Biochemical Pharmacology, Vol. 42, No. 12, pp. R25–R28, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

RAPID COMMUNICATION

INHIBITION OF VITAMIN B_{12}-DEPENDENT METHIONINE BIOSYNTHESIS BY CHLOROFORM AND CARBON TETRACHLORIDE

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(Accepted 15 October 1991)

Cobalamin-dependent 5-methyltetrahydrofolate--homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) is inactivated by nitrous oxide in intact cells [1-3]. The oxide intercepts a cob(I)alamin species which occurs as a reactive intermediate in the methyltransferase reaction (Fig. 1) [4].

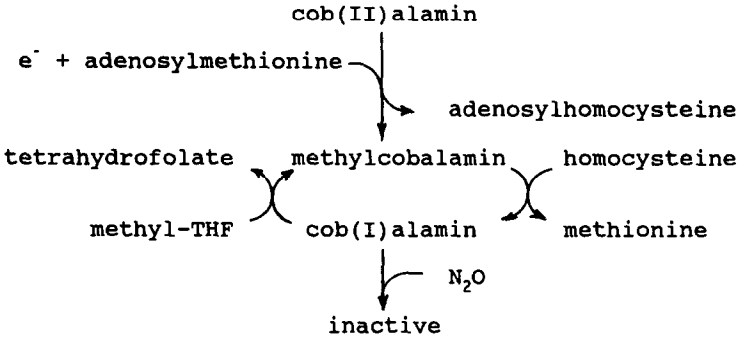

Fig. 1. Reactions of methionine synthase.

Other inhalation anesthetics can react with reduced cobalamin *in vitro* to afford covalent organometallic adducts [5-7]. For instance, although the structures of the adducts are not established [8], chloroform and carbon tetrachloride can covalently modify the cobalt atom of cobalamin *in vitro* in the presence of reducing agents [5,6], and reaction with a cob(I)alamin-like compound is proposed as the mechanism by which bacterial methane biosynthesis is inhibited by chloroform [5]. By means of an auxotrophic strain of *Escherichia coli* lacking a cobalamin-independent pathway for the *de novo* synthesis of methionine [3], the possibility was thus tested that chloroform and related compounds can, like nitrous oxide, block cobalamin-dependent methionine synthesis in intact cells.

MATERIALS AND METHODS

Prototrophic *E. coli* 14948 and auxotrophic *E. coli* 14169 [3] were obtained from the American Type Culture Collection. Working cultures were grown overnight in minimal medium supplemented with 0.2% casein hydrolysate and then resuspended in cold minimal medium. Minimal medium contained 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.2% D-glucose, 0.1% $(NH_3)_2SO_4$, 0.04% sodium citrate, 0.005% $MgSO_4$, and 0.0005% $FeSO_4$.

Methoxyflurane (from Heechung Yang, Abbott Laboratories) and halothane were

distilled to remove preservatives; chloroform was washed with water and dried over $MgSO_4$. Sevoflurane was from D. Ryan Cook and Peter J. Davis, Children's Hospital of Pittsburgh.

The requisite volumes of liquid agent or gas were injected into sealed culture bottles (190 mL) and dispersed at 23°. Small volumes of $CBrCl_3$ or 1-bromopropane were delivered after dilution into noninhibitory quantities of CH_2Cl_2. Bottles were then injected with 2.0 mL of inoculated medium and agitated in the dark at 37°. Large vials were injected with small volumes of inoculated medium in order to buffer changes in partial pressures of oxygen and volatile agent under the brief culture conditions. Growth was judged through increased absorbance at 660 nm by means of a Perkin-Elmer lambda 3A spectrophotometer.

RESULTS AND DISCUSSION

Prototrophic *E. coli* can grow on medium free of both corrins and methionine by virtue of an accessory methyltransferase (EC 2.1.1.14) lacking a cobalamin cofactor [2,3]. *E. coli* 14169 is an auxotroph requiring either vitamin B_{12} or else methionine for lack of the accessory methyltransferase [3]. Exposure of the auxotroph to an atmosphere containing low levels of chloroform, carbon tetrachloride, or bromotrichloromethane resulted in aqueous levels which blocked growth in the absence, but not in the presence, of exogenous L-methionine. In contrast, growth of a prototrophic strain was not inhibited significantly by these vapors. A representative experiment is outlined in Table 1. The vapors are thus selective inhibitors of vitamin B_{12}-dependent methionine synthesis. The data of Table 1 were obtained near the stationary phase of growth. In other experiments, the initial vapor concentrations were determined at which the turbidity achieved at 37° in 8 hr (during the exponential phase of growth of untreated bacteria) was half that of untreated bacteria. A 50% reduction in optical density achieved in 8 hr occurred with initial gas-phase concentrations of 0.12% $CHCl_3$, 0.06% CCl_4, or 0.01% $CBrCl_3$. That concentration of $CHCl_3$ is below levels administered to higher organisms for anesthetic purposes. For instance, the alveolar gas concentration of $CHCl_3$ at which half of treated rodents lose the righting reflex is 0.84% [9]. In the presence of L-methionine at an initial concentration of 0.075 mM, the gas-phase levels of polyhalomethanes required for 50% inhibition of optical density achieved in 8 hr increased to 3.5, 1.9, and 0.11%, respectively. A mixture of other amino acids (L-asparagine, L-arginine, L-glutamic acid, glycine, L-histidine, L-proline, and L-tryptophan each at 100 mg/L [10]) did not similarly reverse growth inhibition under the conditions of Table 1.

Isolated methionine synthase is inactivated *in vitro* by 1-iodopropane and by 1-bromopropane [4]. Therefore, the less reactive 1-bromopropane was tested against intact cells (Table 1). 1-Bromopropane was less selective than the polyhalomethanes as an inhibitor of methionine biosynthesis. That is, the initial gas-phase concentrations required for 50% inhibition of optical density achieved at 8 hr were 0.08 and 0.15%, respectively, in the absence and presence of exogenous methionine (0.075 mM).

The chloroform-like compounds dichloromethane and 1,1,1-trichloroethane were not inhibitory at gas-phase concentrations of 2.0% (Table 1). Other agents noninhibitory at 2.0% were the anesthetics trichloroethylene ($CHCl=CCl_2$), sevoflurane [CHF_2-O-$CH(CF_3)_2$], methoxyflurane (CH_3-O-CF_2-$CHCl_2$), isoflurane (CHF_2-O-$CHCl$-CF_3), enflurane (CHF_2-O-CF_2-$CHClF$), and halothane (CF_3-$CHBrCl$) (data not

Table 1. Inhibition of vitamin B_{12}-dependent growth of *E. coli* by halocarbons

Agent	A_{660} (cm^{-1})		
	Auxotroph		Prototroph
	-Met	+Met	-Met
None	1.67	1.70	1.70
0.89% $CHCl_3$	0.00	1.70	1.64
0.49% CCl_4	0.00	1.65	1.66
0.02% $CBrCl_3$	0.00	1.68	1.66
0.12% $n\text{-}C_3H_7\text{-}Br$	0.25	1.69	1.67
2.00% $CH_3\text{-}CCl_3$	1.68		
2.80% CH_2Cl_2	1.73		

Prototrophic *E. coli* 14948 was cultured in minimal medium. The medium for auxotrophic *E. coli* 14169 was supplemented with 10 nM cyanocobalamin and, when present, 0.075 mM L-methionine. Aliquots of inoculated broth were incubated at 37° for 14 hr in bottles initially containing the indicated concentration of halocarbon vapor in air.

shown). Unlike nitrous oxide, the last three anesthetics do not inactivate methionine synthase significantly in mammals [11] although enflurane and halothane do yield photolabile organocobalt compounds upon nonenzymatic reaction with cobalamin in the presence of reducing agents *in vitro* [7]. Other compounds which covalently modify cob(I)alamin *in vitro* [8] but which, at 2.0% levels, did not inhibit growth of E. coli 14169 were acetylene, Freon 11 (CCl_3F), and Freon 12 (CCl_2F_2). Furthermore, at aqueous concentrations of 1.0 mM, there was no inhibition by chloral hydrate nor its metabolites trichloroacetate and 2,2,2-trichloroethanol, nor by 2,2-dichloroacetamide ($CHCl_2\text{-}CO\text{-}NH_2$), examined because of the dichloroacetamido moiety of chloramphenicol [7]. In all negative cases, turbidometrically judged growth in 8 hr at 37° was at least 95% of control.

It is interesting that *E. coli* 14169 was not inhibited significantly by 2.0% 1,1,1-trichloroethane despite close chemical similarity to chloroform and carbon tetrachloride. This observation raises the possibility that carbenes (or organocobalt carbenoid species) derived through two-electron reductions of the effective polychloromethanes [8,12] may be responsible for the inhibition of bacterial growth. Thus, the carbene derived from 1,1,1-trichloroethane is expected to rapidly rearrange to the less reactive compound vinyl chloride (Fig. 2) while rearrangements are not available to the one-carbon carbenes [12].

$$CCl_3\text{-}CH_2\text{-}H \xrightarrow{2e^-} :CCl\text{-}CH_2\text{-}H \longrightarrow H\text{-}CCl{=}CH_2$$

Fig. 2. Proposed rearrangement of carbene derived from trichloroethane.

Carbene rearrangements may be responsible for the failure of several of the tested compounds to inhibit the bacterium. Furthermore, the methionine-reversible inhibition of microbial growth by polyhalomethanes may involve inhibition of methionine biosynthesis as well as methyltransferase-mediated release of cytotoxic carbenes or other reactive products into free solution.

Chloroform and carbon tetrachloride are extensively metabolized in mammals

to chemically reactive products capable of inactivating diverse macromolecules in a virtually indiscriminant fashion [13]. Inhibition of methionine biosynthesis by these agents is thus unlikely to occur in a highly selective manner in mammals. In *E. coli*, however, low levels of the halocarbons prevented vitamin B_{12}-dependent methionine synthesis without significantly inhibiting other processes required for rapid growth in simple medium containing methionine. Inhibition of methionine synthesis (and of the methionine cycle [1]) may thus prove to contribute to the toxicity of the chlorocarbons to higher organisms, particularly in tissues poor in cytochrome P450. Furthermore, the results may pertain to antimicrobial effects of chlorinating agents (including leukocyte-generated hypochlorite) and to ecological effects of halocarbons. It is likely that some electrophilic/oxidant drugs and other bioactive agents in addition to N_2O [1-3] and, as shown here, $CHCl_3$, CCl_4, $CBrCl_3$, and 1-bromopropane will similarly prove capable of perturbing cobalamin function in intact cells.

Acknowledgements--T.A.A. is supported by the Parker B. Francis Young Investigator Award from the Foundation for Anesthesia Education and Research. The study was supported in part by NSF Grant DMB 85-05498 awarded to Prof. R.H. Abeles, Brandeis University.

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