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METHAEMOGLOBIN FORMATION INDUCED BY THIOLS

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Abstract—The ability of various thiols to promote an oxidation of haemoglobin has been investigated. Methaemoglobin is found to represent the main oxidation product. Following the appearance of this pigment, however, trace amounts of a greenish precipitate are also regularly formed. The results indicate that oxygen is the ultimate electron acceptor, and that the thiols act by facilitating a one-electron transfer from the ferrous ion of haem to oxygen. Under these processes free radicals seem to be generated. The possibility of a haemoglobin–peroxide–thiol complex as an intermediate has been considered. Neither hydrogen peroxide nor any oxidation products of the thiol seem to be directly involved in the mechanism of oxidation. The results are briefly discussed in relation to the haemolytic effect of certain drugs on erythrocytes deficient in glucose-6-phosphate dehydrogenase.

IT IS well known that several reducing substances in the presence of oxygen bring about an oxidation of haemoglobin.[1] This oxidation usually leads in part to methaemoglobin in part to green oxidation derivatives of haemoglobin. The latter products constitute a group of compounds in which the tetrapyrrole nuclei have become oxidized.[2, 3] Although all the products demonstrate an increased absorption of red-infrared light, their spectra differ slightly depending upon the agent used for their preparation (sulfhaemoglobin,[4] pseudohaemoglobin,[5] choleglobin[6]). Sometimes the oxidation of the haem group is accompanied by a denaturation of the globin residue, leading to precipitation.[1] The exact mechanisms for the above modes of oxidative degradation are still unknown.

Drugs or metabolic products of such, which are known to cause methaemoglobinaemia[7] with or without simultaneous Heinz body formation,[8] in general seem to be reducing substances. Within the last years a number of these drugs are found to induce haemolytic anaemia in a group of patients with a deficient activity of the glucose-6-phosphate dehydrogenase in their red cells.[9, 10] The oxidizing properties of these drugs on haemoglobin seem to be related to their haemolytic effect.[11–13] This relationship, therefore, has led to a renewed interest in the oxidative breakdown of haemoglobin by such chemical agents.

Recently we have shown that the thiol, diethyldithiocarbamate, when added to erythrocytes, leads to a disappearance of the intracellular GSH as well as to an inhibition of the glucose metabolism.[14] A methaemoglobin-forming ability of diethyldithiocarbamate was found to be essential in the mechanism underlying these changes. In the present paper we report in detail on the oxidizing properties on haemoglobin of diethyldithiocarbamate as well as of other thiols.

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EXPERIMENTAL

Materials

Diethyldithiocarbamate was obtained from E. Merck, AG, Darmstadt, Western Germany, and cysteamine from Fluka, AG, Chemische Fabrik, Buchs, Switzerland. Cystamine was prepared by oxidation of cysteamine with I_2. The disulphide was purified by recrystallization from an ethanol–HCl–ether solution. ^{35}S-labelled cystamine was purchased from The Radiochemical Centre, Amersham, Bucks., England. ^{35}S-labelled cysteamine was obtained by adding trace amounts of the radioactive disulphide to a solution of inactive thiol immediately before use. Other chemicals used were commercial products of high purity.

Methods

Washed human erythrocytes were prepared as previously described.[14] Cells containing methaemoglobin were obtained by treatment with amyl nitrite.[15] In experiments with intact erythrocytes, the washed packed cells were suspended in a phosphate-buffered saline, pH 7·4, containing (mequiv/l.): Na^+ ion, 163; K^+ ion, 6·7; Cl^- ion, 110; HPO_4^{2-} ion, 33. Haemolysate was made by twice freezing and thawing the washed erythrocytes suspended in a phosphate-buffered saline, pH 7·4, containing (mequiv/l.): Na^+ ion, 67; K^+ ion, 103; Cl^- ion, 103; HPO_4^{2-} ion, 33. Before use the haemolysate was freed from stroma by centrifuging at 20,000 × *g* for 20 min.

All experimental incubations were performed at 37°. Anaerobic conditions, when wanted, were obtained as previously described.[14] Methaemoglobin was measured by the method of Horecker and Brackett[16] after centrifuging the diluted samples at 20,000 × *g* for 10 min. The total haemoprotein was determined spectrophotometrically as cyanmethaemoglobin by the method of Ceilous.[17] Absorption spectra were recorded by a Zeiss RPQ 20A recording spectrophotometer.

Globin-bound ^{35}S-cysteamine was determined by precipitating the haemolysate with 10% trichloracetic acid; after washing twice, the precipitate was dissolved in 0·2 N NaOH, and suitable aliquotes of this solution were plated for radioactive counting in an end-window GM counter. To exclude any attachment of cysteamine to the haem group, the haem was in certain instances split from the globin by re-suspending the precipitate in an acetone–HCl (1%) solution. No difference was found between the radioactivity of the globin residue and the complete haemoglobin. The presence of small amounts of proteins other than haemoglobin in the haemolysate were not corrected for.

In order to detect the formation of possible oxidation products of cysteamine during the incubation, the supernatants from the trichloracetic acid precipitations were subjected to high voltage paper electrophoresis. This was carried out in a phtalate buffer, pH 2, both with and without 0·02 M $HgSO_4$ as described by Eldjarn and Pihl.[18] This method permits a separation of the thiol and the disulphide as well as of other oxidation products. The distribution and the amounts of radioactive components on the paper electrophoretogram were determined in a Frieske and Hoepfner strip counter.

The ability of diethyldithiocarbamate to initiate an oxidation of sulfite to sulfate by atmospheric oxygen was examined by measuring the oxygen uptake in a conventional Warburg apparatus. These experiments were conducted in the same buffer as used for the haemolysate preparation, but the buffer contained in addition $1·3 \times 10^{-3}$ M EDTA to prevent autoxidation of sulfite.

RESULTS

Haemoglobin-oxidizing properties of various thiols in the presence of oxygen

Thiols are in general reducing substances. In accord with this property we find that in haemolysate a number of thiols reduce methaemoglobin (preformed by the amyl nitrite treatment) to haemoglobin (Fig. 1). Following the initial reduction, however, a reformation of this pigment takes place (Fig. 1). The ability of thiols to promote an

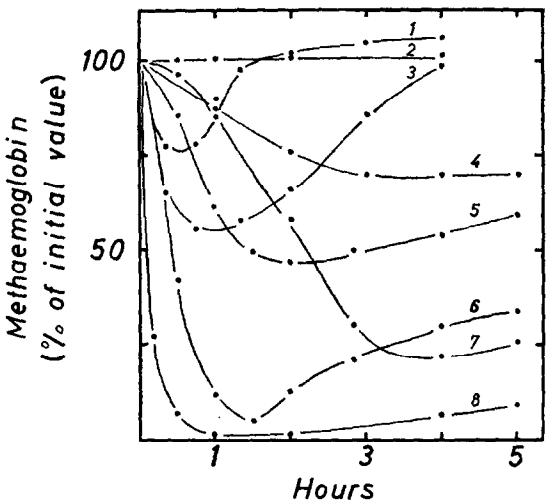

FIG. 1. Methaemoglobin-reducing ability of various thiols. Haemolysates, in which about 70 per cent of the total haemoprotein (approx. conc.: 5·5 g/100 ml) initially was methaemoglobin, were incubated in the presence of the thiol (10^{-2} M) as follows: curve 1, diethyldithiocarbamate; curve 2, ergothioneine; curve 3, dimethyldithiocarbamate; curve 4, glutathione; curve 5, cysteamine; curve 6, penicillamine; curve 7, thioglucolate; curve 8, dithiopentaerythrite. Methaemoglobin concentrations are expressed as per cent of the initial concentration.

oxidation of haemoglobin to methaemoglobin is more clearly demonstrated when initially no methaemoglobin is present in the haemolysate (Fig. 2). It will be seen that the formation of the pigment proceeds almost linearly for hours, but at a different rate depending upon the thiol used. A delay period for the appearance of methaemoglobin is revealed in the case of most of the thiols tested. This may not mean that no oxidation occurs in this period. More likely the methaemoglobin formed is subjected to an immediate re-reduction by the thiol. The rate of this reduction will gradually decrease as the thiol is oxidized by its interaction both with methaemoglobin (Fig. 1) and with atmospheric oxygen,[19] thus in turn allowing an accumulation of methaemoglobin to take place.

No significant amounts of soluble oxidation products of haemoglobin other than methaemoglobin could be detected in the haemolysate, as judged from their absorption spectra. However, following the appearance of methaemoglobin, trace amounts of a greenish precipitate regularly appears. This product, which represents a minor fraction of the total amount of haemoglobin oxidized, was subjected only to some preliminary studies. The precipitate formed in the presence of cysteamine is found to be soluble in 0·1 N NaOH and in 0·1 N HCl. At acid pH a solution of this pigment demonstrates a faint absorption band at 602 mμ and a Soret band at 393 mμ. At

alkaline pH the Soret band moves to 405 mμ, and the absorption maximum at 602 mμ broadens out. Upon reduction of the pigment with $Na_2S_2O_4$ the solution demonstrates three distinct absorption maxima, at 607, 557 and 525 mμ, all of which show a red shift of about 20 mμ when carbon monoxide is bubbled through the solution. The addition of oxygen reconverts the reduced pigment to its original form. The spectro-

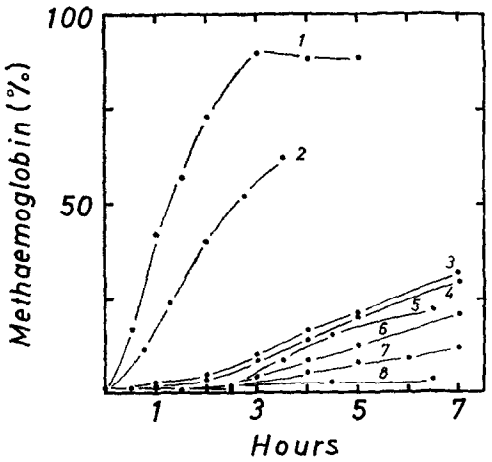

FIG. 2. Methaemoglobin-forming ability of various thiols. Haemolysates, initially free from methaemoglobin, were incubated in the presence of the thiol (10^{-2} M) as follows: curve 1, diethyldithiocarbamate; curve 2, dimethyldithiocarbamate; curve 3, penicillamine; curve 4, cysteamine; curve 5, thioglocolate; curve 6, cysteine; curve 7, glutathione; curve 8, no thiol added. The methaemoglobin concentrations are expressed as per cent of the total haemoprotein present (approx. conc.: 6·5 g/100 ml).

photometric properties of this green precipitate correspond essentially to those of the precipitate formed in the presence of ascorbic acid[6] although the absorption maxima differ slightly. It seems reasonable to believe that the precipitate is a denatured oxidation product of haemoglobin, the porphin rings of which are intact and the iron is in its ferric state.

Role of oxygen in the oxidation

It seems obvious that the thiols as such will not be able to oxidize haemoglobin directly. Accordingly, we find that the presence of oxygen is essential in the oxidation (Fig. 3). Oxygen probably represents the ultimate electron acceptor. In an atmosphere of 20% O_2 and 80% CO, no methaemoglobin formation takes place in the presence of either diethyldithiocarbamate or cysteamine. Under these conditions only carboxyhaemoglobin will be present (about 99·9 per cent). This observation may merely demonstrate a higher resistance of carboxyhaemoglobin against oxidation. It may suggest, however, that it is the oxygen combined to haemoglobin which carries out the oxidation.

Role of disulphides and of other possible oxidation products of thiols

As mentioned the thiols will gradually become oxidized during the incubation. The possibility therefore exists that an oxidation product of the thiol rather than the thiol

as such is responsible for the methaemoglobin formation. Paper electrophoretic studies revealed no such products except the disulphide and its mixed disulphide with GSH. The disulphide, however, may be expected to interact with groups or linkages essential in protecting the iron of haemoglobin against autoxidation.[20] Disulphides are known to react with free —SH groups of proteins by mixed disulphide formation.[21]

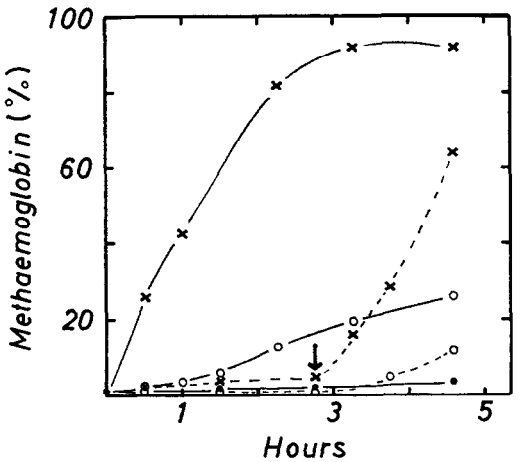

FIG. 3. Significance of oxygen in the thiol-induced oxidation of haemoglobin. Haemolysates (total haemoprotein conc.: 7·1 g/100 ml) were incubated with diethyldithiocarbamate (×), or with cysteamine (○) in concentrations of 10^{-2} M, in an atmosphere of air (continuous curves) or of nitrogen (broken curves). ●, No thiol added. In the anaerobic incubations oxygen was admitted after $2\frac{3}{4}$ hr of incubation (the arrow).

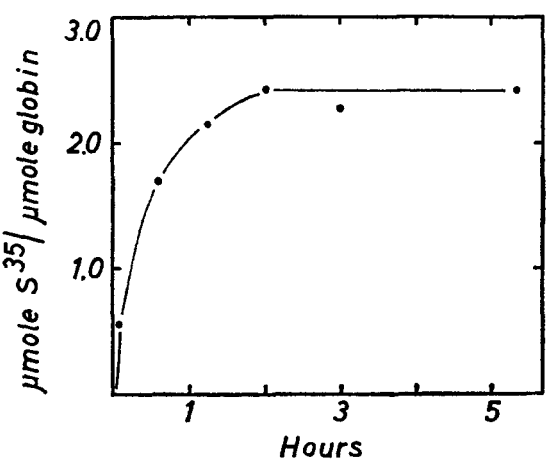

FIG. 4. Amounts of radioactive cysteamine which become attached to globin as a function of time of the incubation. Haemolysates (total haemoprotein conc.: 5·8 g/100 ml) were incubated in the presence of ^{35}S-labelled cysteamine (10^{-2} M).

That such a disulphide interaction occurs even when the thiol is added to haemolysates, is demonstrated by experiments with ^{35}S-labelled cysteamine (Fig. 4). During the first 1½ hr of incubation increasing amounts of radioactive sulphur become attached to the globin, reaching a maximum value of about 2·3 μmoles per μmole globin. This is in agreement with previous observations, using other SH-blocking agents, showing that haemoglobin possesses two especially reactive —SH groups.[22] In intact erythrocytes on the other hand, Eldjarn *et al.*[23] were able to demonstrate only one —SH group which freely entered into exchange reaction with cystamine.

Figure 5 shows that cystamine is less efficient than its corresponding thiol in promoting the oxidation of haemoglobin. This observation indicates that the disulphide itself does not play an essential role in the methaemoglobin formation. The fact that cystamine at all gives rise to methaemoglobin may be explained by the formation of cysteamine via exchange reactions with GSH and with protein —SH groups. This

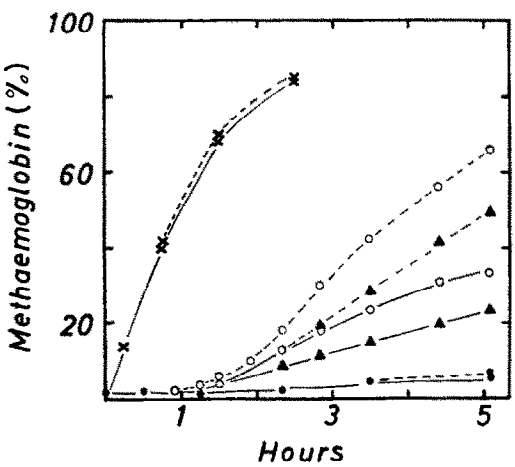

FIG. 5. Effect of EDTA on the methaemoglobin-forming ability of the thiols diethyldithiocarbamate and cysteamine, and of the disulphide cystamine. Haemolysates (total haemoprotein conc.: 7·0 g/100 ml) were incubated with (broken curves) and without EDTA (continuous curves) as follows: ×, diethyldithiocarbamate (10^{-2} M); ○, cysteamine (10^{-2} M); ▲, cystamine (5×10^{-3} M); ●, control.

assumption is supported by the observation that the methaemoglobin-forming effect both of cysteamine and of cystamine is increased by the presence of EDTA (Fig. 5). This agent, by its chelating properties, inhibits autoxidation of cysteamine. Accordingly, in the case of diethyldithiocarbamate, which does not undergo autoxidation,[24] EDTA was found indifferent.

Generation of free radicals during the oxidation

The above results indicate that the thiols act by facilitating the one-electron transfer from the ferrous ion of haemoglobin to oxygen. During such a reaction a generation of free radicals would be expected. In an attempt to demonstrate these, we made use of the ability of free radicals to initiate certain chain reactions. The autoxidation of sulfite to sulfate has previously been recommended as a suitable test system for the

detection of free radicals.[25–27] Figure 6 shows that some oxidation of Na_2SO_3, as measured by the oxygen consumption, takes place in the presence of diethyldithiocarbamate. The addition of a small amount of haemolysate, however, greatly accelerates this oxidation. When only diethyldithiocarbamate is present, negative manometric values are obtained, owing to the fact that this thiol decomposes to diethylamine and to the volatile carbon disulphide.[24]

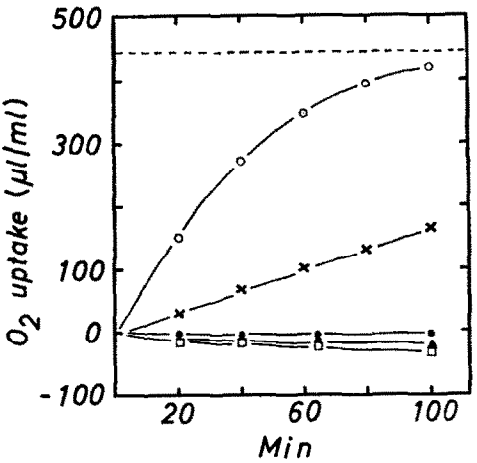

FIG. 6. Initiation of the aerobic oxidation of sulfite by diethyldithiocarbamate (ASH) with and withou haemolysate present. The incubation flasks, the contents of which totalled 3 ml, contained: ○, Na_2SO_3 (40 µmoles) + ASH (11 µmoles) + haemolysate (corresponding to 100 µl washed packed erythrocytes). ×, Na_2SO_3 (40 µmoles) + ASH (11 µmoles). ▲, ASH (11 µmoles) alone. □, ASH (11 µmoles) + haemolysate (100 µl). ●, Na_2SO_3 (40 µmoles) + haemolysate (100 µl). The broken line indicates the volume of oxygen needed to bring about a complete oxidation of 40 µmoles of sulfite.

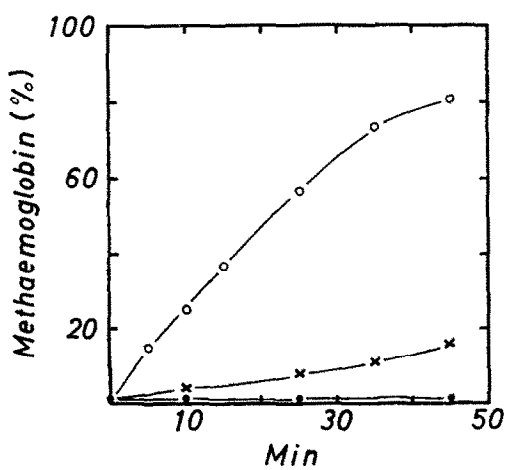

FIG. 7. Stimulating effect of Na_2SO_3 on the methaemoglobin-forming ability of diethyldithiocarbamate (ASH). Haemolysates (total haemoprotein conc.: 4·8 g/100 ml) were incubated as follows: ○, ASH ($3·8 \times 10^{-3}$ M) + Na_2SO_3 ($1·3 \times 10^{-2}$ M); ×, ASH ($3·8 \times 10^{-3}$ M); ●, Na_2SO_3 ($1·3 \times 10^{-2}$ M); or nothing added.

Sodium sulfite alone does not give rise to the formation of methaemoglobin. However, when Na_2SO_3 in addition to diethyldithiocarbamate is present in the haemolysate, the rate of methaemoglobin formation is much higher than when only the thiol is present (Fig. 7). Under these conditions an oxidation of sulfite simultaneously occurs (Fig. 6), a process which is known to involve a radical chain reaction.[26]

Methaemoglobin formation by diethyldithiocarbamate in normal erythrocytes and in erythrocytes with deficient glucose-6-phosphate dehydrogenase

The experiments presented above are all carried out with haemolysates. The methaemoglobin-forming ability of thiols, however, has also been observed in solutions of crystalline human haemoglobin (purchased from Nutritional Biochemicals Corporation) and in intact erythrocytes. Pure haemoglobin in the presence of thiols was oxidized more rapidly than the haemoglobin in haemolysates, and a larger fraction of it underwent denaturation and precipitation. Solutions of crystalline haemoglobin, however, were unsuitable as test-system since the pure pigment was significantly autoxidized and denatured even in the absence of thiols. In the case of intact cells, a few experiments have been carried out with erythrocytes from a patient with deficient activity of the glucose-6-phosphate dehydrogenase. This seemed of interest according to the view outlined in the introduction. Figure 8 shows that the presence

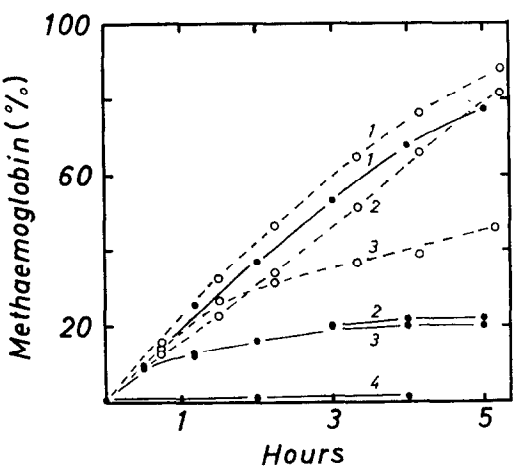

FIG. 8. Accumulation of methaemoglobin in erythrocytes with and without substrate in the presence of diethyldithiocarbamate. Washed normal erythrocytes (continuous curves), or erythrocytes from a patient with glucose-6-phosphate dehydrogenase deficiency (broken curves) were preincubated for 10 min with substrate (initial conc.: $5{\cdot}6 \times 10^{-3}$ M) and without substrate. Diethyldithiocarbamate (ASH) in a concentration of $7{\cdot}5 \times 10^{-3}$ M was added at zero time. Curve 1, ASH without substrate. Curve 2, ASH + glucose. Curve 3, ASH + inosine. Curve 4, nothing added.

of diethyldithiocarbamate in a concentration of $7{\cdot}5 \times 10^{-3}$ M causes rapid accumulation of methaemoglobin in normal as well as in the pathological erythrocytes. In normal cells this accumulation will be counteracted by the presence of glucose or inosine. In cells with deficient activity of the glucose-6-phosphate dehydrogenase glucose hardly offers any protection, and inosine offers only partial protection, against the

methaemoglobin accumulation. In these cells the glucose metabolism is probably inhibited via an inhibition of the hexokinase at a mechanism previously reported.[14] It may be mentioned that in normal erythrocytes an inhibition of the glucose metabolism was obtained only at diethyldithiocarbamate concentrations above $1{\cdot}3 \times 10^{-2}$ M.[14] The impaired reduction of methaemoglobin in the presence of inosine probably demonstrates the insufficient activity of the glucose-6-phosphate dehydrogenase, since the pentose phosphate shunt accounts for the greater part of the pigment reduced when diethyldithiocarbamate is present.[14] On the other hand, the results do not exclude the possibility that a partial inhibition also below the hexokinase level takes place.[14]

DISCUSSION

A formation of methaemoglobin from haemoglobin is brought about in one of several ways: (1) by autoxidation; (2) by the direct action of added oxidants; (3) by the action of certain hydrogen donors in the presence of oxygen.[7] The thiol-provoked formation of methaemoglobin obviously is brought about according to the last mentioned mode.

The mechanism underlying the oxidation of haemoglobin induced by reducing substances is as yet not fully understood. Hydrogen peroxide formed by autoxidation of the compounds has been considered responsible.[3, 28, 29] Keilin and Hartree have demonstrated that this agent when generated continuously by the enzymatic oxidation of glucose, leads to oxidation of haemoglobin.[30] When hydrogen peroxide is added as such, significant breakdown occurs only when the catalase is inhibited simultaneously, e.g. by cyanide.[3, 30] Catalase has also been found to protect against the methaemoglobin-forming effect of X-rays,[31] and to slow down the coupled oxidation of ascorbic acid and oxyhaemoglobin.[32] In the latter reaction Lemberg *et al.*[33] have postulated a ferrous haem–hydrogen peroxide complex to be an intermediate. In the case of thiols, however, the oxidation of haemoglobin can hardly be ascribed to the formation of hydrogen peroxide for the following reasons: (1) Diethyldithiocarbamate, which is the most potent of the thiols tested, does not react spontaneously with oxygen,[24] and consequently will not give rise to hydrogen peroxide. (2) EDTA, which slows down the autoxidation of thiols, was found to increase the methaemoglobin-forming ability of autoxidizable thiols. (3) When using autoxidizable thiols such as cysteamine, the methaemoglobin formation is revealed at a time of incubation when only a fraction of the cysteamine is left for further hydrogen peroxide generation.

In the oxidative breakdown observed in the presence of certain hydrazine derivatives, stable or semi-stable autoxidation products of these compounds have been proposed to be the immediate electron acceptors.[11, 12] The present study reveals no oxidation products of cysteamine except its disulphide. This agent, however, apparently is incapable of oxidizing haemoglobin directly. The methaemoglobin formation which occurs when cystamine is added (Fig. 5) is most likely explained by the generation of the corresponding thiol through the disulphide interaction with GSH and with protein —SH groups. On the basis of the present observations, therefore, it seems reasonable to believe that it is the thiol itself which is involved in the mechanism of oxidation.

The following theory for the methaemoglobin-forming ability of thiols seems consistent with the data presented. When oxygen is linked to the iron of haem, two of its electrons are shared with the iron, thus increasing the electrophilic properties of the

oxygen. Thiols, therefore, even when they do not react with atmospheric oxygen (e.g. diethyldithiocarbamate) may react with oxygen of oxyhaemoglobin. This reaction probably results in an intermolecular peroxide complex (Fe—OO—SR), which most likely is highly unstable and decomposes under intramolecular oxidation of the ferrous haem ion. In this decomposition a generation of free radicals (hydroxyl- or peroxide radicals?) may occur. The thiol may either be generated, or oxidized, the latter possibly as a consequence of the radicals formed.[34] Support for this theory is to be seen in the following. The formation of the haem–peroxide–thiol complex corresponds to the reaction which has been postulated for the autoxidation of thiols. In the latter case, the primary step is believed to be an addition of a molecule of oxygen with the formation of a thiol–peroxide complex (RS—OO). The reaction involved in the decomposition of the Fe—OO—SR complex may be correlated to certain iron-catalyzed processes which occur when hydrogen peroxide is used as oxidant (Fenton's reagent).[35] In these reactions free hydroxyl- and/or peroxide radicals have been demonstrated to be intermediates, and the iron to be oxidized from the ferrous to the ferric state. That radicals are indeed generated in the thiol-induced oxidation of haemoglobin is suggested by the results of Fig. 6. In this connexion it should be mentioned that Rostorfer and Cormier obtained evidence for the formation of of hydrogen peroxide or, more likely, radicals in the interaction between phenylhydrazine and oxyhaemoglobin.[29]

It will be apparent from the above that in the case of thiols such as cysteamine, methaemoglobin appears in the solution only after the greater part of the thiol has become oxidized. Consequently, only trace amounts are needed to provoke the oxidation. In spite of this fact, however, it is difficult to explain the observation that the formation of methaemoglobin may proceed for hours (Fig. 2), since one would expect a complete oxidation of the thiol to take place. One explanation may be that a catalytic concentration is maintained by a continuous interaction of the corresponding disulphide with protein —SH groups, possibly made available through a gradual denaturation. The latter is supported by the results of Snow[22] demonstrating that the number of titrable —SH groups of human haemoglobin increases from 2·2 to 6·0 when the protein is denaturated with sodium dodecyl sulfate. Allen and Jandl have shown that the haemoglobin precipitated in the presence of phenylhydrazine binds more $GS^{35}H$ than does the still-soluble haemoglobin.[12] The need for a continuous regeneration of the thiol may also explain the fact that we observed an increasing rate of methaemoglobin formation by increasing the concentration of cysteamine, although the pigment never appeared before the greater part of the thiol had become oxidized. Thus, at higher concentration of the thiol, a corresponding higher concentration of its disulphide will be formed, which in turn may increase the disulphide interaction with protein —SH groups. It should be stressed that the amount of thiol thus formed may not be expressed by the amount simultaneously bound to proteins, since the disulphide interaction may not only give rise to mixed disulphides but also to intramolecular disulphides.

Most of the reducing substances which are reported to bring about an oxidation of haemoglobin, in addition to methaemoglobin lead to other oxidation products which remain in solution. Whereas thiols such as dimercaptopropanol[36, 37] and thioglycolic acid[3] possess this ability, cysteine and GSH have been found by Lemberg *et al.*[38] to be almost inactive in this regard. Consistent with the latter observations the thiols here

tested does not lead to the formation of detectable amounts of soluble oxidation products except methaemoglobin.

Recently we have shown that diethyldithiocarbamate might lead to an inhibition of the carbohydrate metabolism of erythrocytes, and that this inhibition was preceded by an oxidation of the intracellular GSH.[11] The mechanism underlying these changes was found to involve an oxidation of the thiol to its disulphide (disulfiram) by the action of the methaemoglobin formed. Disulfiram in turn inhibited primarily the hexokinase, but at higher concentrations also other enzymes of the glucose metabolism were affected. High concentrations of diethyldithiocarbamate were needed since the erythrocytes were found to possess a considerable disulfiram-reducing capacity via their glutathione–glutathione reductase system. In the present report it is shown that erythrocytes deficient of the glucose-6-phosphate dehydrogenase is less resistant against this poisoning, apparently owing to a reduced capacity of their glutathione-glutathione reductase system. Our data therefore indicate that the sequence of changes which takes place in these cells, and which takes place also in normal cells but at higher concentrations of diethyldithiocarbamate, is as follows: (1) diethyldithiocarbamate-induced formation of methaemoglobin; (2) oxidation of diethyldithiocarbamate to disulfiram by methaemoglobin; (3) oxidation of GSH by disulfiram; (4) blocking of protein —SH groups by disulfiram and by GSSG; (5) inhibition of enzymes. One would expect that these changes ultimately may lead to haemolysis through an interaction with processes essential in maintaining the cell integrity. It should be noted that a similar sequence of events has been observed upon the addition of acetylphenyl hydrazine to erythrocytes.[11, 12, 39] Furthermore, a decrease in GSH[10, 41] as well as an appearance of methaemoglobin[13] are observed to be a prehaemolytic phenomenon also *in vivo*.

Since disulfiram in the organism is reduced to diethyldithiocarbamate,[14] this drug, when used in the treatment of alcoholism, will probably tend to precipitate haemolysis in patients suffering from glucose-6-phosphate dehydrogenase deficiency.

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