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The Mechanisms of Intestinal Absorption of the Carcinogen MNNG (*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine)[1] (42729)

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Abstract. We studied the characteristics and mechanisms of MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) intestinal absorption and the interaction between bile acids and fatty acids and MNNG absorption rate *in vivo* in male Sprague–Dawley rats. We perfused a segment of the proximal small bowel with a physiological solution containing MNNG to assess its basic kinetics and the influence of some physiological and dietary factors on carcinogen absorption. We found that MNNG was absorbed by simple passive diffusion. Transport of MNNG was the highest at pH 6.0. The addition of the bile salt, taurocholate by itself, greatly increased MNNG absorption, while the addition of the long-chain unsaturated fatty acids, oleic and linoleic, decreased the rate of absorption of MNNG. The phospholipid lecithin addition to the perfusate did not change the rate of MNNG absorption. Induction of dietary vitamin A deficiency (serum vitamin A level decreased from 40.9 to 13.7 μg/dl) did not change the absorption rate of MNNG. These studies demonstrate that bile acids, dietary fatty acids, and the pH of the intestinal content can modify the rate of absorption of this carcinogen by the small intestine. Since initial intestinal absorption determines serum levels and subsequent reabsorption and enterohepatic cycling determines long-term lumenal levels, serum levels, and total body content, factors which modify the rate of intestinal absorption of MNNG could also modify its carcinogenicity.

Since MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) is formed by nitrosation of *N*-methyl-*N'*-nitroguanidine in the stomach (1, 2), both its total body concentration and its subsequent intestinal lumenal concentrations would depend on its initial rate of absorption by the small intestine and subsequent intestinal reabsorption and enterohepatic cycling. Since both initial absorption and subsequent enterohepatic cycling depend on the underlying rate and characteristics of MNNG intestinal absorption, we investigated the influence of intestinal pH, bile acids, and fatty acids on the absorption rate of MNNG using well-established and validated intestinal perfusion methods (3–5). In addition, since vitamin A has been shown to decrease incidence of some tumors in man (6) and to counteract the effects of some carcinogens (7), we also investigated the possible influence of dietary vitamin A intake on the intestinal absorption rate of MNNG.

Materials and Methods. The basic intestinal perfusion solution contained 0.1 m*M* MNNG and tracer amounts of inulin (a nonabsorable marker to correct for nonspecific fluid shifts) in a Krebs phosphate buffer at pH 6.0. Changes in the perfusate were made by modifying the pH or MNNG concentration, adding fatty acids, sodium taurocholate, or lecithin to the perfusate solution (3).

Male Sprague–Dawley rats ranging in age from 4 to 8 weeks were given free access to feed (Lab-Blox, 8604-00, Wayne Laboratory Animal Diet) and water. In order to study the role of dietary intake of vitamin A, a separate group of rats was given either a control diet composed of vitamin-free casein 20%, sucrose 32.5%, DL-methionine 0.3%, corn starch 32.5%, alphacel 5.0%, corn oil 5.0%, mineral mix 3.5%, vitamin mix 1.0%, and choline bitartrate 0.2% (ICN Nutritional Biochemicals) or the same diet without vitamin A.

After ether anesthesia, an inflow tube was inserted into the small bowel distal to the bile

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duct entry site. Encircling ligatures prevented the entry of endogenous bile into the perfused segment. An outflow glass cannula was then inserted 32 cm distal to the inflow tubing. The jejunal segment was flushed with 10 ml saline followed by 10 ml air. The peritoneal cavity was then closed and the rat was placed in a Plexiglas restraining cage and allowed to awaken. The rat's body temperature was maintained at 37°C by a forced air heating unit attached to a feedback temperature controller. A syringe pump was used to infuse the perfusate solution at a constant flow rate of 0.5 ml/min. Samples of the perfusate were taken and volumes were measured at 20-min intervals for a period of 2 hr. At the end of the experiment the perfused intestine was dried for 48 hr with a 5-g weight suspended at the dependent end of the segment to ensure constant degree of stretching (3–5). The length and weight of the dried segment were recorded.

Triplicate 100-μl aliquots were obtained from each 20-min collection of the perfusate and placed in separate scintillation vials containing 4 ml of Ultra-fluor (National Diagnostics). All counts were carried to 1.5% counting error. [^{14}C]MNNG and [^{3}H]inulin counts were separated using standard curves prepared by counting of a series of correspondingly labeled toluene standards in both channels. ^{14}C spillage into the ^{3}H channel averaged 10 ± 2% while the reverse spillage was negligible. All ^{3}H counts were corrected for ^{14}C spillage prior to absorption calculations. The amount of MNNG absorbed was calculated from its steady-state disappearance rate from the perfusate using standardized methods and calculations (3–5). The final data were expressed per unit weight and length of the bowel since there is a lack of agreement as to the ideal denominator for expressing intestinal absorption data. The dilution or concentration of each sample secondary to intestinal secretion or absorption of water was corrected by the nonabsorbable indicator [^{3}H]inulin (4, 5). The 20-min absorption values from each animal were combined with values of absorption of other animals in each specific absorption experiment and a final mean ± SEM for each experimental condition was tabulated (3–5). Statistical comparison was evaluated with Student's *t* test.

Results and Discussion. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine is a well-established gastrointestinal carcinogen. Following oral administration MNNG can induce tumors in the stomach and the small intestine (8–11). When given rectally to both germ-free and conventional rats either alone (12) or together with promoters such as cholesterol, cholesterol metabolities, and bile acids (13, 14), MNNG has been shown to cause colonic carcinomas as well. Man may be exposed to MNNG-like compounds through lumenal nitrosylation with dietary nitrite of naturally occurring guanidine compounds such as L-arginine or creatine. This could produce MNNG-like carcinogenic compounds in the presence of acid in the stomach (1, 2, 15).

Direct gastric mucosal exposure to MNNG could result in tumor formation probably without requiring systemic absorption of the compounds (11). However, the formation of tumors in other locations such as the small bowel, colon, liver, or abdominal mesentery does require intestinal absorption of MNNG followed by exposure of the tissues through the circulation as well as from the lumenal side. The intestinal absorption of MNNG will determine both its initial and subsequent serum concentrations as MNNG undergoes enterohepatic cycling and its long-term lumenal concentration (16). Thus, the absorptive mechanisms and intralumenal factors which modify MNNG absorption may affect the carcinogenicity of MNNG or compounds similar to it, by modulating the initial rate of intestinal absorption and subsequent reabsorption and enterohepatic cycling.

We used a well-defined and carefully studied perfusion model in our laboratory (3–5) in order to study the intestinal absorption of MNNG in the unanesthetized rat. We perfused the proximal small intestine with MNNG in a wide range of concentrations (2.25 μM–1 mM). The absorption rate of MNNG was linear with its perfusate concentration (Table I) indicating that passive diffusion of MNNG is the predominant mechanism of its intestinal absorption. Since passive diffusion is primarily driven by the concentration of MNNG in the intestinal lumen our findings indicate that the small intestine is unable to restrict the absorption

TABLE I. INFLUENCE OF MNNG CONCENTRATION ON ITS ABSORPTION RATE

MNNG concentrations (M)	No. rats	MNNG absorption	
		nmole/g/hr	nmole/100 cm/hr
10^{-3}	3	43,505.9 ± 2691.7	49,406.9 ± 15,725.1
10^{-4}	3	2,724.3 ± 122.0	4,355.5 ± 789.3
2.25×10^{-6}	3	52.6 ± 2.6	85.9 ± 2.6
		$r = 0.99$	$r = 0.99$
		Intercept = −838	Intercept = −304

Note. Values are means ± SE. Six samples were assayed per rat with the final value for absorption being the mean of all values in each series. The slope, intercept, and r values were derived by least-squares analysis of absorption rate versus concentration.

of MNNG with increasing lumenal concentrations. This finding would predict that carcinogenicity would be dose dependent over a wide range of concentrations and fits well with epidemiological and experimental data available from a wide range of studies (1, 2, 8–14, 17, 18).

We investigated the influence of intralumenal pH on the intestinal absorption of MNNG. Because MNNG is susceptible to decomposition in alkaline pH of 7.5 or above (1), we performed our experiments at pH 6.5 or below. Intestinal absorption rate of MNNG was maximal at lumenal pH of 6.0 (Table II). MNNG absorption decreased at more acidic pH of the perfusate probably due to partial ionization of MNNG at lower pH (18). Ionization of MNNG will prevent its absorption through the lipid pathways and diminish its rate of intestinal absorption. This finding suggests that even though nitrosoguanidine compounds are formed in the stomach in an acidic medium, their systemic absorption may be more efficient at the more alkaline environment of the small intestine. Thus, small intestinal absorption of MNNG-like compounds may be an important factor in the general carcinogenicity of these compounds and could explain tumor formation in areas other than the stomach.

TABLE II. INFLUENCE OF INTESTINAL PERFUSATE pH ON MNNG ABSORPTION RATE

pH	No. rats	MNNG absorption	
		nmole/g/hr	nmole/100 cm/hr
6.5	3	1882.8 ± 72.2	3215.0 ± 128.7
6.0	3	2724.3 ± 122.0*	4355.5 ± 789.3*
5.5	3	1530.0 ± 87.9*	2987.9 ± 207.9*

Note. Values are means ± SE of six determinations per rat with the final value determined by pooling of all the data at each pH.

* $P < 0.05$ when compared to the next higher pH.

High saturated fatty acid diets have been shown to increase the tumor incidence associated with the administration of some carcinogens (18). Therefore, we investigated the influence of fatty acids with different degrees of saturation on the intestinal absorption of MNNG. In this set of experiments we added the bile acid taurocholate in a 10 mM concentration to the basic buffer perfusate at pH 6.0. We compared the absorption of MNNG when solubilized in 10 mM taurocholate micelles to its absorption following the addition of fatty acids and lecithin to the taurocholate perfusate. The absorption rate of MNNG in the presence of the monounsaturated oleic or the polyunsaturated linoleic acid decreased significantly when compared to its absorption with taurocholate only (Table III). Likewise, the natural phospholipid lecithin also diminished the absorption of MNNG on a weight basis (Table III). The most likely mechanisms which could explain the decrease in MNNG absorption following fatty acid additions are increased micellar solubility of MNNG and increased size of the micellar particles. In the presence of bile acids, MNNG with its lipid solubility would partition into the bile acid micelles. In order to be absorbed, MNNG would have to leave the micelles and penetrate the absorptive enterocytes as a monomer. The addition of fatty acids to the intestinal perfusate will cause expansion of the micelles, increase their diame-

TABLE III. INFLUENCE OF LIPID ADDITIONS TO THE INTESTINAL PERFUSATE ON MNNG ABSORPTION RATE

Add lipid	No. rats	MNNG absorption	
		nmole/g/hr	nmole/100 cm/hr
None	3	3353.1 ± 159.7	5646.0 ± 401.3
Oleic acid	3	1718.6 ± 43.7*	3877.8 ± 173.2*
Linoleic acid	3	1961.7 ± 121.8*	3970.3 ± 881.5*
Lecithin	3	2507.8 ± 85.3*	5018.7 ± 143.4

Note. Values are means ± SE of three rats with six determinations per rat. To solubilize the added fatty acid, 10 m*M* sodium taurocholate was added to the perfusate. MNNG concentration was kept at 0.1 m*M* and the perfusate pH at 6.0.

* $P < 0.05$ when compared to absorption rate in the absence of fatty acids.

ter, and increase the micellar affinity for MNNG. These changes would shift the partitioning of MNNG away from the absorptive epithelium to the micelles resulting in the observed decrease in absorption rate. (Table III). The increased micellar size following fatty acid additions would also slow the rate of micellar penetration of the unstirred water layer and would contribute to the overall decrease in absorption rate of MNNG.

Thus, the present experiments indicate that increased unsaturated fatty acid intake could decrease the intestinal absorption rate of carcinogens with high lipid solubility. This observation is not in conflict with the known metabolic effects of high fatty acid diets which could increase tumor incidence by mechanisms that are not connected with the intestinal absorption rate of the carcinogens. Thus, our observation of decreased absorption of MNNG following the addition of unsaturated fatty acids may not necessarily diminish the carcinogenicity of a compound if the fatty acids act as promoters because of metabolic interactions between the fatty acids and the carcinogen which is unrelated to the effects of fatty acids on the carcinogen's intestinal absorption rate.

In order to assess the effect of the addition of bile acids to the perfusate by themselves on the absorption of MNNG, we added the common conjugated bile acid sodium taurocholate to the intestinal perfusate. This series of experiments is somewhat artificial since under normal physiological conditions, bile acids would not be present in the intestinal lumen by themselves without some fatty acids. Nonetheless, these experiments were designed to clarify the role of bile acids in the intestinal absorption of MNNG. Taurocholate addition increased the absorption rate of MNNG by the small intestine (Table IV). Since bile acids can change the intestinal permeability characteristics (20–22), and have also been shown to increase the carcinogenicity of MNNG (13, 14), our data (Table IV) suggest that the enhanced carcinogenicity of MNNG by bile acids may be partly due to bile acid-induced increased absorption of MNNG by the small bowel.

Bile acid addition to the intestinal perfusate in the absence of fatty acids (as would take place in the large bowel) could promote

TABLE IV. INFLUENCE OF BILE ACID ON MNNG ABSORPTION RATE

Taurocholate (m*M*)	No. rats	MNNG absorption	
		nmole/g/hr	nmole/100 cm/hr
0	3	1882.8 ± 72.2	3215.0 ± 128.7
10	3	3353.1 ± 159.7*	5646.0 ± 401.3*

Note. Values are means ± SE of six determinations per rat. Studies were done at ph 6.0 and at 0.1 m*M* MNNG concentration.

* $P < 0.01$ when compared to baseline studies without taurocholate.

TABLE V. INFLUENCE OF DIETARY VITAMIN A DEFICIENCY ON MNNG ABSORPTION RATE

Vitamin A serum levels (μg/100 ml)	No. rats	MNNG absorption	
		nmole/g/hr	nmole/100 cm/hr
40.9 ± 5.6	3	1620.2 ± 84.1	3179.1 ± 175.5
13.7 ± 2.3*	6	1315.7 ± 14.9	2546.7 ± 157.7

Note. Values are means ± SE of six determinations per rat. The rats on vitamin A-deficient diet showed clinical signs of vitamin A deficiency after 12 weeks on the diet. Perfusions were done with 0.1 m*M* MNNG at pH 6.0.
* Significant ($P < 0.05$) difference from baseline.

better transport of MNNG across the intestinal unstirred water layer. This layer, which is adjacent to the lipid absorptive cell membrane, is a barrier to diffusion of lipid-soluble compounds. Monomer forms of MNNG would traverse this layer at a much slower rate than would micellar particles containing MNNG (19, 20, 22). By adding bile acids to the perfusate, we allowed MNNG to incorporate into micellar particles and hence to traverse the unstirred water layer at a much faster rate, thereby increasing its rate of intestinal absorption. Thus, bile acids or other detergent molecules would enhance the absorption of MNNG-like carcinogens by the intestine which could be significant even in the absence of fatty acids as would occur in the colon.

Increased dietary vitamin A levels or dietary supplementation with vitamin A precursors, such as beta carotene, has been shown to diminish the incidence of some tumors in man (6, 7) or experimental animals (23–27). We wondered whether the dietary status of vitamin A could also affect the absorption rate of MNNG because of the known requirement of vitamin A for epithelial structure, integrity, and function (16). Therefore, we tested the influence of dietary vitamin A status on the absorption rate of MNNG. We created clinical and biochemical vitamin A deficiency in our animals by feeding them a diet deficient in vitamin A for 3 months. Once the animals became clinically deficient, their serum vitamin A level was found to be less than half of its level in pair fed control animals. When MNNG absorption was assessed in the control as well as vitamin A-deficient animals, no change in the absorption rate of MNNG was found (Table V). Thus vitamin A deficiency does not appear to influence the absorption rate of this carcinogen in rats and could not explain the increase in the incidence of some tumors with vitamin A deficiency.

These studies demonstrate that intestinal absorption of a carcinogen can vary under different lumenal conditions. Bile acids, fatty acids, and the pH of the intestinal lumen can influence the rate of intestinal absorption of some carcinogens. Since initial intestinal absorption and reabsorption during enterohepatic cycling can control the lumenal and serum concentrations and total body accumulation of carcinogens these factors could affect the duration and intensity of exposure of tissues to ingested environmental carcinogens and may play some role in the rate of induced malignancy.

1. Mirvish SS. Formation of *N*-nitroso compounds: Chemistry, kinetics, and *in vivo* occurrence. Toxicology Appl Pharmacol **31**:325–351, 1975.
2. Gibson GG, Ioannides C. Safety Evaluation of Nitrosatable Drugs and Chemicals. London, Taylor and Francis Ltd, 1981.
3. Hollander D. Intestinal absorption of vitamins A, E, D, and K. J Lab Clin Med **97**(4):449–462, 1981.
4. Hollander D, Dadufalza VD, Sletten EG. Does essential fatty acid absorption change with aging? J Lipid Res **25**:129–134, 1984.
5. Hollander D, Rim E, Morgan D. Intestinal absorption of 25-hydroxy vitamin D-3 in the unanesthetized rat. Amer J Physiol **236**:E441–E445, 1979.
6. Moon RC, Grubbs CJ, Sporin MB, Goodman DG. Retinyl acetate inhibits mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea. Nature (London) **267**:620, 1977.
7. Sporin MB, Dunlop NM, Newton DL, Smith JM. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogues (retinoids). Fed Proc **35**(6):1332–1338, 1976.
8. Fujimura S, Kogure K, Sugimura T, Takayama S. The effect of limited administration of *N*-methyl-

N'-nitro-*N*-nitrosoguanidine on the induction of stomach cancer in rats. Cancer Res **30**:842–848, 1970.

9. Sugimura T, Fujimura S, Baba T. Tumor production in the glandular stomach and alimentary tract of the rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Cancer Res **30**:455–465, 1970.
10. Volfson NI, Kalinovsky VP, Pliss GB, Seitz JF, Petrov AS. Morphological and biochemical changes induced in rat stomach by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Exp Pathol **17**(5):299–300, 1979.
11. Ahang A, Li Y, Li G, Sun Z. Induction of gastric adenocarcinoma in rats by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and ultrastructural changes of epithelial cells of the pyloric gland region. Sci Sin **24**(3):416–430, 1981.
12. Balish E, Shih CN, Croft WA, Pamukcu AM, Lower G, Bryan GT, Yale CE. Effect of age, sex and intestinal flora on the induction of colon tumors in rats. J Natl Cancer Inst **58**(4):1103–1106, 1977.
13. Reddy BS, Watanabe K. Effect of cholesterol metabolites and promoting effect of lithocholic acid in colon carcinogenesis in germ-free and conventional F-344 rats. Cancer Res **39**(5):1521–1524, 1979.
14. Reedy BS, Watanabe K, Weisburger JH, Wynder EL. Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F-344 rats. Cancer Res **37**(9):3238–3242, 1977.
15. Endo H, Takahaski K, Kinoshita N, Utsunoniya T, Baba T. An approach to the detection of possible etiologic factors in human gastric cancer. Gann Monogr Cancer Res **17**:17–29, 1975.
16. Hollander D. Retinol lymphatic and portal transport. Influence of pH, bile and fatty acids on transport. Amer J Physiol **239**:G210–G214, 1980.
17. Lawley PD, Thatcher CJ. Methylation of deoxyribonucletic acid in cultured mammalian cells by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Biochem J **116**:693–707, 1970.
18. Nigro ND, Singh DV, Cambell RL, Pak MS. Effect of dietary beef fat on intestinal tumor foration by azomethane in rats. J Natl Cancer Inst **54**(2):439–442, 1975.
19. Thomson ABR, Dietschy JM. The role of the unstirred water layer on intestinal permeation. In: Czaky TZ, Ed. Pharmacology of Intestinal Permeation. II. New York/Berlin, Springer-Verlag, pp165–269.
20. Thomson ABR, Rajatte R. Effect of dietary modification on the enhanced uptake of cholesterol in diabetic rats. Amer J Clin Nutr **37**:244–252, 1983.
21. Saunder DR. Regional differences in the effect of bile salts absorption by rat small intestine *in vivo*. J Physiol **250**:373, 1975.
22. Thomson ABR, Dietschy JM. Intestinal lipid absorption: Major extracellular and intracellular events. In: Johnson LR, Ed. Physiology of the Gastrointestinal Tract. New York, Raven Press, pp1147–1220, 1981.
23. Chopra DP, Wilkoff LJ. Reversal by vitamin A analogues (retinoids) of hyperplasia induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in mouse prostate organ cultures. Natl Cancer Inst **58**(4):923–929, 1977.
24. Felix EL, Loyd B, Cohen MH. Inhibition of the growth and development of a transplantable murine melanoma by vitamin A. Science **189**:886–888, 1975.
25. Hauswirth JW, Brizuela BS. The differential effects of chemical carcinogens on vitamin A status and on microsomal drug metabolism in normal and vitamin A-deficient rats. Cancer Res **36**:1941–1946, 1976.
26. Newberne NM, Rogers AE. Rat colon carcinomas associated with aflatoxin and marginal vitamin A. J Natl Cancer Inst **50**(2):439–448, 1973.
27. Rettura G, Schittek A, Hardy M, Levenson SM, Demetriou A, Seifter E. Brief communication: Antitiumor action of vitamin A in mice inoculated with adenocarcinoma cells. J Natl Cancer Inst **54**(6):1489–1491, 1975.

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