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INACTIVATION OF B_{12} AND FOLATE COENZYMES BY BUTYL NITRITE AS OBSERVED BY NMR: IMPLICATIONS ON ONE-CARBON TRANSFER MECHANISM

M. Abu Khaled[a*], Charles L. Watkins[b] and Carlos L. Krumdieck[a]

Department of Nutrition Sciences[a] and Chemistry[b]
University of Alabama at Birmingham
Birmingham, Alabama 35294

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The effects of butyl nitrite, a frequently used recreational drug, on methyl cobalamin and 5-methyl tetrahydrofolate were investigated by using ^{1}H and ^{31}P NMR spectroscopies. While no effect could be observed in organic solvents, strong interactions of butyl nitrite with the methyl cobalamin and 5-methyl tetrahydrofolate were found to occur in water. Butyl nitrite decomposes in water generating H^{+} and NO_2^{-}. The former protonates to give the "base-off" configuration of methyl cobalamin while the $Co\text{-}CH_3$ bond is cleaved. Similarly, the methyl group at the 5N position and the pyrazine ring of 5-methyl tetrahydrofolate were found to be affected by butyl nitrite. The overall interaction of butyl nitrite with both coenzymes shows displacement of the methyl group and derivatization or destruction of the coenzymes that may lead to deficiencies of both B-12 and/or folates.

The frequent use of organic nitrite inhalants has been recently reported in homosexual men with Kaposi's sarcoma (1). Exposure by inhalation to volatile alkyl nitrites is also a common occurrence in humans as a result of their presence in tobacco smoke. The ill effects of chronic exposure to these compounds have not been thoroughly investigated, although their potential mutagenicity (2,3) and deleterious effects on T-lymphocyte function (4) and interferon production (3) have been noted. The mechanisms of these effects are currently unknown. Coenzymes such as methyl cobalamin (Me-Cbl) and 5-methyl tetrahydrofolate (5MTHF) are essential cofactors for growth and proliferation of mammalian cells, a deficiency of which may affect the lymphatic systems (5-7). We postulated that the volatile alkyl nitrites possessing strong oxidating activity (8) could inactivate Me-Cbl and 5MTHF by oxidation as in the case of nitrous oxide inhalation (9,10). In this report we present the

*To whom correspondence should be addressed.

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results of an in vitro study of the interactions of butyl nitrite with both Me-Cbl and 5MTHF by the use of nuclear magnetic resonance spectroscopy (NMR). Butyl nitrite, a volatile liquid, was selected as the model alkyl nitrite because of its widespread use and commercial availability.

Materials and Methods

Both Me-Cbl and 5MTHF were purchased from Sigma and were used without further purification. Butyl nitrite was purchased from the Kodak Co. All the samples for NMR experiments were prepared in degassed deuterated solvents under nitrogen and subdued light. All the NMR experiments were performed on a Nicolet NMC-300 wide bore NMR Spectrometer equipped with a multinuclear probe and operating at a probe temperature of 30°C. ^{1}H spectra were obtained at 300.12 MHz using a pulse width of 8 sec for a 90° magnetization vector with a delay time of 1 sec. The 121 MHz ^{31}P spectra were obtained by ^{1}H broad band decoupling by using a pulse width of 40 sec for a 90° magnetization vector with a delay time of 3 sec. Spectral data were collected and processed by using the Nicolet NMCFT-1280 computer program. The ^{31}P spin-lattice relaxation (T_1) values were determined by using a modified inversion recovery method which incorporates 90° pulse phase shifting and composite 180° pulses to correct for phasing and pulse timing errors (11,12). In each experiment at least ten delay times were chosen, the largest delay time being at least five times T_1. The T_1 data were analyzed by non-linear least squares regression procedures using the three-parameter equation of Levy and Peat (13).

Results

Although butyl nitrite is essentially insoluble in water, addition of 10 µl of butyl nitrite to a 0.5ml sample of 10 mM Me-Cbl dissolved in deuterated water (D_2O) shows a considerable, almost instantaneous, effect on the ^{1}H NMR and optical absorption parameters with a concomitant drop in the pD of the solution to 2.0. The ^{1}H NMR spectra of the reaction mixture as a function of time are given in Figure 1. The assignments of the ^{1}H NMR resonances were taken from Hensens et. al. (14). Observable spectral changes of interest are indicated by asterisks. It can be seen in Fig. 1f. that after 12 hours the signal due to the Co-CH_3 group at -0.39 ppm has totally disappeared while a new signal appears at 3.35 ppm which corresponds to the CH_3 group of methanol. The ^{31}P spectra of Me-Cbl with butyl nitrite are given in Figure 2 as a function of time. Similar interesting reaction phenomena are also observed as in Figure 1. The upfield shift at -0.68 ppm (Fig. 2B) corresponds to the ^{31}P signal of Me-Cbl in acidic medium as reported earlier

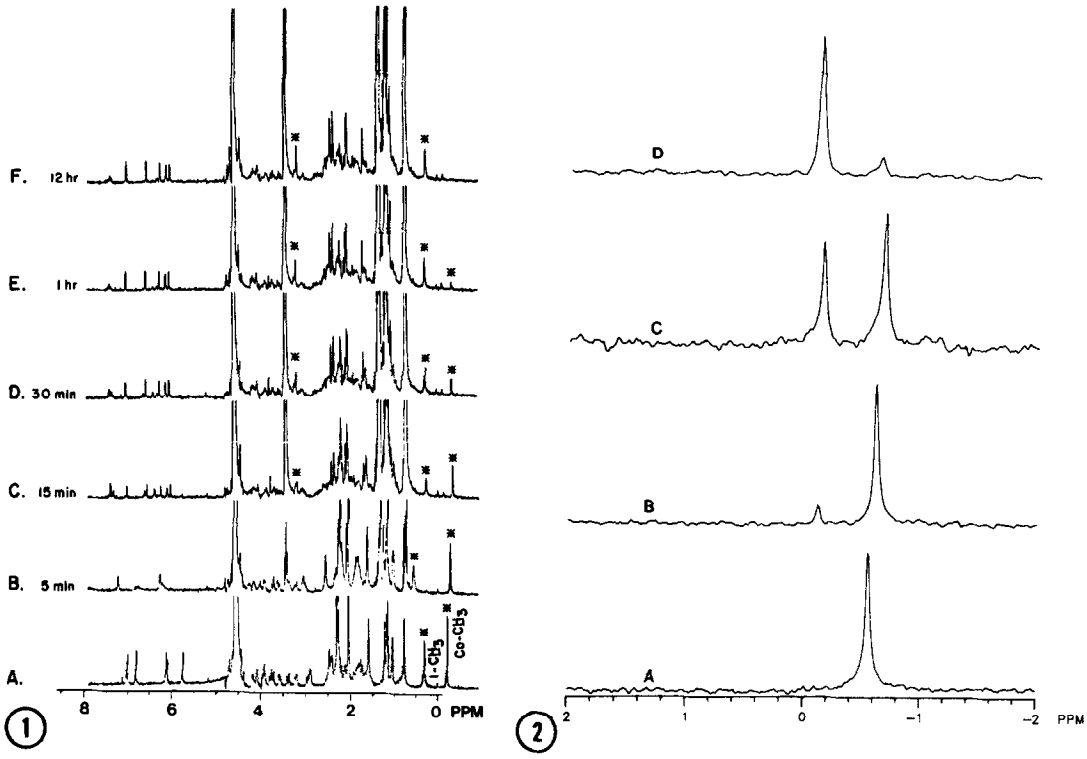

Fig. 1: The 300 MHz ^{1}H NMR spectra of Me-Cbl with butyl nitrite as a function of time. Note that the changes in resonances of interest are indicated by asterisks.

Fig. 2: The 121 MHz ^{31}P NMR spectra of Me-Cbl: A) in D_2O, B) 5 minutes after adding butyl nitrite, C) after 30 minutes, D) after 12 hours.

(15). After 30 minutes, however, another lower field signal appeared at -0.12 ppm (Fig. 2C) at the expense of the high field signal at -0.68 ppm. After 12 hours the high field signal had almost disappeared while the lower field signal became more intense (Fig. 2D). Also, ^{31}P spin-lattice relaxation time (T_1) values were obtained for (a) Me-Cbl and (b) Me-Cbl in the presence of butyl nitrite since the T_1 of the ^{31}P resonance provides information on the coordination states of the benzimidazole (Bz) ring with the central Co atom in B_{12} (16).

The interactions of butyl nitrite with 5MTHF in D_2O were monitored by following the ^{1}H NMR spectra as a function of time. The assignments of all the resonances were taken from Poe et al. (17). Figure 3 shows the effect of butyl nitrite on 5MTHF after 15 minutes and contains the chemical structure of

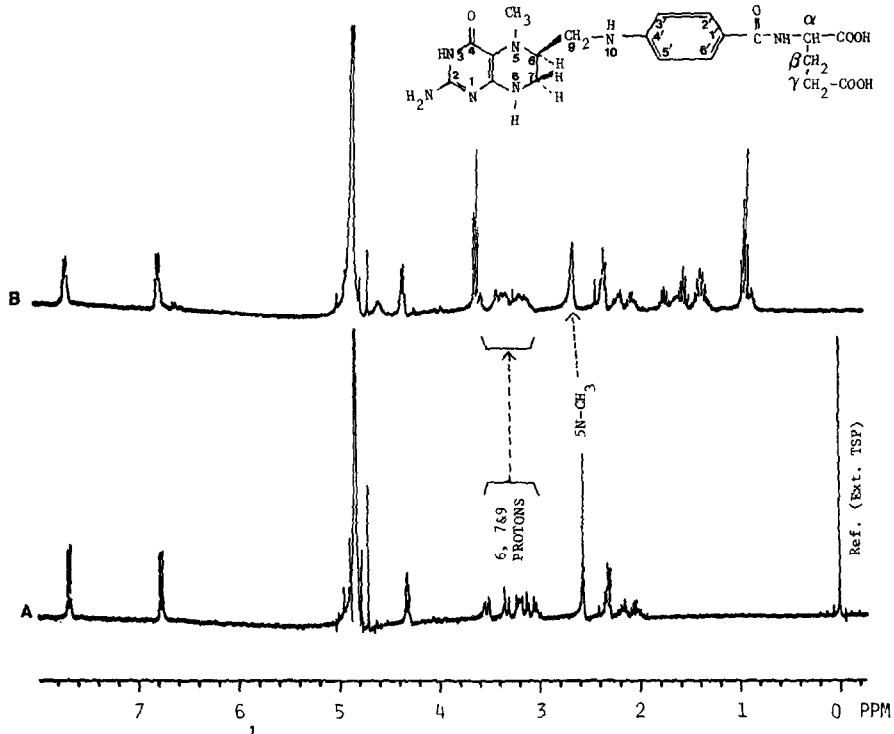

Fig. 3: The 300 MHz ^{1}H NMR spectrum of 5N-methyl tetrahydrofolate (5MTHF) A) in D_2O and B) with butyl nitrite. After 15 minutes the molecular structure of 5MTHF with numbering system is given.

5MTHF. After 4 hours very complex ^{1}H NMR spectra were obtained (not shown here) indicating total decomposition of 5MTHF.

Discussion

Brodie and Poe (18) first demonstrated the dissociation of the 5,6-dimethylbenzimidazole (Bz) ring from the central Co atom in B_{12} by ^{1}H NMR spectroscopy. The configuration with a cobalt-dissociated Bz ring is termed "base-off" in contrast to the cobalt-coordinated form, called "base-on". The ring current of the Bz moiety, in the "base-on" state, provides an intrinsic perturbation of the chemical shifts of protons which "lie below" the plane of the corrin ring. Based on such perturbations, Hensens et. al. (14) also noticed the chemical shift changes of the $1\text{-}CH_3$ and the $Co\text{-}CH_3$ protons in Me-Cbl at acidic pHs and established that the $1\text{-}CH_3$ proton resonance shifted 127 Hz downfield while the $Co\text{-}CH_3$ proton signal moved 31 Hz upfield at 270 MHz observation frequency. In the present study similar

spectral properties of these two groups of protons have been observed, i.e., the 1-CH_3 signal moved almost 145 Hz downfield while the Co-CH_3 signal went 40 Hz upfield at 300 MHz (see Figs. 1A & B). Supported by these previous reports (14,18) we, therefore, conclude that the initial effect observed after addition of butyl nitrite to an aqueous solution of Me-Cbl is the transformation of the "base-on" configuration to the "base-off" state of the cobalamin brought about by the ensuing acidification of the media.

While in the "base-off" state prevailing in acid solution, the Me-Cbl was seen to undergo further modifications on prolonged exposure to butyl nitrite. These modifications are clearly indicated by the spectral changes depicted in Figures 1C to 1F. While the spectrum obtained after 5 minutes (Fig. 1B) satisfies the conditions as induced by acidic environment, after 15 minutes, however, the 1-CH_3 signal at 0.42 ppm started reappearing and the Co-CH_3 signal at -0.39 ppm started diminishing in intensity (see Fig. 1C). Simultaneously, a small signal at 3.35 ppm, which exactly corresponds with the ^1H resonance of the CH_3 protons of methanol, evolved. The spectral changes were complete after 12 hours. At this time the methanol signal at 3.35 ppm had fully evolved while the Co-CH_3 signal at -0.39 ppm had disappeared. Concomitantly, the downfield shifted 1-CH_3 signal reappeared at its original position of 0.42 ppm. Of particular interest in this experiment is the loss of the methyl group from the central Co atom of Me-Cbl as it reacts with butyl nitrite.

The results obtained by employing ^{31}P NMR (Fig. 2) are significant since similar spectral changes as discussed above can also be found to occur when Me-Cbl is reacted with butyl nitrite. The upfield shift from -0.55 ppm to -0.68 ppm in (Fig. 2B) is in good agreement with all previous reports (15,16) indicating the "base-off" state of Me-Cbl in an acid medium. The downfield signal at -0.12 ppm due to the interaction of Me-Cbl with butyl nitrite (Fig. 2C) becomes a prominent signal after 12 hours (Fig. 2D). In order to understand the characteristic of this signal at -0.12 PPM, the

spin-lattice relaxation time (T_1) was measured since it provides information on the molecular motion of a compound or part of a compound. Misra et. al. (16) used the T_1 parameter to further distinguish the "base-off" form of B_{12} from its "base-on" state. In the case of the "base-off" form the Bz ring has more motional freedom and thus the T_1 value of the ^{31}P is higher than when the ring is coordinated to the Co atom. In accordance with this we observed a longer T_1 (1.84 sec) for the ^{31}P signal at -0.68 PPM compared to Me-Cbl in D_2O (base-on, T_1 = 1.05 sec). On the other hand, the ^{31}P signal at -0.12 ppm, obtained after complete reaction with butyl nitrite, gives a much shorter T_1 value (0.84 sec). Thus, the cobalamin is again in the "base-on" form. The reappearance of the 1-CH_3 proton resonance at its original position of 0.42 ppm (see Fig. 1F) also lends support to this notion. It has been suggested (2) that butyl nitrite decomposes in water to yield $CH_3(CH_2)_3OH + H^+ + NO_2^-$. Nucleophilic substitution of the NO_2^- ion may lead to the formation of nitrito cobalamin (NO_2-Cbl). We, therefore, tentatively propose that the ^{31}P NMR signal at -0.12 ppm (Fig. 2D) is due to NO_2-Cbl formed by displacing the methyl group from the central Co atom in Me-Cbl when reacted with butyl nitrite.

Addition of butyl nitrite to 5MTHF initially causes broadening of the 5N-CH_3 and 6 and 7-H pyrazine ring protons as shown in Fig. 3. This broadening indicates that the reduced pyrazine ring of 5MTHF is being affected by the oxidant butyl nitrite leading to the decomposition of the coenzyme. The oxidation of reduced folates into a variety of byproducts is well established in the literature (19). The instantaneous effect of butyl nitrite on 5MTHF may initially be the displacement of the methyl group from the 5N position and as the reaction proceeds a variety of oxidized byproducts result which give rise to complex ^1H NMR spectra. The significance of this experiment is the loss of the one-carbon fragment, namely the 5N-methyl group, with the subsequent destruction of the coenzyme. Frequent butyl nitrite inhalation entails therefore the risk of B_{12} and/or folate deficiency with consequent inpairment of DNA replication and immune function (5).

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References

1. Curran, J.W., Morgan, W.M., Hardy, A.M., Jaffe, H.W., Darrow, W.W. and Dowdle, W.R. (1985) Science 229, 1352-1357.
2. Jorgensen, K.A. and Lawesson, S.-O. (1982) N. Engl. J. Med. 307, 893-894.
3. Hersh, E.M., Reuben, J.M., Bogerd, H., Rosenblum, M., Bielski, M., Mansell, P.W.A., Rios, A., Newell, G.R. and Sonnenfeld, G. (1983) Cancer Res. 43, 1365-1371.
4. Goedert, J.J., Wallen, W.C., Mann, D.L., Strong, D.M., Neuland, C.Y., Green, M.H., Murray, C., Fraumen, J.F., Jr., and Blattner, W.A. (1982) Lancet i: 412-416.
5. Nauss, K.M. and Newberne, P.M. (1981), Adv. Exp. Med. Biol. 135, 63-91.
6. Kano, Y., Sakamoto, S., Hida, K., Tsuboyama, A. and Takaku, F. (1981) Clin. Chim. Acta, 109, 69-76.
7. Lumb, M., Perry, J., Deacon, R. and Chanarin I. (1981) Am. J. Clin. Nutr. 34, 2412-2417, 1981.
8. Haley, T.J. (1980) Clin. Toxicol. 16, 317-329.
9. Koblin, D.D., Waskell, L., Watson, J.E., Stokstad, E.L.R. and Eger, E.I., II. (1982) Anesth. Analg. 61, 7578-7584.
10. Deacon, R., Lumb, M., Muir, M., Perry, J. and Chanarin, I. (1979) In Vitamin B_{12}: Proc. 3rd Env. Symp. Vit. B_{12} and Intrinsic Factor, Zurich Switzerland, pp 1054-1060.
11. Cutnell, J.D., Bleich, H.E. and Glasel, J.A. (1976) J. Magn. Reson. 21, 43-51.
12. Freeman, R., Kempsell, S.P. and Levitt, M.H. (1980) J. Magn. Reson. 38, 453-459.
13. Levy, G. and Peat, I. (1975) J. Magn. Reson. 18, 500-509.
14. Hensens, O.D., Hill, H.A.O., Thornton, J., Turner, A.M. and Williams, R.J.P. (1976) Phil. Trans. R. Soc. Lond. B273, 353-357.
15. Satterlee, J.D. (1979) Biochem. Biophys. Res. Commune. 89, 272-278.
16. Mishra, P.K., Gupta, R.K., Goswami, P.C., Venkatasubramanian, P.N. and Nath, A. (1981) Biochem. Biophys. Acta 668, 406-412.
17. Poe, M., Hensens, O.D. and Hoogsteen, K. (1979). J. Biol. Chem. 254, 10881-10884.
18. Brodie, J.D. and Poe, M. (1972) Biochem. 11, 2534-2542.
19. Blakley, R.L. (1969). The biochemistry of folic acid and related pteridines. American Elsevier Publishing Co., New York.