PROPERTIES OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE AND ITS ACTION ON *Bacillus subtilis* TRANSFORMING DNA

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SUMMARY

The stability of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was measured by a spectrophotometric assay as a function of pH, temperature, and buffer. The effect of MNNG was then tested on *Bacillus subtilis* transforming deoxyribonucleic acid (DNA). Conditions which lead to MNNG decomposition cause inactivation of transforming DNA. However, transforming DNA is not inactivated under conditions of MNNG stability, e.g. 0.05 M Tris-maleate buffer (pH 6).

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) inactivates transforming deoxyribonucleic acid (DNA; 1,2). Above pH 5.5, this is probably a result of methylation (3,4) since MNNG decomposes to N-nitrourea and a methyldiazonium ion (5,6). Below pH 5.5, inactivation may occur by deamination because MNNG decomposes to N-methyl-N'-nitroguanidine and nitrous acid (7,8). At pH 5.5, MNNG appears to be chemically stable but nevertheless *in vivo* it mutagenizes the replication point in *Escherichia coli* (9,10) and *Staphylococcus aureus* (11) implying that the *in vivo* action of MNNG at pH 5.5 may depend on the presence of a replication point. Consequently, non-replicating DNA, e.g. *in vitro* transforming DNA, might be refractory to MNNG at pH 5.5, but not at pH 7.

This report describes the stability of aqueous MNNG solutions as a function of pH, temperature and buffer. These data provide criteria for determining the stability of MNNG under any specific set of mutagenesis conditions. The inactivation of *Bacillus subtilis* transforming DNA was then tested under both conditions of MNNG stability and conditions that lead to MNNG decomposition.

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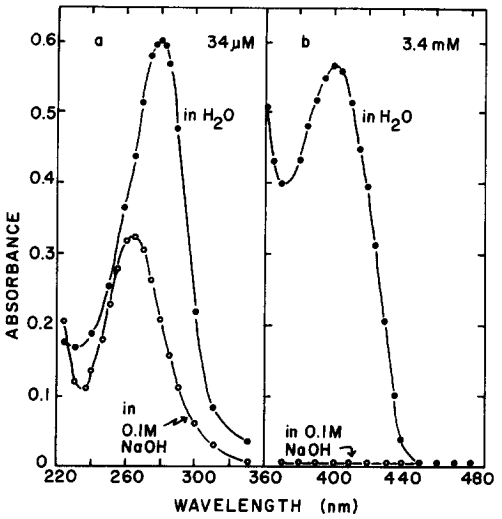

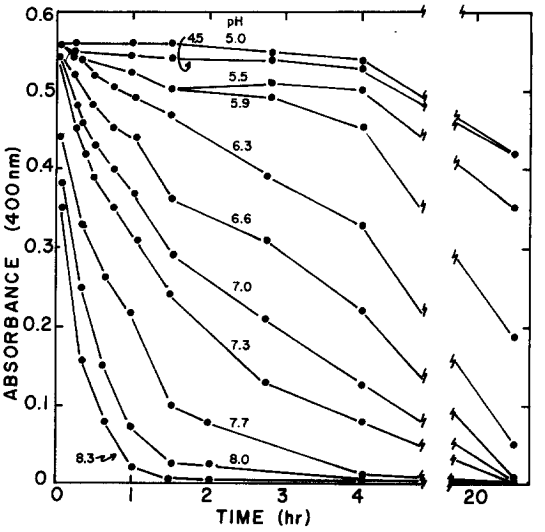

Fig. 1 Spectra of MNNG.

Fig. 2 pH Dependence of MNNG Decomposition. MNNG (1 mg/ml) was diluted with an equal volume of 0.2 M sodium phosphate buffer and incubated at 37° in the dark. At intervals, the absorbance at 400 nm and the pH were measured.

MATERIALS AND METHODS

MNNG (Aldrich Chemical Co.) was dissolved in distilled water and stored at 0°C in the dark. All manipulations were conducted in subdued light (< 20 foot-candles) and spectra were measured on a Zeiss PMQ spectrophotometer. Ultraviolet and visible spectra of MNNG in water at final concentrations of 5 μg/ml (34 μM) and 500 μg/ml (3.4 mM) respectively, are shown in Fig. 1. There are absorption maxima at 280 nm and 400 nm with molar extinction coefficients of 1.77×10^4 and 167.9 M^{-1} cm^{-1}, respectively. In the presence of 0.1 M NaOH, the ultraviolet spectrum changes to one similar to N-nitrourea (12), but the visible spectrum decreases to zero. The absorbance at 400 nm is linear with concentration and is not affected by the presence of either salmon sperm DNA (160 μg/ml) or bovine serum albumin (400 μg/ml). Thus the absorbance at 400 nm provides a convenient assay for MNNG without interference from either protein or DNA. Also the absorbance at 400 nm allows the use of commonly available colorimeters; with a Bausch and Lomb Spectronic 20, the absorbance of MNNG (500 μg/ml) at 400 nm is 0.56 and it is linear with concentration.

Transforming DNA was isolated from B. subtilis 168, strain BC-30 (trp-2, ery-1), as described elsewhere (13,14). Samples of DNA (30 μg/ml) were incubated with MNNG (1 mg/ml) in 0.1 M phosphate, 0.05 M Tris-maleate, or 0.05 M Tris buffers. Cysteine was added at a final concentration of 1 mg/ml and standard saline citrate (SSC) was 0.15 M NaCl-0.015 M Na citrate. Controls, lacking MNNG, for each sample were treated identically. After 4 hr at 37°C in the dark, the absorbance at 400 nm and the pH were measured. All samples were dialyzed in 3.0 l of SSC (pH 5) at 0°C for 20 hr and then assayed for transformation. The DNA concentration was measured by Burton's method (15) and ranged from 25 to 30 μg/ml in all the samples. Each sample (0.1 ml) was tested for sterility and

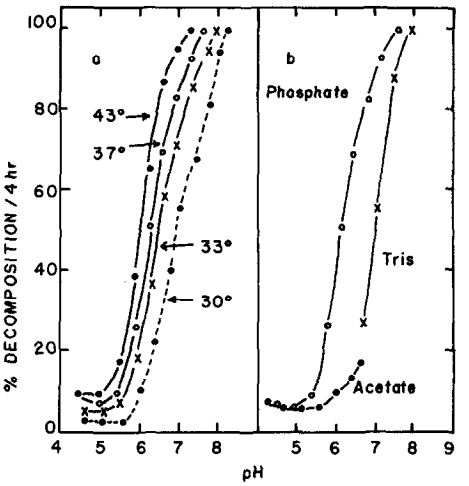

Fig. 3 MNNG Decomposition as a Function of Temperature and Buffer. Conditions as in Fig. 2. (a) 0.1 M phosphate buffer with variable temperature and pH (b) 0.1 M buffer at 37°C with variable pH.

found to have less than 10 colony forming units. Transformation (14,16) was tested with 0.1 ml of each sample on B. subtilis 168 strain BC-100 (purA16, leu-8, metB5). Transformants to leucine prototrophy were counted on minimal plates (16) plus adenine, methionine, and tryptophan and total cell numbers were measured on minimal plates plus adenine, methionine, tryptophan and leucine. The number of transformants were normalized by calculating transformants/10^6 cells/μg DNA and % inactivation was calculated by comparing the decrease in transformation frequency of the MNNG-treated DNA sample with its control.

RESULTS and DISCUSSION

The decomposition of MNNG as a function of pH, temperature and buffer was measured by loss in absorbance at 400 nm (Fig. 2). The decomposition of MNNG in 0.1 M phosphate buffer at different pH's appears to obey first order kinetics with rate constants varying between 1.5×10^{-4} min^{-1} at pH 5 to 5.2×10^{-2} min^{-1} at pH 8.2. These rates of decomposition are dependent on temperature and type of buffer as shown in Fig. 3. Also at a given pH, the rates increase with increasing concentration of buffer (data not shown). Similar results have been reported by Lawley and Thatcher (17) except they used 10% ethanol as a solvent which might be deleterious to in vivo mutagenesis.

The stability of MNNG under conditions used for in vitro treatment of

Table 1

DECOMPOSITION OF MNNG IN DIFFERENT BUFFERS

		% Decomposition[1]		
Buffer	pH	2 hr	4 hr	20 hr
0.1 M acetate (Cerdá-Olmeda et al.; 9)	5.5	2	6	16
0.05 M Tris-maleate (Adelberg et al.; 19)	6.0	6	15	51
Glucose minimal salts medium (Anagnostopoulos and Spizizen; 16)	7.0	67	93	100
Standard saline citrate	5.0	2	6	20
Standard saline citrate	5.9	2	6	20
Standard saline citrate (Marmur; 13)	6.6	9	16	42

[1]The percent decomposition of MNNG (500 μg/ml) was determined after incubation at 37° in the dark as described in Fig. 2 and 3.

transforming DNA and *in vivo* treatment of bacteria is shown in Table 1. MNNG is most stable in 0.1 M acetate (pH 5.5) and standard saline citrate (pH 5 or 5.9).

The inactivation of transforming DNA by MNNG in different buffers was tested. As shown in Table 2, conditions that cause MNNG decomposition, e.g. 0.1 M phosphate (pH 7.3), inactivate transforming DNA. If MNNG does not break down, e.g. in 0.1 M phosphate (pH 5.5), little inactivation occurs. Cysteine has been previously reported (4,5,17,18) to catalyze the methylation of DNA by MNNG at pH 6. As shown in Table 2, it causes degradation of MNNG at pH 5.6 resulting in inactivation of transforming DNA. Thus there is a direct correlation between MNNG decomposition and *in vitro* inactivation of DNA.

The mechanism of action of MNNG *in vivo* at pH 5.5 may be methylation of DNA because of endogenous cysteine in the cell (18). Since non-replicating

Table 2

EFFECT OF MNNG ON TRANSFORMING DNA[1]

uffer	pH	% Decomposition of MNNG/4 hr	Transformants/ 10^6 cells/μg DNA[2]	% Inactivation
hosphate	5.5	9	236 (255)	8
hosphate	7.3	86	291 (1370)	79
hosphate + cysteine	5.6	94	70 (318)	78
ris-maleate	6.0	7	1950 (2390)	14
ris	7.5	68	135 (1740)	92
SC	5.0	0	900 (942)[3]	5
SC	6.6	12	1145 (1230)	7

See the Materials and Methods

[2]Numbers in parentheses represent the transformation frequencies for the individual controls of each sample.

[3]For samples treated in SSC, DNA concentration was not measured therefore transformation frequencies were not corrected for DNA.

DNA can be mutagenized at pH 5.5 in the presence of cysteine, the action of MNNG may not depend on any unique properties of the DNA at the replication point. Rather the preferential mutagenesis of the replication point could be the result of a concentration of sulfhydryl groups from the replication proteins at that site, e.g. polymerases and DNA unwinding proteins.

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REFERENCES

1. A. Terawaki and J. Greenberg, Biochim. Biophys. Acta 95 (1965) 170.
2. H. Kolb and F. Kaudewitz, Mutation Res. 10 (1970) 85.

3. D.R. McCalla, Biochim. Biophys. Acta 155 (1968) 114.
4. V.M. Craddock, Biochem. J. 111 (1969) 615.
5. F. Lingens, J. Rau and R. Süssmuth, Z. Naturforschg. 23 (1968) 1565.
6. R. Haerlin, R. Süssmuth and F. Lingens, FEBS Letters 9 (1970) 175.
7. F.K. Zimmerman, R. Schwair and U. Laer, Z. Verebungsl. 97 (1965) 68.
8. D.R. McCalla, A. Reuvers and R. Kitai, Canad. J. Biochem. 46 (1968) 807.
9. E. Cerdá-Olmeda, P.C. Hanawalt and N. Guerola, J. Mol. Biol. 33 (1968) 705.
10. N. Guerola, J.L. Ingraham and E. Cerdá-Olmeda, Nature New Biol. 230 (1971) 122.
11. R.A. Altenbern, J. Bacteriol. 95 (1968) 1642.
12. M. Piskorz and T. Urbanski, Bull. Acad. Polon. Sci. 11 (1963) 615.
13. J. Marmur, J. Mol. Biol. 3 (1961) 208.
14. J.C. Copeland and J. Marmur, Bacteriol. Rev. 32 (1968) 302.
15. K. Burton, Biochem. J. 62 (1956) 315.
16. C. Anagnostopoulos and J. Spizizen, J. Bacteriol. 81 (1961) 741.
17. P.D. Lawley and C.J. Thatcher, Biochem, J. 116 (1970) 693.
18. R. Süssmuth, R. Haerlin and F. Lingens, Biochim. Biophys. Acta 269 (1972) 276.
19. E.A. Adelberg, M. Mandel and G. Chen, Biochem. Biophys. Res. Comm. 18 (1965) 78