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# STUDY OF METHYL BROMIDE REACTIVITY WITH HUMAN AND MOUSE HAEMOGLOBIN

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**ABSTRACT**

A study has been carried out on *in vitro* reactivity of human and mouse hemoglobin spectrophotometrically at physiological pH, using different protein to reagent ratios. Hemoglobin side chains were modified with different concentrations of methyl bromide on agro-soil fumigant. To ascertain if the site of alkylation was the reactive sulfhydryl group present at cystein – 93 on the β chain of hemoglobin (β-93 cys), a spectrophotometric measurement using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) was used to measure the free sulfhydryl groups before and after treatment of hemoglobin with various amounts of methyl bromide. The results show that the methylbromide reacted substantially with both human and mouse hemoglobin at β-93 cys.
The decrease in the number of sulfhydryl groups (SH) per hemoglobin molecule on addition of various concentrations of methylbromide ranges from 5.10 to 2.35 $\pm$ 0.01 and 5.01 to 0.93 $\pm$ 0.01 for human and mouse hemoglobin, respectively.
The results showed a dose-dependent decrease in the number of sulfhydryl group indicating that hemoglobin can serve as a biomarker of human occupational exposure to methy bromide fumigants.

**Keywords:** Hemoglobin, sulfhydryl groups, methylbromide, Cysteine, histidine.

## INTRODUCTION

Disinfestation of soil and composite is essential in modern intensive horticultural production (Commission of the EU Scientific Committee on Pesticides, 1985). High capital investment and high labour costs in such intensive horticulture necessitate a high degree of crop specialization, which involve the growing of only one or very few kinds of crops with year round production and very limited crop rotation (Commission of the EU Scientific Committee on Pesticides, 1985). Under these conditions, disease organisms and pests build-up in the soil and become a major limiting factor if not controlled. Control of soil-borne diseases and pests can be achieved by disinfestation of the soil or composite and by the use of chemicals such as methyl bromide (Hine, 1969).

Methyl bromide is a highly toxic gas. The highly toxic nature of methyl bromide coupled with its lack of odour presents hazards to applicators. This gas is widely used as a fumigant of soil and composite in areas out of doors and indoors. Fumigation with methyl bromide is the most widely used method of soil disinfestation in protected cropping and is effective for the control of a wide spectrum of pests and diseases. It is also a strong methylating agent and a powerful lipophilic solvent which easily penetrates and dissolves various rubber materials. Methyl bromide is easily absorbed in the lungs and through the skin. Both as a liquid and as a gas, it can cause itching, burning, severe pulmonary irritation and blistering upon contact with the skin (Johnson, 1945).

The acute effects in man and experimental animals after accidental or experimental inhalation have been reported (Van den Oever, *et al*, 1982; Smart 1990). A concentration of 40,000 mg/m$^{-3}$ has caused lung oedema, cyanosis, coma and death after only a few minutes exposure while exposure to 800 mg/m$^3$ for few hours could be tolerated without severe effects (Johnson, 1945; Hine, 1969).

Reports have shown that inhalation of sub-lethal concentrations of methylbromide may lead to severe and sometimes irreversible effects on the central nervous system with the following symptoms: headache, nausea, drowsiness, "pseudo" drunkenness tremors, hypersensitivity to noise, vision disturbance, speed defects and insomnia (Van den Oever, *et. al.* 1982; Hine, 1969; Johnson, 1945).

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Hine (1969) observed that after chronic or sub-chronic exposure, an increased bromide content is found in blood and urine and that the content of thiol moieties albumen and globulin in the blood is changed (Torkelson, and Rowe, 1982; Murphy, 1990).

Methyl bromide methylates the SH groups of cysteine, glutathione and several SH containing enzymes. Methylation of the SH group essential to cellular-oxidation has been suggested as the possible mechanism for the neurological effects of the compound (Murphy, 1980, Torkelson and Rowe, 1982; Buckell, 1949).

Occupational exposure hazards to methyl bromide occurs for a variety of occupations such as farmers and those living in the immediate neighbourhood of soils to which this fumigant has been applied. The mutagenic properties of methyl bromide were previously demonstrated by many workers in bacteria and barley and its alkylating properties are known (IARC, 1986; Ehrenberg, *et. al.* 1974; Djalali-Behzad, *et. al.* 1981; Moriya, *et. al.* 1983; Simmon, *et. al.* 1977; Ferranti, *et.al.* 1996). Reports have shown that methyl bromide alkylates human hemoglobin *in-vivo* and *in-vitro* at physiological pH and that the site of addution is specifically at the highly nucleophilic – SH group of β-93 Cys (Ferranti, *et. al.* 1996). Hemoglobin, the oxygen protein of erythrocytes is currently used as a biomarker for chemical exposure in the work place to measure the amounts of certain carcinogens in the body in order to determine whether an individual has suffered genetic damage and to estimate the possible risk of cancer from carcinogens (Bergmark, *et. al.* 1993; Farmer, *et. al.* 1986).

It has been shown that methyl bromide reacts readily with nucleophillic sites of hemoglobin including the N-terminal valine, cysteine, lysine and histidine (Osterman-Golkar, *et. al.* 1984; Farmer, *et. al.* 1986; Bergmark, *et. al.* 1993; Ferranti, *et. al.* 1996).

At present, very little is known about amino acid reactivity towards methyl bromide within protein and most of the experiments have been carried out on free amino acides or model peptides (Dunkelburg, 1980). We have therefore studied the *in-vitro* reactivity between Methyl bromide and hemoglobin spectrophotometrically to determine if methyl bromide alkylates human and mouse hemoglobin at physiological pH and if the site of methylation is specifically at the highly nucleophilic sulfhydryl group of β--93. This work is also aimed at determining whether there is a relationship between the exposed conditions and the extent of methylation.

## MATERIALS AND METHODS

Blood from normal human donors was obtained from the Blood Bank, University College Hospital, Ibadan in Nigeria. Mouse blood was obtained from the Veterinary Hospital, University of Ibadan. The 5,5-dithiobis (2-nitrobenzoic acid (DTNB) used was a product of Sigma St. Louis, USA. Methyl bromide (98% purity) was a product of Merck & Co. Inc. Manufacturing Chemists Rahway, N.J., USA. 99% Absolute Ethanol was used.

Hemoglobin was prepared according to normal laboratory procedures (Antonini and Brunori, 1971). Samples were deionized by passage through Sephadex $G_{25}$ (medium). The solution of methylbromide for *in-vitro* measurement was prepared by bubbling gaseous Methyl bromide (MeBr) in cold pure ethanol. The concentration of the MeBr solution was determined from the increase in the weight. Hemoglobin concentration of 4μM tetramer in phosphate buffer 7,4, I=0.05M was used throughout the experiment. 1 mM ethanoic MeBr was prepared as stock solution. The hemoglobin concentrations were determined by Drabkins method as modified by Van Kampen and Zijlstra (1982), 3.0 mM DTNB stock solution was prepared for use.

The alkylating capability of MeBr towards sulfhydryl groups was assessed by a spectrophotometric technique with DTNB. DTNB permits the determination of sulfhydryl group in simple compounds and has been used to monitor the disappearance of free SH groups in hemoglobin following exposure to alkylating agents (Okonjo and Okia, 1983). The hemoglobin was reacted with equimolar – 2, -5,-10,-20 and –30 fold excess MeBr over human hemoglobin. Both reaction mixtures were maintained at room temperature for one hour and analysed for protein SH content according to the method of Okonjo and Okia (1993). All reactions were carried out in triplicate at room temperature and in phosphate buffer pH 7.4 of ionic strength 0.05M. The sulfhydryl concentrations were determined by dividing the Absorption Maxima Δmax by the molar extinction coefficient 13,000 $m^{-1}$ $cm^{-1}$ assumed for 5 thio-2 nitro-benzoate (TNB) the product of the reaction. The ratios of hemoglobin-SH concentration to

hemoglobin were calculated by dividing the appropriate values. This procedure was repeated for mouse hemoglobin using –5, -10, -20, -50, -100, and –150 fold excess MeBr over mouse hemoglobin, which was obtained with capillary tube at the Veterinary Physiology Department, University of Ibadan in Nigeria.

The above procedure was carried out for the control experiment without the addition of methyl bromide.

## RESULTS AND DISCUSSION

Human and mice hemoglobin reacted with Methyl bromide (MeBr) *in-vitro* at pH 7.4 resulted in the decrease in the number of sulfhydryl group per hemoglobin molecle. Following the reaction between MeBr and hemoglobin, there was a clear dose – dependent decrease in sulfhydryl concentration with increasing amounts of MeBr (Figures 1 and 2).

In order to investigate if hemoglobin might serve as a biomarker of exposure for MeBr encountered in the workplace, human hemoglobin was alkylated at physiological pH by the MeBr to ascertain if the site of alkylation was the reactive sulfhydryl present at cysteine – 93 (β-93 Cys) on the β-chain of hemoglobin a spectrophotometric investigation using DNTB was used to measure the free sulfhydryl groups before and after treatment of MeBr, using Cecil Spectrophotometer, Model CE 1021, 1000 Series. Results indicate that the MeBr reacted with the sulfhydryl group and gave a dose-dependent decrease in the sulfhydryl to hemoglobin ratio as measured by this method (Tables 1 and 2). A similar experimental design has been used to monitor the disappearance of free sulfhydryl groups after exposure to a number of different alkylating agents *in-vitro*, including styrene oxide (Neis, *et. al.* 1984). With five fold excess MeBr to human hemoglobin, the sulfhydryl concentration had decreased to 89.5% of the control, at the highest dose of 150 fold excess, the sulfhydryl concentration was 41% of the control (Figure 4). After reaction of MeBr with mice hemoglobin, the sulfhydryl concentration decreased to 91% of the control, with two fold excess MeBr to hemoglobin. At the highest does of 30 fold excess, the sulfhydryl concentration was only 17% of the control (Figure 3) indicating extensive modification of the two reactive sulfhydryl groups. The number of SH group 5.7 and 5.5 for human and mouse (Tables 3 and 4) hemoglobin determined in this study, in the absence of MeBr, is reasonable under experimental conditions if compared with the 6.0 found in the native hemoglobin.

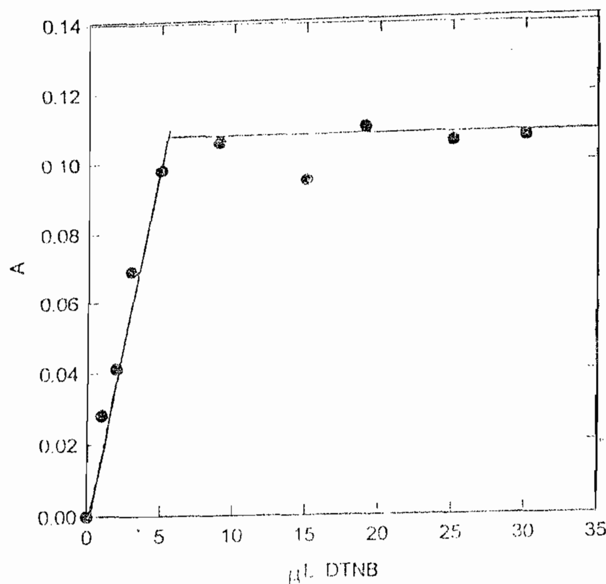


Figure 1 Titration of human hemoglobin with DTNB; change in absorbance at 412nm, ΔA as a function of the volume of DTNB mixed with 3 ml of 50 fold excess MeBr over hemoglobin. Conditions: Hemoglobin concentration, 4μM; stock DTNB concentration 3.0 mM; phosphate buffer pH 7.4 (ionic strength 0.05M, added salt, NaCl). ΔA values were corrected for dilution. The maximum change in absorbance ΔAmax is 0.105. Number of sulfhydryl groups per tetramer = 3.7.

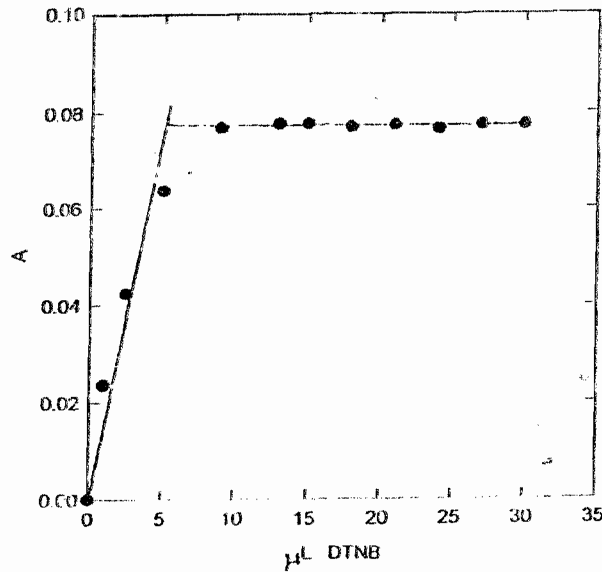


Figure 2: Titration of human hemoglobin with DTNB; change in absorbance at 412nm, ΔA as a function of the volume of DTNB mixed with 3 ml of hemoglobin. Conditions: Hemoglobin concentration, 4μM; stock DTNB concentration 3.0 mM, phosphate buffer pH 7.4 (ionic strength 0.05M, added salt, NaCl). ΔA values were corrected for dilution. The maximum change in absorbance ΔAmax is 0.0775. Number of sulfhydryl groups per tetramer = 5.7.

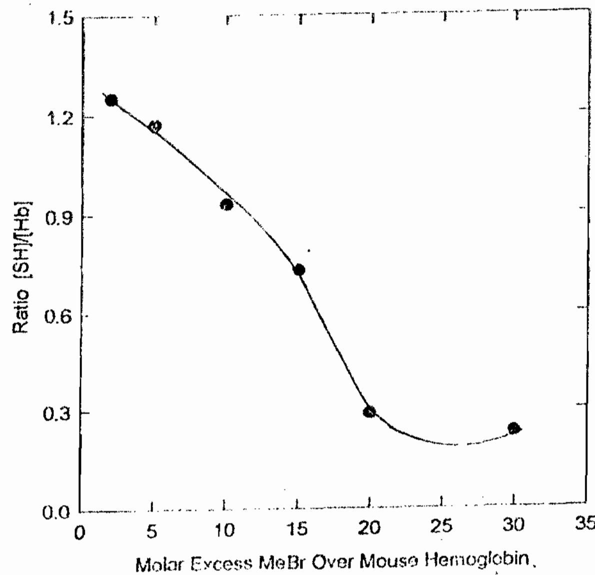


Figure 3: The ratio of sulfhydryl to mouse hemoglobin concentration following treatment with 2-, 5-, 10-, 20-, and 30-fold MeBr. Measurements were made spectrophotometrically with DTNB at pH 7.4, phosphate buffer (ionic strength 0.05M ). [Hb] = 4 x $10^{-6}$M. Number of -SH group per tetramer of the control experiment = 5.5.

The results of Tables 1 and 2 show that the sulfhydryl groups of mouse hemoglobin react more with MeBr than with human hemoglobin indicating that although MeBr alkylates human and mouse hemoglobin, β-93 cys is not probably the major alkylating target.

This study thus demonstrate that MeBr chemically reacts with both human and mouse hemoglobin at physiological pH. The reaction of MeBr produced a decrease in the human and mouse hemoglobin sulfhydryl concentration (down to 41% of control with a 150 fold excess MeBr with human hemoglobin and 16% of the control with 30 fold excess MeBr with mouse hemoglobin: Tables 1 and 2). This observation confirms the validity of this method and that the sulfhydryl groups were indeed free and reactive.

The spectrophotometric experiment determined specifically that β-93 cys was not the major alkylation target by MeBr in Mouse

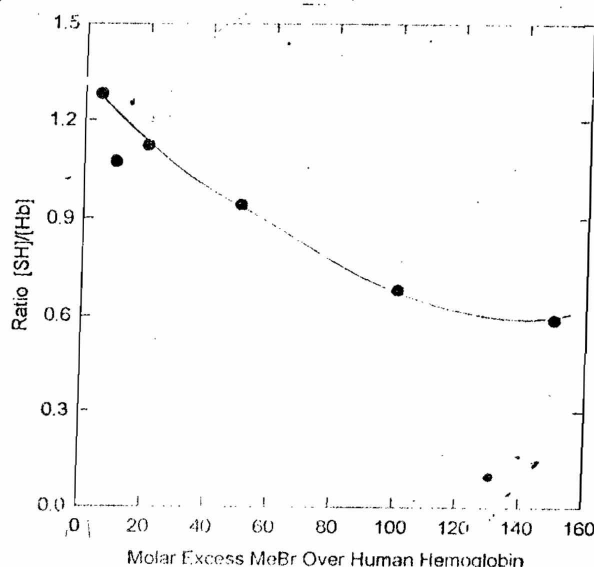


Figure 4: The ratio of sulfhydryl to human hemoglobin concentration following treatment with 5-, 10-, 20-, 50-, 100-, and 150-fold excess MeBr. Measurements were made spectrophotometrically with DTNB at pH 7.4, phosphate buffer (ionic strength 0.05M ) [Hb] = 4 μM. Number of -SH group per tetramer of the control experiment = 5.7.

Table 1: Reaction of Mouse hemoglobin showing the molar concentrations of SH group and hemoglobin after treatment with different concentrations of MeBr.

| Molar Excess MeBr | [SH] x $10^{-6}$ | [SH]/[Hb] | % of the control |
|---|---|---|---|
| 2 | 5.01 | 1.25 | 91.09 |
| 5 | 4.69 | 1.17 | 85.27 |
| 10 | 3.72 | 0.93 | 67.64 |
| 15 | 2.91 | 0.73 | 52.90 |
| 20 | 1.16 | 0.29 | 21.09 |
| 30 | 0.93 | 0.23 | 16.91 |

Titration of mouse hemoglobin was made spectrophotometrically with DTNB, conditions (Hb) = 4μM, Phosphate Buffer 7.4, I = 0.05M, Number of SH per tetramer for the control experiment = 5.5

hemoglobin (Tables 1 and 2 compared). Performing an identical alkylation experiment with human hemoglobin did not produce an analogous decrease in the number of free SH group per mole of hemoglobin with increasing amount of MeBr (Tables 1, 2 and Figs. 1 and 2). This strongly suggests that β-93 cys of hemoglobin is not the only target for alkylation by the MeBr. It should be noted, however, that in all strains of hemoglobin, primary structure differs from that of human hemoglobin (Kleinschmidt and Spouros, 1987). Hemoglobin from certain strains of BALB/C and Mus Musculus contain cysteine at position 13 on the β-chain (apart from that at position 93 on the β-chain) and is 10 times more reactive than β-chain cys in human hemoglobin toward ethylene oxide (Segerback, 1994). The presence of a reactive cysteine at the 13 position could explain the measurable levels of alkylation on cysteine by MeBr in this report. Reports have also shown that the α-chain cysteine has not been accessible to alkylating agents (Erve, *et. al.* 1996).

The β-93 cys sits in the heme pocket

Table 2: Reaction of human hemoglobin with MeBr showing the ratio of the molar concentrations of SH group and hemoglobin after treatment with molar excess MeBr.

| Molar Excess MeBr | [SH] x $10^{-6}$ | [SH]/[Hb] | % of the control |
|---|---|---|---|
| 5 | 5.10 | 1.28 | 89.47 |
| 10 | 4.28 | 1.07 | 75.09 |
| 20 | 4.48 | 1.12 | 78.60 |
| 50 | 3.74 | 0.94 | 65.61 |
| 100 | 2.72 | 0.68 | 47.72 |
| 150 | 2.35 | 0.59 | 41.23 |

The spectrophotometric titration of the hemoglobin was carried out with 5,5'-dithiobis (2-nitrobenzoate), (DTNB). Number of SH per hemoglobin tetramer the control experiment = 5.7

which has substantial hydrophobic character that allows some aromatic alkylation species to react at β-93 cys (Ring, *et. al.* 1998).

However analysis by tandem fast atom bombardment mass spectrometry (FAB/MS) has shown that cysteine, residues, the main reactive sites, were fully methylated in hemoglobin exposed to a 10 fold excess of MeBr differently from other residues including histidines and that only specific residues (the N-terminal amino group of both α and β globins (not cystein 112) in the β-chain) were reactive indicating a different accessibility to reaction of the SH groups in the protein chain (Ferranti, *et. al.* 1996). The hemoglobin side chains were selectively modified and the degree of modification at each site is a function of the position of the single amino acid residue within the protein quarternary structure, raising the possibility that alterations of structure and functional properties of human hemoglobin following exposure to alkylating agents may be mediated through such covalent protein modifications.

Hemoglobin modification by MeBr includes the binding site of 2,3-diphosphoglycerate (2,3 DPG) (Ferranti, *et. al.* 1996). The co-factor binds electro-statically to the β-chain of deoxy-hemoglobin at a specific site in the entrance of the central cavity along the dyad axis of symmetry (Bunn and Forget, 1986).

The phosphate groups of 2,3 DPG form salt bonds with the β-N-terminal group and the imidazole of β-2 and β-143 histidine residues all of which are methylated with MeBr (Ferranti, *et. al.* 1996). As a consequence of methylation of even one of these sites, the transition between deoxy and oxy-hemoglobin can altered, α Tyr 140 and β- Tyr 145 are among the few Tyr residues susceptible to MeBr action, together with Aspartic 94 (the only reactive aspartic residue) and histidine 146 in the β-chain. Modification of one or more of the above residues as a consequence of exposure to MeBr or similar compound would lead to stabilization of the oxygenated form of hemoglobin with subsequent alteration of the functional properties and clinical effects earlier enumerated.

This work illustrates the importance of protein tertiary structure surrounding a functional group in addition to an existence of a reliable relationship between the exposure conditions and the extent of alkylation of a particular amino acid residues.

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