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Hemoglobin-Glutathione Relationships in Trout Erythrocytes Treated with Monochloramine

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Studies of the effects of monochloramine (NH_2Cl), a widely used drinking- and waste-water disinfectant, on human (EATON et al. 1973) and fish (BUCKLEY 1977) erythrocytes *in vivo* have indicated oxidation of hemoglobin (Hb), and secondary to oxidant damage, hemolysis. In human erythrocytes *in vitro*, NH_2Cl causes inhibition of hexose monophosphate shunt activity, critical to maintaining glutathione in reduced form (EATON et al. 1973). Studies of the interaction of reduced glutathione (GSH) with endogenous and exogenous oxidants pertinent to Hb oxidation and to other sensitive compounds, particularly those of the cell membrane, have revealed different patterns of oxidation of Hb and GSH that may be oxidant-specific (MILLER & SMITH 1970). The following report describes the relationship between Hb and GSH in NH_2Cl-treated rainbow trout erythrocytes.

MATERIAL AND METHODS

Phosphate-buffered saline (PBS) (10 mM, pH 7.4, 137 mM NaCl, 2.7 mM KCl) plus glucose (5.6 mM) was prepared in deionized water. Solutions of NH_2Cl were prepared immediately before use by the addition of equimolar amounts of sodium hypochlorite (NaOCl) and ammonia (from standardized NaOCl and NH_4Cl) to PBS + glucose, and measured amperometrically (APHA 1976).

Reduced glutathione was measured by the method of BEUTLER et al. (1963) with standards prepared in 0.01% EDTA and verified by measurement of the molar extinction coefficient. The reaction of NH_2Cl and GSH in PBS + glucose (cell suspension medium) was determined by spectrophotometric titration of excess GSH. Measurements were made immediately after mixing and addition of reagents as the reaction was very rapid at 20°C. Hemoglobin was measured in 0.45 µm-membrane-filtered hemolysates by the cyanmethemoglobin method, which measured the total of all pigments present, from the absence of significant difference ($P = 0.01$, Student t-test) in Hb content between control and

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NH_2Cl-treated erythrocyte suspensions. Methemoglobin (MHb) was measured in 0.45 µm-membrane-filtered hemolysates by the method of EVELYN & MALLOY (1938) and expressed as a percentage of the total oxyhemoglobin (HbO_2) + MHb (HARLEY & MAUER 1960). Denatured Hb, herein collectively called sulfhemoglobin (SHb), was estimated from the loss of the total of HbO_2 + MHb in NH_2Cl-treated vs. control hemolysates (also called "loss of intact Hb" by HARLEY & MAUER 1960) and correlated with measurements of SHb extinction (E) at 620 nm (EVELYN & MALLOY 1938) (see Fig. 2).

Washed erythrocytes (minus buffy coat) were prepared from blood drawn from unanesthetized rainbow trout, Salmo gairdneri ($\bar{x}$ = 410 g), and suspended in cold PBS + glucose. Control and experimental suspensions, expressed as molar ratios of NH_2Cl to Hb (trout Hb: tetramers of α and β chains with same overall molecular structure as mammalian Hb (BRUNORI 1975)) were prepared by the addition of 0.5 ml of erythrocytes with known Hb content to 14.5 ml of 15°C PBS + glucose with known NH_2Cl content (zero in controls). Immediately after mixing, erythrocytes were washed twice in 30 volumes of cold PBS + glucose + 1% heat-inactivated fetal calf serum (as a protective measure against the trauma of washing) and analyzed. Recovery of Hb and GSH during prolonged incubation was investigated and reported elsewhere (BUCKLEY 1981).

Hemolysate was prepared for dialysis for removal of GSH by lysis of washed erythrocytes (GSH content 0.84 mM) in deionized water, centrifuged for removal of stroma, frozen once in liquid nitrogen, and dialysed against 100 volumes of PBS at 4°C for 16 - 18 h with two changes of dialysate. The dialysed hemolysate was then filtered (0.45 µm membrane) prior to use (GSH content, not detectable).

RESULTS

Erythrocytes

GSH. The relationship between depletion of GSH and concentration of NH_2Cl, expressed as a molar ratio to initial GSH, is presented in Fig. 1. The percentage of the initial GSH remaining after NH_2Cl treatment is calculated from the regression equation with a 4/1 molar ratio of NH_2Cl to GSH:

$$GSH = 100.8 + (-5.08)\ 4 = 80\ (\pm 5.1)\% \quad (1)$$

$$GSH = 1.0\ (0.8) = 0.8\ (\pm 0.015)\ mM \quad (2)$$

Of the original GSH, 0.8 mM remains and 0.2 mM has been oxidized. The standard error of the estimate (S.E.E.) is contained in the answer.

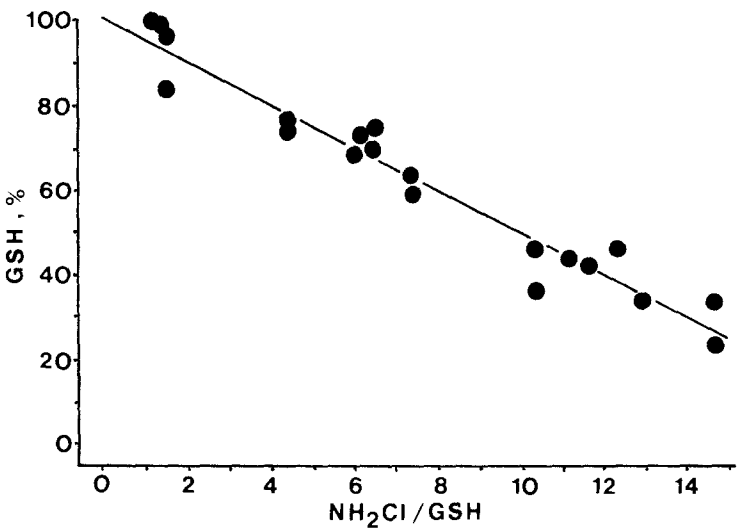

Fig. 1. Erythrocytes
Y = 100.8 + (-5.08) X
r = - 0.973
Syx = 5.1

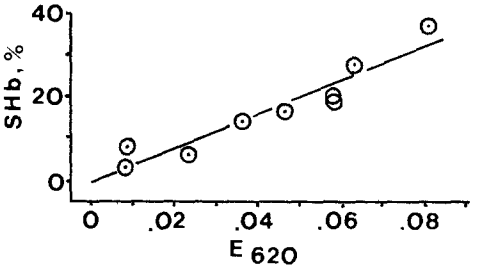

Fig. 2. Hemolysates
Y = 0.5 + 404 X
r = 0.944
Syx = 3.4

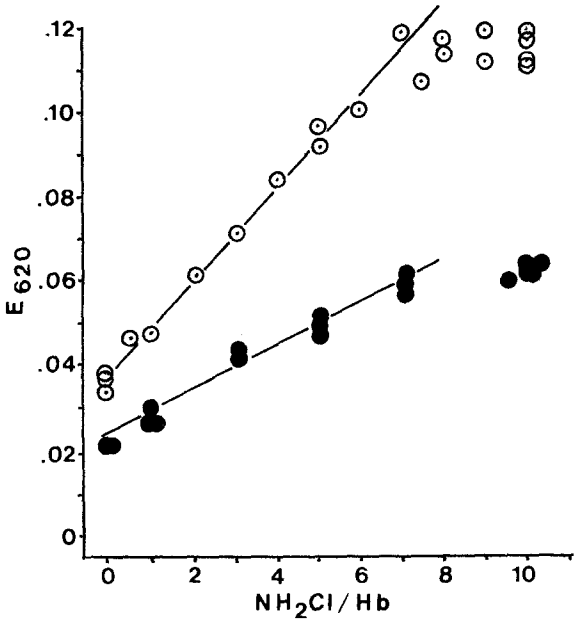

Fig. 3.

Erythrocytes ●
Y = 0.024 + 0.005 X
r = 0.985
Syx = 0.003

Dialysed
Hemolysates ⊙
Y = 0.037 + 0.011 X
r = 0.999
Syx = 0.001

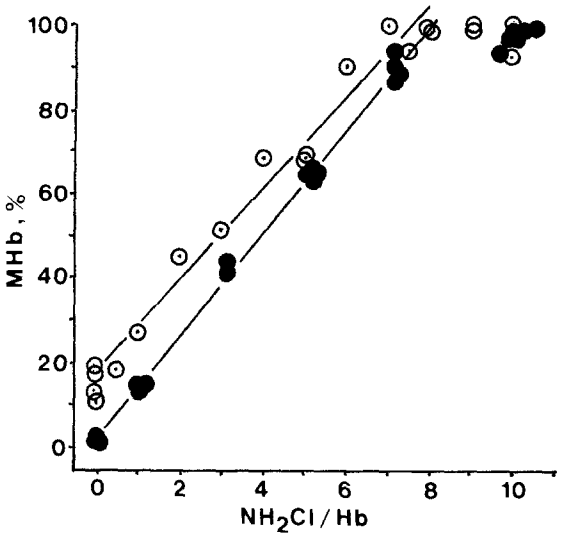

Fig. 4.

Erythrocytes ●
Y = 2.4 + 12.2 X
r = 0.999
Syx = 1.7

Dialysed
Hemolysates ⊙
Y = 17.6 + 10.8 X
r = 0.991
Syx = 4.8

The slopes are significantly different at $\alpha = 0.05$ but not at $\alpha = 0.025$

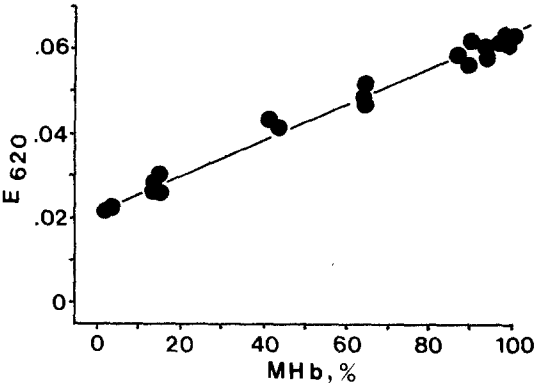

Fig. 5.

Erythrocytes treated with NH_2Cl
Y = 0.022 + (4.2×10^{-4}) X
r = 0.995
Syx = 0.003

SHb. The percentage of the SHb formed was estimated from the regression equations in Figs. 2 and 3.

$$SHb,\% = \frac{(slope)(slope_h)(NH_2Cl/Hb)(slope_e)(NH_2Cl/_{Hb})}{(slope_h)(NH_2Cl/Hb)} \quad (3)$$

Subscripts e and h denote erythrocytes and hemolysate (Fig. 3), respectively. Eq. (3) simplifies to:

$$SHb,\% = (slope)(slope_e)(NH_2Cl/Hb) \quad (4)$$

For example, with a 4/1 molar ratio of NH_2Cl to Hb:

$$SHb = 404\ (0.005)(4) = 8\ (\pm\ 1.4)\% \quad (5)$$

$$SHb = (1.0)(0.08) = 0.08\ (\pm\ 0.014)\ mM \quad (6)$$

MHb. The relationship between MHb formation and NH_2Cl treatment is shown in Fig. 4. The percentage of MHb of the total undenatured Hb (HbO_2 + MHb) is calculated from the regression equation. For example, with a 4/1 molar ratio of NH_2Cl to Hb:

$$MHb = 12.2\ (4) = 48.8\ (\pm\ 1.7)\% \quad (7)$$

Of the total undenatured Hb, approximately 49% is MHb and 51% is unreacted or HbO_2. The amount of MHb is calculated from the original 1.0 mM Hb and the 0.08 mM SHb (Eq. 6) as follows:

$$MHb = (1.0 - 0.08)(.49) = 0.45\ (\pm\ 0.008)\ mM \quad (8)$$

It follows that the amount of unreacted Hb is:

$$Hb = (1.0 - 0.08)(.51) = 0.47\ (\pm\ 0.009)\ mM \quad (9)$$

Equation for Hb oxidation and GSH depletion. The stoichiometry of the reaction of NH_2Cl with GSH and Hb in erythrocytes is outlined in the following equation, in which the product values (mM) are from Eqs. 6, 8, 9, and 2, respectively (S.E.E. omitted for clarity):

$$4.0\ NH_2Cl + 1.0\ Hb + 1.0\ GSH \rightarrow 0.08\ SHb + 0.45\ MHb + 0.47\ Hb + 0.8\ GSH + 0.2\ (GSSG, RSSG) \quad (10)$$

The oxidized glutathione most likely takes the form of GSSG and/or RSSG, where the latter consists of mixed disulfides with globin (ALLEN & JANDL 1961, SRIVASTAVA & BEUTLER 1970).

Hemolysate

The amounts of MHb and SHb formed in dialysed hemolysate were calculated from the regression equations in Figs. 2, 3, and 4, in the same manner as for erythrocytes, with a molar ratio of NH_2Cl to Hb of 4/1 for consistency with previous calculations:

$$SHb = 404\ (0.011)(4) = 18\ (\pm\ 1.6)\% \quad (11)$$

$$SHb = 1.0\ (0.18) = 0.18\ (\pm\ 0.016)\ mM \quad (12)$$

$$MHb = (10.8)(4) = 43\ (\pm\ 4.8)\% \quad (13)$$

$$MHb = (1.0 - 0.18)(.43) = 0.35\ (\pm\ 0.034)\ mM \quad (14)$$

$$Hb = (1.0 - 0.18)(.57) = 0.47\ (\pm\ 0.027)\ mM \quad (15)$$

NH_2Cl - GSH reaction. The reaction of reduced glutathione was approximately equimolar with the initial concentration of NH_2Cl (slope = - 0.94, r = - 0.999).

DISCUSSION

The empirically derived equation for the oxidation of Hb and depletion of GSH by NH_2Cl in rainbow trout erythrocytes provides a quantitative basis for examining the relationship between reactants and products during rapid oxidative changes in erythrocytes. Using the results of preceding calculations, the proportion of NH_2Cl consumed in the formation of

the products can be estimated. The depletion of 0.2 mM of GSH required approximately 0.2 mM of NH_2Cl. A portion of the other 3.8 mM NH_2Cl (4.0 - 0.2 = 3.8) reacted with Hb to form 0.45 mM MHb and 0.08 mM SHb. It is likely that the iron of SHb was oxidized to the ferric state; therefore, 2.12 mM NH_2Cl (4 (0.45 + 0.08) = 2.12) was consumed in the oxidation of iron. The remaining 1.68 mM NH_2Cl (4.0 - (0.2 + 2.12) = 1.68) was consumed in the formation of 0.08 mM SHb and, presumably, other products. The amount of NH_2Cl required to form 0.08 mM SHb in erythrocytes can be estimated from: (1) 0.18 mM SHb formed by 1.88 mM NH_2Cl (4.0 - (4 (0.35 + 0.18)) = 1.88) in hemolysate (Eqs. 12, 14), and (2) the ratio of the slopes of the regression lines in Fig. 3.

$$NH_2Cl = (0.08)(1.88)(2.2)/0.18 = 1.84 \text{ mM} \quad (16)$$

The fair agreement between the two estimates, 1.68 and 1.84, indicates that: (1) unmeasured cytosolic oxidation products, including noniron heme reactions, presumed to be present, accounted for an unknown fraction of the NH_2Cl (implicated in the formation of SHb) to approximately the same extent in both erythrocytes and hemolysate and, (2) reactions with cell membrane compounds were minimal. The latter conclusion is supported by only slight (3.8%) hemolysis when incubation was prolonged (BUCKLEY 1981).

The production of SHb prior to complete (100%) MHb formation raises the question of equivalence to oxidation of the four hemes per tetramer. Perhaps, as is the case with human Hb (ITANO & ROBINSON 1956), incomplete oxidation results in a mixture of tetramers in all stages of completeness of heme oxidation. The multiplicity of trout Hbs, with their differing properties (BRUNORI 1975), could contribute to the presumed differential sensitivity to oxidation. The relationship shown in Fig. 5 in which MHb and SHb increase in concert, is consistent with differential sensitivity to oxidation. The physical basis may be that single heme oxidation induces conformational changes primary to oxidation of globin groups critical to denaturation. Conversely, oxidation of these critical groups could alter quaternary and tertiary structure, thereby increasing the susceptibility of heme to oxidation. Finally, it is also possible that simultaneous MHb and SHb formation is simply due to the very reactive nature of NH_2Cl and iron, and noniron reactions occur indiscriminately as NH_2Cl diffuses into the cell. The first explanation is consistent with the hypothesis that MHb production is an essential step in the formation of Heinz bodies (ALLEN & JANDL 1961, HARLEY & MAUER 1961), which

correlate with the appearance of SHb (ALLEN & JANDL 1961). However, this relationship is in question because of reports of an absence of correlation between MHb and the oxidative precipitation of Hb (BEUTLER & BALUDA 1962, RENTSCH 1968).

Glutathione, present in 0.8/1 molar ratio with Hb, did not exert a complete protective effect toward Hb, as evidenced by Eq. 10 and by its reciprocal relationship with MHb ($r = -0.960$) and SHb ($r = -.912$). This may be a characteristic of exogenous oxidants, which diffuse rapidly into cells owing to high lipid solubility and inactivate (EATON et al. 1973) or bypass (JACOB & JANDL 1966) metabolic defenses adequate for endogenous oxidants. The oxidant damage to human erythrocytes by acetylphenylhydrazine (APHZ) has been explained by its ability to bypass glutathione peroxidase despite persisting GSH (JACOB & JANDL 1966), to the extent that Hb is more susceptible to oxidation than GSH (DESFORGES 1962). In several species of mammals, GSH and Hb were oxidized in concert in APHZ-treated erythrocytes (KURIAN & IYER 1977). In this work also, GSH did not retard MHb formation (Fig. 4) but may have acted to retard SHb formation (Fig. 3). Reduced glutathione has been shown to block precipitation of Hb by APHZ treatment (ALLEN & JANDL 1961). The inhibition of 0.1 mM SHb (Eq. 12 minus Eq. 6) probably did not lie solely in the extra 0.2 mM NH_2Cl (Eq. 10) consumed by cellular GSH, because 1.68 to 1.84 mM NH_2Cl is implicated in the formation of a slightly lesser amount of SHb (0.08 mM). It is possible that, as has been reported for human hemoglobin (ALLEN & JANDL 1961, SRIVASTAVA & BEUTLER 1970), the 0.2 mM GSH reacted with 0.2 mM of the critical (β chain) globin sulfydryl groups, forming mixed disulfides and preventing oxidative denaturation of 0.1 mM SHb. Nonextinction at 620 nm of these mixed disulfides is critical to this explanation.

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