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Mutagenicity of Dimethylglycine When Mixed with Nitrite: Possible Significance in Human Use of Pangamates (40815)

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Pangamic acid (vitamin B_{15}) has been widely sold as a dietary supplement in the United States, with sales rocketing after a popular magazine extolled its virtues (1). It has been pointed out, however, that the Food and Drug Administration regards this material as "not an identifiable substance . . . not a vitamin nor a provitamin . . . in man or animals . . . no medical, nutritional, or other usefulness for these substances has been established" (1). The reference to "not an identifiable substance" derives from the fact that a number of different formulations have been sold under this name, most containing either a dichloroacetate salt or dimethylglycine hydrochloride (1). In recent studies using the Ames *Salmonella*/mammalian microsome mutagenicity test (2), dichloroacetate proved to be mutagenic (3). There is a 90% concordance between mutagenicity and carcinogenicity (4) and dichloroacetate subsequently proved to be directly toxic to animals and man (5).

The current studies were conducted to determine possible mutagenicity of dimethylglycine hydrochloride, which is contained in most pangamate formulations which do not contain dichloroacetate. Because of prior evidence (6) that this substance was nitrosatable to nitrososarcosine, a weak carcinogen, and dimethylnitrosamine, a potent carcinogen, the mutagenicity of dimethylglycine was tested before and after incubation with sodium nitrite under controlled conditions.

Materials and methods. *N,N*-dimethylglycine hydrochloride (MW 139.6) was purchased from Sigma Chemical Company, St. Louis, Missouri. Testing for mutagenicity was carried out using plate incorporation techniques described by Ames *et al.* (2) using the five standard mutants supplied by those workers (TA98, TA100, TA1535, TA1537 and TA1538), with and without the addition of commercially produced 9000*g* supernatant (S9) from rat livers primed with Aroclor 1254 (Bionetics Corporation, Kensington, Md. 20795). The genetic markers, spontaneous revertant rates, and mutagenesis of the organisms to standard mutagens were tested upon receipt of the organisms and twice monthly thereafter, including immediately prior to their use in the tests described here.

Dimethylglycine hydrochloride was subjected to nitrosating conditions as previously described (6) by adjusting a 2 *M* solution to pH 1.5, pH 4.0, and pH 7.0. These solutions were then mixed with sodium nitrite to obtain a final concentration of 1.5 *M* dimethylglycine hydrochloride and 0.5 *M* sodium nitrite, incubated at 37°C for 45 min and then tested as potential mutagens in the assay.

The technique used to prevent residual nitrites from inducing mutagenesis was based on the observation of Fan and Tannenbaum (7) and Archer *et al.* (8), and consisted of incubating the test solutions at pH 4.0 with 1 *M* ascorbate for 1 hr at 25°C while bubbling oxygen through the mixture. Under these conditions, with ascorbate at twice the molar concentration of nitrite, all of the nitrite is consumed by the ascorbate (8). The validity of the method was tested in each experiment by including controls which tested mutagenic effects at equimolar concentrations of nitrite alone, the nitrosated dimethylglycine before ascorbate and oxygen exposure, and nitrite alone exposed to ascorbate and oxygen exposure, and ni-

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trite alone exposed to ascorbate and oxygen under identical conditions to those used for the nitrosated dimethylglycine.

Thus, whenever dimethylglycine was incubated with nitrite to achieve nitrosation, it was present in three times the molar concentration of nitrite, and whenever ascorbate was subsequently added to consume the nitrite under aerobic conditions, its molar concentration was twice that of the nitrite. In all studies, the dose of mutagen added was expressed as the molar concentration of nitrite, with the understanding that any ascorbate present was at twice that concentration and dimethylglycine, if present, was at three times the concentration. The positive control substances used in these studies were sodium azide (Sigma Chemical Company) and 2-anthramine and dimethylnitrosamine (nitrosodimethylamine) obtained from Aldrich Chemical Company, Metuchen, New Jersey 08840.

Results. Preliminary experiments revealed no increase in the number of revertants when using strains TA98, TA1537, and TA1538 to test the dimethylglycine/nitrite mixture, but increased revertants of strains TA100 and TA1535. The remainder of the study was confined to tests using the latter two strains.

Each mutagenesis plate assay was run at each dose with nitrite alone and with the dimethylglycine/nitrite mixture, each tested both before and after consumption of residual nitrite by incubation in ascorbate under aerobic conditions. Some of the data which will not be presented in detail here confirmed the mutagenicity of nitrite for both test organisms (9) and the ability of ascorbate plus O_2 to eradicate this mutagenicity (7–9). Since the ascorbate/nitrite ratio was kept constant, tests of increasing nitrite doses incorporated increasing ascorbate doses, which at different levels decrease and increase revertants to histidine auxotrophy in the *Salmonella* test (8). Thus, the controlled evaluation of mutagenicity of dimethylglycine subjected to nitrosation necessitates that one subtract the number of revertants generated when the organism was incubated with a test solution containing identical doses of nitrite and ascorbate but no dimethylglycine.

Table I shows the number of revertants using strain TA100 incubated with varying doses of the dimethylglycine products, after subtraction of the number of revertants observed when dimethylglycine was omitted from the experiment. Table II shows the corresponding results using strain TA1535. The number of revertants observed in the absence of dimethylglycine, and subtracted in the Table, appears in parentheses.

The data (not shown) indicated that nitrite alone was an extremely potent mutagen for both organisms in the absence and the presence of the S9 supernatant. This mutagenicity was entirely abolished, however, when ascorbate was present at twice the molar concentration of nitrite, and the number of revertants actually fell at increasing doses of these substances, presumably due to the previously described effect of ascorbate. The test solutions containing dimethylglycine, when incubated with the S9 rat liver products, produced

TABLE I. STRAIN TA100 REVERTANTS TO NITROSATED DIMETHYLGLYCINE, CONTROLLED AT EACH NITRITE DOSE WITH PLATE ASSAYS CONTAINING NO DIMETHYLGLYCINE[a]

		$NaNO_2$ dose (μmole per plate)			
		10	50	150	750
DMG/$NaNO_2$ + ascorbate[b]	No S9	110 ± 42 (337)	143 ± 142 (268)	165 ± 164 (234)	—[c] (132)
	+ S9	111 ± 30 (310)	192 ± 87 (238)	323 ± 67 (242)	459 ± 53 (149)

[a] Spontaneous revertants: 226 ± 34. 1 μg sodium azide: 2947 ± 410. 2 μg 2-anthramine + S9: 1877 ± 126.

[b] DMG—dimethylglycine hydrochloride in constant 3:1 molar ratio with $NaNO_2$. Ascorbate—in constant 2:1 molar ratio with $NaNO_2$, and used as detailed in the text.

[c] Revertants in these test plates (containing dimethylglycine) were fewer in number than those in the control plates (containing no dimethylglycine).

TABLE II. STRAIN TA1535 REVERTANTS TO NITROSATED DIMETHYLGLYCINE, CONTROLLED AT EACH NITRITE DOSE WITH PLATE ASSAYS CONTAINING NO DIMETHYLGLYCINE (SHOWN IN PARENTHESES)[a]

		$NaNO_2$ dose (μmoles per plate)			
		10	50	150	750
DMG/$NaNO_2$ + ascorbate[b]	No S9	98 ± 87 (40)	83 ± 87 (66)	96 ± 32 (53)	111 ± 8 (32)
	+ S9	17 ± 8[c] (45)	111 ± 8 (53)	256 ± 77 (36)	366 ± 66 (44)

[a] Spontaneous revertants: 43 ± 15. 1 μg sodium azide: 3013 ± 58. 2 μg 2-anthramine + S9: 1327 ± 66.

[b] DMG—dimethylglycine hydrochloride in constant 3:1 molar ratio with Na NO_2. Ascorbate—in constant 2:1 molar ratio with $NaNO_2$, and used as detailed in the text.

[c] Note that 30 μmoles nitrosated DMG/plate were mutagenic, and 30 μmoles DMG = 4.2 mg, which is much less than the amount of DMG in one tablet of the largest selling "B_{15}" in the U.S. (1).

unequivocal dose-related evidence of mutagenicity. When the solution was not exposed to modification by the rat liver enzyme, there was a slight increase in the number of revertants which provided only weak evidence for direct mutagenicity. Similarly, the commercially purchased dimethylnitrosamine run as a positive control did not evince mutagenicity except when previously incubated with the rat liver supernatant, when we observed an average of 285 revertants of TA100 to 15 mg dimethylnitrosamine per plate, and 53 revertants of TA1535 using the same dose. The weak mutagenicity of this substance was nevertheless unequivocal, since addition of 100 mg to the S9 mixture per plate yielded 1169 revertant colonies using TA100 and 1060 revertant colonies using TA1535.

Discussion. This study confirms that dimethylglycine is nitrosatable (6). The nitrites which can produce this reaction are ubiquitous in nature, and occur in human saliva (10). The concentration of dimethylglycine used in this study was based upon prior studies (6) and was much less than that anticipated after swallowing only one 50-mg tablet of dimethylglycine. The pH conditions in this study are comparable with those such a tablet would be exposed to after ingestion.

Based upon the relative effects of commercially purchased dimethylnitrosamine run as a positive control, far more revertants were produced by the tested quantities of nitrosating mixture (Tables I and II) than would be anticipated from the dimethylnitrosamine generated under the conditions of this study (6). A possible cause for this is mutagenicity of other products of nitrosation of dimethylglycine, such as nitrososarcosine (6). The whole number of revertants produced by synergistic action of these products would be greater than the sum of the parts.

Mutagenicity by nitrite itself has been eliminated by appropriate controls, leaving the presence of dimethylglycine as the only variable. This technique confirmed once again that nitrite is itself mutagenic, and that this effect can be eliminated by exposing it to twice its molar concentration of ascorbic acid and bubbling oxygen through the mixture. The mutagenicity of nitrosated dimethylglycine was stable to this procedure, confirming the report that ascorbic acid appeared to have no effect on the hepatotoxicity in rats of preformed *N*-nitrosamine (9).

This data on mutagenicity of dimethylglycine appears particularly pertinent in view of the recent claim by the largest seller of B_{15} in the U.S. that their product is dimethylglycine, and by the Food and Drug Administration that dimethylglycine is illegal and possibly dangerous (11). Whether the current findings are also pertinent to the small amounts of dimethylglycine present in meat as a catabolic product of choline is unclear. Unlike the commercial B_{15}, which contains dimethylglycine as the hydrochloride (the material used in this study), the dimethylglycine in meat is not hydrochloride. Furthermore, it is intracellularly located and diluted, rather than free and concen-

trated as in the current study and in some commercial "B_{15}" preparations. Part of this data has been reported in abstract form (12).

Summary. Nearly all formulations of the health food product variously tradenamed "B_{15}," vitamin B_{15}, pangamic acid, and pangamate contain either dichloroacetate (DCA) or dimethylglycine hydrochloride (DMG). We previously reported that DCA is mutagenic, and tested DMG in the current study under nitrosating conditions anticipated during human ingestion. It was not mutagenic alone. When DMG was preincubated with sodium nitrite to simulate exposure to salivary and gastric nitrites, and incubated at 37°C for 45 min at pH 4 to simulate the period before gastric emptying, and then tested using appropriate controls, a characteristic dose-related mutagenic effect was observed, presumably due to nitrosation products such as dimethylnitrosamine and nitrososarcosine.

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