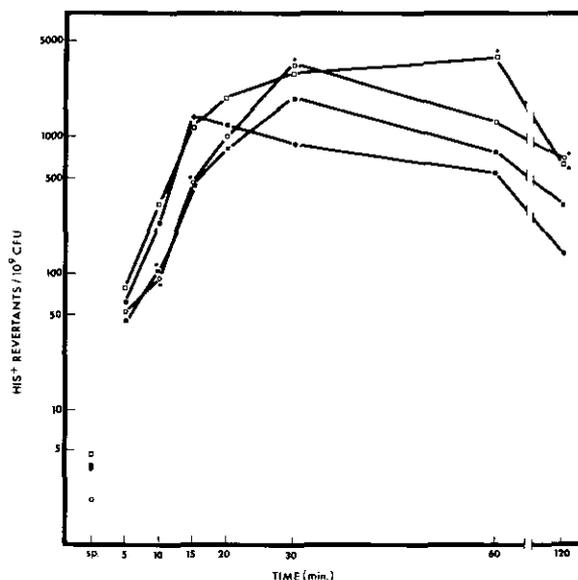


FIGURE 7. Effects of AAN pretreatment on the mutagenicity of 10.0 mg/kg NMU for *S. typhimurium* G-46 in chow-fed mice: (□) AAN=200.0 mg/kg; (■) 100.0 mg/kg; (○) 20.0 mg/kg; and (●) 0 mg/kg. Asterisks (\*) denote  $p < 0.05$  when compared to 0 mg/kg AAN.

mg/kg AAN, although displaced to 30 min, was essentially equivalent to the control.

### Reconstruction Studies

The ratios of *his+ met- / his- met+* (*S. typhimurium* Z-34/*S. typhimurium* G-46) were significantly lower ( $p < 0.05$ ) than the inoculum ratio at 120 min in the untreated group (Table 1). The ratios in the AAN group and the DMNA group did not differ from the inoculum ratio ( $p > 0.05$ ). AAN + DMNA treatment resulted in ratios at 90 and 120 min. that were significantly larger than the inoculum ( $p < 0.05$ ), implying selection for the *his+* revertant.

The determination of the relationship between the treated groups and the inocula is predicated on the inoculum ratio which, in this experimental design, is a constant, lacking variance. The lack of variance, in all probability, is the major factor in the significance of the AAN and DMNA values. In any event, there was no effect over the entire time span of the experiments that

Table 1. Ratios of *S. typhimurium* his+ met- to *S. typhimurium* his- met+ ( $\times 10^{-6}$ ) recovered from the peritoneal cavity of the mouse.

Time after inoculation of cell suspension, min	Ratios <sup>a</sup>			
	Control	AAN	DMNA	AAN + DMNA
0 (Inoculum)	1.99 <sup>b</sup>	1.28	1.82	1.45
60 min	1.91 (1.70-2.13) <sup>c</sup>	1.21 (0.76-1.54)	1.61 <sup>d</sup> (1.40-1.89)	1.70 <sup>d</sup> (1.34-2.17)
90 min	1.79 (1.55-2.06)	1.32 (1.17-1.61)	1.80 (1.26-2.40)	1.70 <sup>e</sup> (1.53-1.92)
120 min	1.69 <sup>e</sup> (1.54-1.84)	1.35 (1.16-1.55)	2.09 (1.78-2.46)	1.55 <sup>e</sup> (1.48-1.62)
180 min	2.03 (1.60-2.67)	1.57 (1.16-2.06)	1.82 (1.51-2.12)	1.77 (1.41-2.09)

<sup>a</sup> Z-34/G-46.

<sup>b</sup> Mean ratio.

<sup>c</sup> 95% confidence limits.

<sup>d</sup> Without DMNA.

<sup>e</sup>  $p < 0.05$  when compared to inoculum.

indicated a selective advantage or disadvantage being conferred upon either the his+ revertant or the parent *S. typhimurium* G-46.

## Discussion

The mutagenicity of a number of carcinogenic nitrosamines for *S. typhimurium* G-46 in the host-mediated assay have been previously reported (1,2,5,7,13). There still remained the need, however, to demonstrate that *in vivo* mutagenicity in the host-mediated assay is truly linked to the metabolic state of the host and not merely to the physical presence of the cells in the animal, and to show that the host-mediated assay can be utilized as a tool to study the metabolism of compounds that are mutagenic.

The results presented here, while not directly indicative of metabolism, demonstrate that factors known to affect the metabolism of DMNA in test animals can also influence its mutagenicity in the host-mediated assay, as well as the mutagenicity of the related *N*-nitroso compounds, NM and NMU.

The reconstruction studies demonstrate that the appearance of his+ revertants of

*S. typhimurium* G-46 in the host-mediated assay is a mutagenic effect rather than the result of selection in the mouse peritoneal cavity (Table 1) and imply that AAN pretreatment depresses DMNA mutagenicity by acting on its metabolism and not on the indicator bacteria.

The significant inhibition of DMNA and NM metabolism by AAN (Figs. 5 and 6) corresponds to the reported inhibitory effects of AAN on DMNA breakdown in rats and its resulting toxic and lethal manifestations (14-18). The depressed DMNA mutation frequency seen at AAN levels of 20.0 and 100.0 mg/kg, indicates a greatly decreased formation (or release) of active mutagens to the indicator cells in the peritoneal cavity.

An interesting effect is the significant difference in the mutagenic responses to DMNA and NM in mice fed a complete semisynthetic diet in place of the commercial chow (Fig. 1). This enhancement of nitrosamine activity by a chow diet had not heretofore been reported, but Brown et al. (19) found that mice and rats fed chow diets had a higher level of 3-methyl-4-monomethylaminoazobenzene *N*-demethylase activ-

ity than animals fed purified diets. Holtzman and Gillette (20) demonstrated an increased level of aniline hydroxylase activity in male rats fed purified diets as compared to chow; but enzyme activity in female rats was unaffected by diet. Marshall and McLean (21) observed that male rats fed a semisynthetic diet had a decreased liver P-450 concentration when compared to rats fed a chow diet. Friedman and Yin (22) reported that the metabolism of aflatoxin B<sub>1</sub> and dimethylaminopyrine in liver slices from male rats maintained on a chow diet was higher than in liver slices from rats maintained on a semisynthetic diet. In addition, McLean and McLean (23) observed an increase in the oral LD<sub>50</sub> of carbon tetrachloride in male rats fed a semisynthetic (30% casein) diet as opposed to a stock (chow) diet.

These results demonstrated that the types of diet employed when studying the metabolism and the *in vivo* effects of *N*-nitrosamines, and of other classes of compounds as well, may be an important consideration in the interpretation of the effects reported in the literature.

On the basis of these observations, studies regarding the metabolic effects of protein-free (or other variable) diets should be related to complete semisynthetic diets only, and not to commercial chow diets, as has been done in a number of studies (23-25). For example, an apparent inhibitory effect seen in animals fed a protein-free (semisynthetic) diet when compared to a chow diet, also might be present if a 20% casein (complete semisynthetic) diet were compared to a chow diet.

The depression of DMNA mutagenicity found in protein-depleted mice (Fig. 2) parallels the depression of DMNA metabolism and toxicity previously reported (11,21,26). NM, however, does not present a clear picture, an apparent initial enhancement followed by a depression (Fig. 3). This may be an indication that NM is metabolized by an enzyme system which is independent of DMNA *N*-demethylase.

The data also demonstrate that the enhancement of DMNA *N*-demethylation by

rats, *in vitro*, following 24 hr starvation or 24 hr casein feeding, as found by Venkatesan et al. (27,28) parallels the enhancement of its mutagenicity in the host-mediated assay, although the starvation-induced enhancement only occurred during the initial 30 min and was followed by a depression. The effects reported here can only be related to metabolism in the mouse by inference, since the corresponding metabolic studies were not performed.

NM mutagenicity was likewise enhanced by both dietary regimens, which would indicate that its metabolism is similarly affected (Fig. 3). One would like to see the NM mutagenicity data parallel that of DMNA; 20% casein inducing a higher level of reversion than 24 hr starvation. That this is not the situation may simply be a reflection that the low level of reversion induced by NM, when compared to DMNA, is unsuitable for determination of relatively small effects.

Another possible explanation for the relative insensitivity of NM in situations which generate a marked response in DMNA and NMU mutagenicity might be derived from its dose-response curve. It can be seen (Fig. 8) that the mutagenicity of NM with respect to dose exhibits a gradual rise at the dose level employed (100.0 mg/kg) as opposed to the rapid increases of DMNA (at 20.0 mg/kg) and NMU (at 10.0 mg/kg). Because of this, a change in the effective concentration of NM by either depressing or enhancing its *in vivo* metabolism, would not markedly alter the mutagenic response of *S. typhimurium* G-46. DMNA and NMU, on the other hand, would exhibit relatively large responses to minor changes in their effective concentrations at the dose levels employed.

The results obtained with NMU in these studies indicate that it did not respond as an unmetabolized control would be expected to. It can be seen that, with the exception of 24 hr starvation, NMU appears to respond in a contrary manner to DMNA and NM: that is, when their mutagenicity is inhibited, NMU mutagenicity is enhanced and vice

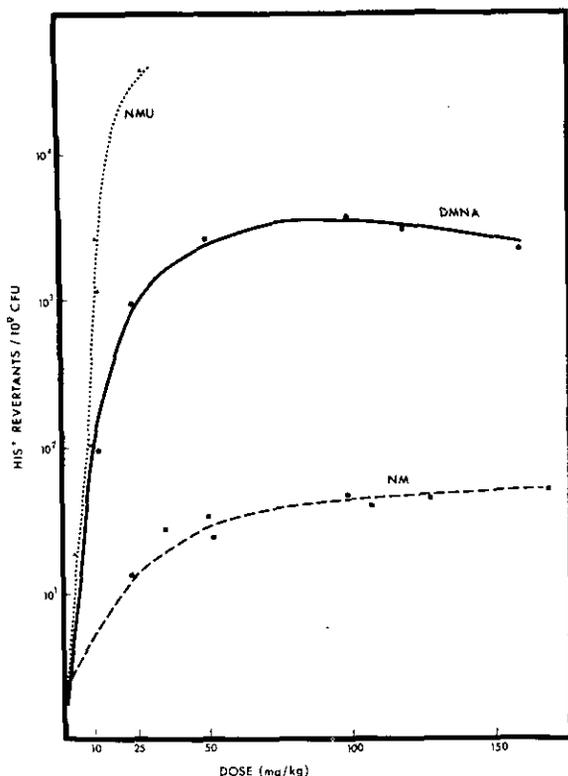


FIGURE 8. Mutagenicity of (●) DMNA, (■) NM, and (▲) NMU for *S. typhimurium* G-46 in the host-mediated assay.

versa. The prolonged mutagenic response seen with AAN treatment and the no-protein diet, while not as clear cut as the inhibition of DMNA, nevertheless, is definitely an enhanced response, possibly due to a lengthened half life of NMU in the mouse.

Also, the marked depression elicited by the 24 hr casein diet which enhances DMNA and NM metabolism and mutagenicity is clearly an inhibition of NMU activity. The apparent detoxification of NMU may be a result of the capability of the liver microsomal oxidase system to metabolize NMU to a nonmutagenic form. Therefore, conditions which enhance its activity, and the mutagenicity of DMNA, such as a 24-hr casein diet, would coincidentally accelerate the breakdown of NMU to a nonmutagenic form. Alternately, depression of this system, by pretreating the mice with AAN or a protein-free diet, could prolong the half

life of the mutagenically-active parent compound, NMU. Whatever the mechanism, the data demonstrate the ability of the metabolically competent mouse to detoxify a mutagenic substance *in vivo*.

The host-mediated assay, because of its design, is relatively insensitive. The proximal mutagen must reach the indicator microorganisms in the peritoneal cavity in sufficient quantity to produce a positive response. Obviously, a large number of factors contribute to the mutagenicity—or lack of mutagenicity—of a compound in the host-mediated assay. These factors are the sum of all the processes which affect the availability of an active compound to the indicator bacteria in the peritoneal cavity. They include: metabolism; tissue distribution; permeability of cell membranes; environmental factors such as nutrition and maintenance conditions; and genetic factors such as animal species, strain and sex. A change in any one of these factors may be sufficient to significantly affect the observed mutagenic response to a specific chemical or class of chemicals.

In addition, a highly active compound may interact with the body tissues so that sufficient active compound might not be available to the peritoneal cavity. The compound may also be too toxic to allow an adequate dose to be given the host animal so that a mutagenic dose can be distributed to the indicator microorganisms. These effects have been observed with both alkylating and intercalating agents (E. Zeiger, unpublished data).

This provides a rationale for bypassing the host mediated assay with compounds active *in vitro*. If a substance is mutagenic *in vitro* and also in the host-mediated assay, no new information is gained from the *in vivo* data since comparative *in vitro-in vivo* quantitation is not possible. On the other hand, if a compound active *in vitro* is not active or only marginally active *in vivo*, a number of explanations are possible, as described above. The concept of metabolic “detoxification”, while attractive, is not indicated in the absence of additional informa-

tion. As has been demonstrated with NMU, detoxification probably is manifested as a quantitative change rather than an all-or-none effect.

The results described here have been interpreted primarily as they might relate to the liver microsomal enzyme system and specifically the oxidative enzymes. Alternatively, the nitrosamines could be metabolized in the liver by additional mechanisms which may not be associated with the microsomal fraction or with the liver. They may also be anaerobically metabolized to the corresponding hydrazides, as was demonstrated for NM by Suss, using a guinea pig liver homogenate under  $N_2$  (29). The mutagenic and carcinogenic effects of a number of hydrazides (30-34) suggest consideration of this alternative.

This is why it is important to relate the mutagenicity of a chemical in the host-mediated assay to the host's metabolic capabilities as defined by *in vivo* and *in vitro* enzyme studies. In fact, this should be a general principle with regard to all *in vivo* toxicological effects, as pointed out by Burns et al. (9) when attempting to calculate "no-effect" levels in laboratory animals and humans. This concept has been demonstrated by the Weisburgers (35) with regard to carcinogens, i.e., that the "no-effect" level in any carcinogenic study is a function of the test system employed, and an alteration in this artificial test system can, and does, reflect an alteration in the dose-response of the animals under test.

This phenomenon has now been demonstrated in regard to the mutagenicity of *N*-nitroso compounds in the host-mediated assay. While no attempt was made to obtain a no-effect level in any of the metabolic systems used, it is obvious that the formation (or the persistence) of the proximal mutagens, *in vivo*, can be affected by alteration of the animals' nutritional state, or by chemical means. Therefore, if a no-effect level for the host-mediated assay response to these compounds is derived, it must be under well-defined conditions, with the understanding that it is not an immutable value.

These results also show, when interpreted in light of the available literature, that the formation of the proximal mutagenic derivative of DMNA parallels the formation of its proximal carcinogenic and hepatotoxic derivatives. This leads to the conclusion that all three *in vivo* effects of DMNA: mutagenicity, carcinogenicity, and hepatotoxicity, may be responses to the same chemical substance. Unfortunately, a similar statement cannot be made at this time regarding NM and NMU, since their metabolism has not been adequately studied. However, their mutagenic responses in the host-mediated assay, following various treatments of the mice, have predictive value for long-term toxicity and carcinogenicity studies.

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