METHEMOGLOBINEMIA AND METHEMOGLOBIN-PRODUCING COMPOUNDS

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I. INTRODUCTION

A great variety of drugs are known to convert hemoglobin into methemoglobin and thereby to impair the blood's capacity to transport oxygen to the tissues. In addition, a number of substances which are used in industrial processes possess this property. Nitrates in food and water which are of themselves innocuous may, by the action of intestinal bacteria, be converted into nitrites and so form methemoglobin. Finally, there are a number of instances, fortunately rare in man, in which, without obvious external cause, methemoglobin is formed from hemoglobin and constitutes a constant hazard to the well-being of the individual.

Without attempting any exhaustive tabulation of the vast literature, the present review is directed towards correlating available information concerning the chemistry of methemoglobin, the biochemical relationships between hemoglobin and methemoglobin, the extent and nature of the physiological impairments produced by methemoglobinemia, the description of the substances which form methemoglobin and the mechanisms by which they do so, and finally the principles and methods to be followed in the treatment of methemoglobinemia.

II. CHEMISTRY OF METHEMOGLOBIN

A. Structure

Methemoglobin is an oxidation product of the normal blood pigment, hemoglobin. The prosthetic group of hemoglobin is variously known as heme, ferroheme (Pauling and Coryell (193); Barron (16)), or ferroporphyrin (Clark (45); Drabkin (65)) and is a complex of iron and protoporphyrin IX, a type III porphyrin. The essential structure of the porphyrins is a nucleus of four pyrrole groups united by four methene, =CH—, linkages. Protoporphyrin IX is 1,3,5,8-tetramethyl-2,4-divinylporphin-6,7-dipropionic acid. The iron in this structure may be considered as coordinated with the four nitrogen atoms, thus leaving two more coordination bonds available. In methemoglobin, iron is present in the ferric state. Methemoglobin is also known as hemiglobin and ferrihemoglobin.

The nature of the linkage between the globin and the prosthetic group in hemoglobin or methemoglobin has received considerable attention. Though there is

much evidence that this combination occurs through a linkage of the iron with the imidazole groups in the globin molecule, the carbamino reaction of hemoglobin does not appear to fit in with such a hypothesis. Clark (47) considers that the exact nature of the bonding between the prosthetic group and the specific protein, globin, is still unknown. This field has been admirably reviewed by Lemberg and Legge (153).

B. Formation of methemoglobin

Methemoglobin may be formed from hemoglobin in a variety of ways. Quite obviously, the essential action is an oxidation of the ferrous to the ferric ion, but such oxidation may be brought about in one of several ways: (a) by the direct action of oxidants; (b) the action of hydrogen donors in the presence of atmospheric oxygen, and (c) autoxidation. In addition, many of the substances which are used to effect this oxidation combine with hemoglobin or methemoglobin.

The ferrous ion of hemoglobin may be oxidized directly by ferric tartrate, ferricyanide, bivalent copper, chlorate, nitrate, quinones, alloxans and dyes of high oxidation-reduction potential (33, 62, 153, 175, 206, 213).

Another mode of oxidation is that of the action of hydrogen donors in the presence of atmospheric oxygen. Chief representatives in this group are the dyes (62, 177). Some of these have oxidation-reduction potentials which are greater than that of methemoglobin, the E_0 of which is 150 millivolts. Such, for example, are phenolindophenol with a potential, E_0, of 250 millivolts. However, a number of the most important methemoglobin-forming dyes such as methylene blue with an E_0 of 10 millivolts have oxidation-reduction potentials below that of methemoglobin.

The oxidizing action of methylene blue may be explained in either of two ways. First, although the E_0 value of methemoglobin is greater than that of methylene blue, the latter is conceived of as interacting with a small fraction of the hemoglobin to form methemoglobin and leucomethylene blue. The latter is then reoxidized to methylene blue by atmospheric oxygen, and a cyclic reaction is set up whereby all the hemoglobin is finally oxidized to methemoglobin. The second explanation is that of Lemberg and his coworkers (153, 154) who have postulated that a hydrogen donor such as leucomethylene blue may undergo autoxidation with the formation of hydrogen peroxide which either oxidizes hemoglobin directly to methemoglobin or else forms an intermediate with oxyhemoglobin; the latter then decomposes to yield methemoglobin:

$$HbO_2 + H_2A \rightarrow Hb \cdot H_2O_2 + A$$
$$Hb \cdot H_2O_2 \rightarrow MetHb$$

The formation of methemoglobin from oxyhemoglobin by substances such as aminophenols, phenylhydroxylamine, phenylhydrazine and hydrazobenzene may be formulated in a similar manner.

A third important mechanism in the formation of methemoglobin from hemoglobin is autoxidation. Neill (184–187) found that various autoxidizable substances present in turpentine, linseed oil, the alcohol-soluble substance of potato

juice, and sterile pneumococcus and other bacterial filtrates were capable of accelerating the formation of methemoglobin. For example, at an oxygen tension of 25 mm. of Hg in the presence of linseed oil, a 4.4 mM solution of hemoglobin yielded 1.1 mM of methemoglobin in two hours. This type of methemoglobin formation is greatly dependent upon the oxygen tension, is negligible at zero oxygen tension and maximal at relatively low tensions, about 25 mm. of Hg, where about half of the hemoglobin is in the form of oxyhemoglobin. At higher oxygen tensions, where a greater proportion of the hemoglobin is in the form of oxyhemoglobin, the rate of formation of methemoglobin is again decreased. It may be noted that spontaneous conversion of hemoglobin to methemoglobin takes place to an appreciable extent in drawn blood or in laked cells. Presumably, this is due to the presence of autoxidizable substances present in blood, for it does not occur when purified crystallized hemoglobin is exposed to air or oxygen.

The dependence of autoxidation of hemoglobin upon the oxygen tension has been formulated more precisely by Brooks (29-31) as follows:

$$\frac{dx}{dt} = k\,[\alpha(a - x)]\,\frac{\mathrm{bp_{O_2}}}{1 + \mathrm{bp_O}}$$

where x is the concentration of methemoglobin, a is the initial concentration of hemoglobin, α is the ratio of hemoglobin to total hemoglobin, and k and b are constants. Legge (153) has interpreted this equation to indicate the spontaneous decomposition of an intermediate, $Hb_4(O_2)_2$, to form methemoglobin.

C. Physical properties of methemoglobin

1. *Spectrophotometric absorption.* One of the most important properties of methemoglobin and one which is utilized in its quantitative estimation is its spectral absorption. The reader will recall that the capacity of a substance to transmit light of various wave lengths may be expressed by the extinction coefficient, $E_{1\,\mathrm{cm.}}^{1\%}$, which represents the ratio of the logarithm of the incidental light, I, to the transmitted light, T, of a 1 per cent solution through a 1 cm. thickness.[1] The ratio can be very easily estimated by determining the transmissions of an appropriate blank solution and a solution of the substance under test. The dilution of the latter must be chosen so that it can be read at a convenient range on the photometer; appropriate calculations to obtain the transmission of a 1 per cent solution through a 1 cm. thickness can then be made. It is also possible to express the extinction as the molar extinction coefficient, E_{mol}, where the concentration of the substance is 1 mole per liter or as the millimolar extinction coefficient, E_{mM}, where the concentration is 1 millimol per liter.

The absorption spectrum of methemoglobin has been the concern of many investigators since the identity of this compound was first established. More recent investigations are those of Hari (93), of Austin and Drabkin (8, 9), of Urbain and Greenwood (230), and of Holden (124). It has been repeatedly noted that the absorption spectrum depends upon the pH. The nature of these changes can

[1] This and subsequent units of extinction may also be designated by the Greek letter, ϵ.

perhaps best be illustrated by the work of Urbain and Greenwood (230) in the visible range of 500 to 660 mμ. For example, the values of E_{mM} at 500 mμ decrease from 8.0 at pH 1.0 to 6.9 at pH 2.5, rise to 9.6 at pH 6.0 and thereafter begin to decrease again. At a wave length of 630 mμ, the value of E_{mM} is 2.4 at pH 1.0, decreases to 2.0 at pH 2.5, then rises to 3.9 at a pH range of 6.0 to 7.0. However, in general, the variation of the value of E_{mM} with pH was different at different wave lengths.

The change in the absorption spectrum has received various explanations. Austin and Drabkin (9) postulated the existence of two forms of methemoglobin, acid and alkaline, which had different absorption characteristics and which were related by the following equation:

$$pH = 8.12 + \log \frac{[\text{MHb alkaline}]}{[\text{MHb acid}]}$$

According to this equation, a 50-50 per cent mixture would be present at pH 8.12, and at pH 9.1 about 91 per cent of methemoglobin would exist in the alkaline form. Holden (124) postulates the existence of alkaline methemoglobin down to pH 6.3, with denaturation of methemoglobin setting in at pH 3.9. Urbain and Greenwood (230) have assumed the existence of several components in addition to alkaline and acid methemoglobins. As the acidity is increased the changes which occur may be conceived as follows: from pH 9.0 to 6.0, a shift from alkaline methemoglobin to acid methemoglobin; from pH 6.0 to 4.5, a shift from acid methemoglobin to some third compound; from pH 4.5 to 2.75, a shift to a compound such as acid hematin; below 2.75, another undefined compound or colloidal substance is formed. Lemberg and Legge (153) have submitted summaries of the values in the literature, on the assumption that there are only two forms of methemoglobin, acid and alkaline:

Acid methemoglobin		
Maxima, mμ	630	500
E_{mM}	3.7–3.8	9.5
Alkaline methemoglobin		
Maxima, mμ	577	540
E_{mM}	8.5	9.7

The absorption of methemoglobin has also been studied in the infra-red and ultraviolet regions. Holden (119, 126) obtained for acid methemoglobin at pH 4.68 a band at 407 mμ with an E_{mM} of 154. For alkaline methemoglobin at pH 10.8, the absorption band occurred at 411 mμ with an E_{mM} value of 90. Horecker (127) did not observe any distinctive absorption bands for methemoglobin in the infra-red region of 700 to 1000 mμ. At pH about 8.9, the value of E_{mM} rose from about 0.30 at 685 mμ to 0.53 at 818 mμ, then decreased to 0.26 at 940 mμ. At pH about 6.3 to 6.7, the value of E_{mM} rose from 0.15 at 700 mμ to about 0.79 at 980 mμ.

2. *Magnetochemical properties.* The magnetic properties of methemoglobin as

well as of related blood pigments have received considerable attention (54, 55, 57, 65, 227). When a substance is placed in a magnetic field, the electrons are so accelerated that a polarization results. The polarization is, of course, opposite to that of the applied field; the substance as a whole tends to be repelled by a magnet and hence the substance is said to be diamagnetic. In contrast, there are other substances which, although rendered diamagnetic when placed in a magnetic field, are also inherently paramagnetic, that is, they are attracted by a magnet. The paramagnetic characteristic of a substance is associated with the presence in these substances of atoms, ions or molecules which contain unpaired electrons. Indeed, the paramagnetic polarization is due to the spin magnetic moments of these electrons and to the moments of their orbital motion.

The principle underlying the measurement of paramagnetic susceptibility is that of "magnetic pull balanced by gravitational pull." Essentially a vertical glass cylinder containing the substance under test is suspended from one arm of a balance and the weight is measured before and after the application of a strong magnetic field to one end of the cylinder. The difference in weight is due to the magnetic field acting on the cylinder. In practice, a cylindrical tube is divided into an upper and lower chamber by a glass partition. The upper chamber is filled with the substance under test, and the lower chamber with pure solvent or with a diamagnetic substance. In the case of hemoglobin derivatives, the establishment of the diamagnetism of carboxyhemoglobin and the determination of the absolute paramagnetic susceptibility of ferrohemoglobin (12,430 $\times$ 10^{-6} c.g.s.u.) permitted the use of the following equation:

$$\chi_m = \frac{\Delta w - \Delta w_{\text{COHb}}}{\Delta w_{\text{Hb}} - \Delta w_{\text{COHb}}} \times 12{,}430 \times 10^{-6} \text{ c.g.s.u.}$$

where Δw, Δw_{Hb} and Δw_{COHb} are the forces measured, in milligrams, for the solution under investigation, for ferrohemoglobin and for carboxyhemoglobin; χ_m is the molal magnetic susceptibility.

The value of the molal magnetic susceptibility of methemoglobin has been investigated in great detail by Coryell, Stitt and Pauling (57) at varying pH levels and ionic strengths. In general, the values for χ decreased from values of about 14,000 to 15,000 $\times$ 10^{-6} c.g.s.u. units in a pH range of about 5 to 7, to a value of about 8,000 $\times$ 10^{-6} c.g.s.u. units at a pH of about 11 to 12. Substitution of the molal magnetic susceptibility χ_m in the equation $u = 2.84 \sqrt{\chi_m T}$ where T is the absolute temperature yields a value for u of 5.80 Bohr magnetons for methemoglobin and one of 4.47 Bohr magnetons for methemoglobin hydroxide. This indicates that there are five unpaired electrons per heme (ionic bands) in methemoglobin, and three in methemoglobin hydroxide.

3. *Other physical properties.* The crystallization of methemoglobin has, of course, permitted more precise studies of the chemical and physical properties of this substance. Methods for obtaining such crystalline preparations have been submitted by Levy (159) and by Dénes (63). These methods involve oxidation of reduced crystalline hemoglobin by an aqueous solution of potassium ferri-

cyanide in slight excess, or of crystalline oxyhemoglobin by an alcoholic or alkaline solution of the ferricyanide. The solubility characteristics of crystalline methemoglobin have been studied by Levy and the X-ray diffraction pattern has been investigated by Boyes-Watson and his associates (27) and by Perutz (195).

D. Chemical interactions and properties

Methemoglobin can combine with a great variety of substances. Most of these interactions consist of a combination of the ferric ion with other ions such as fluoride or cyanide, but changes in the globin part of the molecule are also possible.

1. *Methemoglobin hydroxide.* We have already discussed to some extent the interactions of methemoglobin with hydroxyl ion; at pH levels greater than 7, the hydroxyl ion becomes attached to the sixth bond position of the iron atom, and the spectrum becomes altered, with new maxima appearing at 577 and 540 mμ (153). The addition of sodium hydroxide to methemoglobin may also result in the denaturation of the globin moiety. As we have already noted (100), spectrophotometry yields a pK value of 8.1 for the interaction of methemoglobin with hydroxyl ion: $MetHb + H_2O \rightleftarrows MetHbOH + H^+$. A similar value has been obtained by magnetochemical titration (57).

2. *Methemoglobin Fluoride.* Methemoglobin reacts with fluoride ion to form methemoglobin fluoride, also known as fluoro-methemoglobin (97, 100). Solutions of this compound are red, with absorption bands at 610 mμ and in the green part of the spectrum. Haurowitz (100) found a ratio of one atom of fluorine to one atom of iron in purified preparations of methemoglobin fluoride. Employing spectrophotometric methods, Lipmann (162) determined the value for the dissociation constant at neutral pH, $K = \frac{[MetHb]\ [F^-]}{[MetHbF]}$, to be about 0.015 M. However, the degree of dissociation varies with the pH and decreases in more acid regions. For example, Lipmann (162) observed that at 0.001 M concentration of sodium fluoride, the methemoglobin fluoride was in an undissociated form to the extent of 57 per cent at pH 3.8, of 27 per cent at pH 4.7 and of 6 per cent at pH 6.9.

The values for the dissociation constant of methemoglobin fluroide obtained by spectroscopic methods (105, 162) agree well with those obtained by Coryell and his associates (57), by means of magnetochemical methods. The latter derived the expression

$$\log K_{MetHbF} = -2.23 + 0.59\sqrt{\mu}$$

where u is the ionic strength. At a value of u equal to 0.5, log K equals $-2.23 + 0.4$ or -1.8. K is therefore about 0.015 and in good agreement with the value Lipmann obtained on a solution of swine methemoglobin of unstated ionic strength (162).

3. *Cyanmethemoglobin.* The interaction of methemoglobin with cyanide is of considerable interest since it is this compound which has been considered to underlie the therapeutic effectiveness of methemoglobin in counteracting the

toxic or even lethal action of cyanide (35, 131, 181, 194). As we shall presently see, this interaction is also an important stage in the quantitative determination of methemoglobin. The product has been variously designated as cyanmethemoglobin, ferrihemoglobin cyanide, or hemiglobin cyanide (153). It has also been erroneously referred to as cyanhemoglobin, a term which should more properly be reserved for the possible existence of a compound of hemoglobin with cyanide (68). Cyanmethemoglobin was discovered by Kobert in 1891 (151). Zeynek (246) crystallized this compound and found that it contained one atom of cyanide per atom of iron.

The study of the interaction of methemoglobin with cyanide has been somewhat confused by the fact that potassium or sodium cyanide has usually been employed as the source of cyanide ion. Since the pK value of hydrocyanic acid is 9.1, these salts give an extremely high concentration of hydroxyl ion and hence provide the conditions for the formation of methemoglobin hydroxide as well as of cyanmethemoglobin. It is, therefore, necessary to consider the role of pH in the interaction of methemoglobin with cyanide ion. The various aspects of this interaction have been formulated as follows by Lemberg and Legge (153):

$$MetHb + HCN \rightleftarrows MetHbCN + H^+ \cdots a$$
$$MetHbOH + HCN \rightleftarrows MetHbCN + H_2O \cdots b$$
$$MetHbOH + CN^- \rightleftarrows MetHbCN + OH^- \cdots c$$

Reactions *a* and *c* are sensitive to pH while reaction *b* is not.

The absorption spectrum of cyanmethemoglobin has been studied by a number of investigators (8, 73, 99). The outstanding feature of the spectrum of cyanmethemoglobin is a marked absorption band at 540 mμ. This characteristic is revealed most obviously and simply when a solution of carefully neutralized cyanide is added to methemoglobin; the color changes abruptly from brown to a bright red.

The formation of cyanmethemoglobin is the basis of the therapeutic and prophylactic actions of certain compounds in cyanide poisoning (35, 43, 131, 181, 194). The mechanism of this antidotal action may be formulated in terms of the following competing equilibria:

(1) Ferricytochrome oxidase + cyanide ion
$\rightleftarrows$ ferricytochrome oxidase cyanide

(2) Methemoglobin + cyanide ion $\rightleftarrows$ cyanmethemoglobin

In accordance with these equilibria, the presence of methemoglobin in the blood of an animal, *before* cyanide is introduced, should decrease the toxicity of this agent. On the other hand, the production of methemoglobinemia *after* the administration of a lethal dose of cyanide may result in the liberation of free ferricytochrome oxidase from its combination with cyanide, and in the restoration of the normal sequence of oxidation-reduction reactions in the tissues.

Chen and his coworkers (43) developed a treatment for cyanide poisoning which consisted of the intravenous injection of sodium nitrite and sodium thiosulfate. The sodium nitrite led to the production of methemoglobin and the sodium thiosulfate helped in the elimination of cyanide through the production

of thiocyanate. Jandorf and Bodansky (133) showed that when methemoglobinemia was induced in dogs by means of p-aminopropiophenone, the degree of protection against subsequent inhalation of hydrocyanic acid was proportional to the degree of methemoglobinemia. Albaum, Tepperman and Bodansky (3) studied the *in-vitro* competition between methemoglobin and cytochrome oxidase in brain homogenates for cyanide ion. It was shown that methemoglobin can reverse the cyanide inhibition of cytochrome oxidase activity.

4. *Hydrogen peroxide methemoglobin.* Im 1900 Kobert (152) showed that when hydrogen peroxide was added to a solution of acid methemoglobin, the color turned from brown to red and the absorption spectrum of methemoglobin was replaced by three bands at 500–513 mμ, 545–558 mμ and 584–600 mμ. Kobert reported that when the solution was allowed to stand, the brown color of methemoglobin reappeared. Haurowitz (99) found that hydrogen peroxide reacted more rapidly with acid than with alkaline methemoglobin and that hydroxyl and cyanide ion could displace the hydrogen peroxide from its combination with methemoglobin. Keilin and Hartree (145) observed that the addition of hydrogen peroxide to a solution of pure methemoglobin at a pH of 5.8 to 6.5 caused the appearance of two new absorption bands, namely, at about 589 mμ and 545 mμ. The band at about 500 mμ noted by previous investigators is due to unchanged methemoglobin. This interaction requires one molecule of H_2O_2 per atom of methemoglobin iron. Ethyl hydrogen peroxide forms a similar compound with methemoglobin with absorption bands at the same place in the spectrum (145).

Hydrogen peroxide methemoglobin is not a stable compound. On standing in solution, it rapidly reverts to methemoglobin (98, 145); for example, about 70 per cent free methemoglobin is found after thirty minutes of standing and about 87 per cent after sixty to seventy minutes. Para-phenylenediamine, benzidine, traces of sodium hyposulfite, but not catalase, greatly accelerate the reaction; neither oxygen nor hydrogen peroxide is evolved in the course of this breakdown. This appears to be due to a peroxidase-like activity of methemoglobin; the hydrogen peroxide is used in the oxidation of substances such as p-phenylenediamine or even methemoglobin itself. In this connection, it may be noted that when catalase is absent from a solution of oxyhemoglobin or when catalase activity is suppressed by the addition of small amounts of azide or cyanide, then hydrogen peroxide causes the formation of methemoglobin. It may be appreciated that the addition of hydrogen peroxide to oxyhemoglobin may lead to a succession of reactions in equilibrium, namely, the formation of methemoglobin, the combination of methemoglobin with hydrogen peroxide, the reversion to methemoglobin itself as well as the partial oxidation of methemoglobin, through a peroxidase reaction, to other compounds.

The nature of the oxidation products of methemoglobin caused by hydrogen peroxide has been of considerable interest in connection with a study of the nature of bile pigments. Lemberg and his associates (155) have shown that the green pigment, choleglobin, can be formed by the action of hydrogen peroxide directly on methemoglobin or even indirectly from hemoglobin. Holden has similarly obtained a green basic compound of the bile-pigment type, a haematin and a porphyrin (123, 125).

5. *Methemoglobin azide.* In the course of an extensive study of the physiological actions of azide, Smith and Wolff (219) in 1904 observed that this ion formed a definite compound with methemoglobin, similar to that which cyanide ion formed. Keilin (143) found that the addition of sodium azide to methemoglobin at a pH of 5.6–6.4 caused a change to a red-colored solution with the appearance of two absorption bands at 575.0 mμ and 542.5 mμ. The dissociation constant of methemoglobin azide is about 1.2×10^{-4} and the compound contains 1 molecule of azide per atom of methemoglobin iron. The mean value of the magnetic susceptibility is consistent with the possibility that this compound has one unpaired electron per iron atom, with covalent bonds (56).

6. *Methemoglobin hydrosulfide.* Hydrogen sulfide reacts with methemoglobin at an acid pH, 5.6–5.8, to form methemoglobin hydrosulfide, a red-colored compound which has absorption bands at 545 mμ and 578 mμ. Keilin (144) found that the dissociation constant of this compound was about 1.3×10^{-5}. Coryell and his associates (57) observed that methemoglobin hydrosulfide underwent rapid auto-reduction to ferrohemoglobin, a phenomenon not mentioned by Keilin. In spite of the rapid breakdown of the hydrosulfide, Coryell (57) obtained values for the magnetic susceptibility which indicated the existence of one unpaired electron per iron atom, with covalent bonds.

7. *Nitric oxide methemoglobin.* There has been considerable controversy concerning the combination of nitric oxide with methemoglobin. Haurowitz (100) reported that nitric oxide combined only with reduced hemoglobin in the complete absence of oxygen, and that methemoglobin was reduced by nitric oxide to hemoglobin. In contrast, Anson and Mirsky (6) considered that nitric oxide oxidized hemoglobin to methemoglobin, and that the latter compound then reacted with additional nitric oxide. Keilin and Hartree (146) reconciled these two opposing viewpoints by showing that reduced hemoglobin when mixed with nitric oxide formed a red-colored solution with absorption bands at 536 mμ and 574.5 mμ. Methemoglobin, upon reaction with nitric oxide, yielded a solution with absorption bands at 568 mμ and 531 mμ. The nitric oxide-methemoglobin compound is unstable and reverts rapidly to methemoglobin.

8. *Other compounds of methemoglobin.* Methemoglobin has been shown to combine with a number of other substances, namely, fulminate (15), thiocyanate (140), cyanate (109, 214), ethanol (56), ammonia (56), imidazole (204), and oxidation products of aniline (135). The interaction of acid methemoglobin with thiocyanate is not sensitive to pH; the pK value of this reaction is 3.14. The interaction of alkaline methemoglobin with thiocyanate is pH dependent, and the pK value is 9.8. The magnetic susceptibilities of the compounds formed with ethanol and with ammonia have been determined by Coryell and Stitt (56). Imidazole methemoglobin has one unpaired electron and hence covalent bonds. This compound is not a very firm one; the pK value is 2.7 at pH 7 (204).

E. Oxidation-reduction equilibrium between hemoglobin and methemoglobin

In addition to the reactions of methemoglobin which we have described above, it is necessary to consider the oxidation-reduction equilibrium between hemoglobin and methemoglobin. As we shall show subsequently, methemoglobin is

reduced *in vivo* to hemoglobin by a variety of substances. Reduction *in vitro* can also be accomplished by a number of compounds such as ascorbic acid (236), reduced diphosphopyridine nucleotide (89), and the leuko forms of dyes (177).

The oxidation of hemoglobin to methemoglobin and the reduction of methemoglobin to hemoglobin raise the question whether this is a reversible system and, if so, what its oxidation-reduction potential is. This aspect has been investigated in some detail by Conant and his associates (51–53). After some attempts which yielded approximate values, Conant was able to make fairly precise measurements of the potentials on crystallized horse methemoglobin against an inert platinum electrode, obtaining a value of 152 ± 5 millivolts for E_0 at pH 7.0, in the electrochemical equation

$$E = E_0 + \frac{RT}{n} \ln \frac{[\text{MHb}]}{[\text{Hb}]}$$

where E_0 represents the potential of an equimolar mixture of hemoglobin and methemoglobin. The ionic strength, u, in these experiments was 0.3. Further determinations at a higher ionic strength, $u = 1.6$, yielded a somewhat lower value, about 29 millivolts for E_0 at pH 7.0. Employing a spectrophotometric method, Conant and Scott (53) obtained a value of 150 millivolts at pH 6.9, essentially in agreement with the value obtained by the electrochemical method.

The oxidation-reduction potential of this system has also been investigated by a number of other workers (14, 104, 106, 108, 209–211), particularly with respect to the effect of pH. Barnard (14) also obtained a value of 153 millivolts for E_0 at pH 7.07 at a total concentration of dog methemoglobin of 3.52 mM per liter, and an increase of about 75 millivolts with each increase of one pH unit. Havemann and Wolff (108) studied the effect of pH upon the oxidation-reduction potential, found a value of about 95 millivolts at a pH of 7.0 and a change of about 70 millivolts per pH unit.

The oxidation-reduction potential of the methemoglobin-hemoglobin system has also been investigated by means of dye indicators. In this connection, it may be recognized that a dye with a relatively high oxidation-reduction potential reduces little methemoglobin, whereas dyes with low potentials convert methemoglobin to hemoglobin very effectively. For example, a 90 per cent reduced solution of indigosulfonate ($E_0 = -125$ millivolts) reduced methemoglobin completely, whereas under similar conditions a 90 per cent reduced dichlorphenolindophenol ($E_0 = +217$ millivolts) reduced only 70 per cent of the methemoglobin (209, 210). Essentially this method consists in determining the proportion of methemoglobin which is reduced in the absence of oxygen by mixtures of the reduced and oxidized forms of various dyes. The potentials of these dye mixtures can be calculated from the following equation:

$$E = E_0 + 0.03 \log \frac{\text{oxidized form of dye}}{\text{reduced form of dye}}$$

where E_0 is the potential of a 50–50 per cent mixture of these forms at pH 7.0.

The E_0 value of the methemoglobin-hemoglobin equilibrium may then be calculated as follows:

$$E_{O,\,(MetHb)/(Hb)} = E_{dye} - 0.06 \log \frac{MetHb}{Hb}$$

Employing this equation, Schmidt (210) obtained a value for E_0 of 202 to 214 millivolts, which was higher than that recorded by others. Havemann and Heubner (106) contended that this value was erroneouly high. Schmidt had estimated the amount of reduced hemoglobin formed through the action of the dye by determining the amount of carbon monoxide that could be combined chemically with the hemoglobin-methemoglobin mixture. According to Havemann and Heubner (106) such a technique causes a displacement of the equilibrium, MetHb $\rightleftarrows$ Hb, to the right, and consequently the values for the amount of hemoglobin and the value of E_0 for the methemoglobin-hemoglobin system are too high.

It was appreciated by Conant and his associates (51–53) that the methemoglobin-hemoglobin system was to be classed among those sluggish oxidation-reduction systems in which stable potentials were not quickly established and periods ranging up to several hours were necessary for the electrode to come into equilibrium with the system. For example, Conant and Pappenheimer (52) found that, in their most satisfactory determinations, about half an hour was necessary for establishment of a stable equilibrium; on the other hand, there were many runs in which it was not possible to attain equilibrium, and the results had to be discarded.

The sluggishness and uncertainty which characterize the establishment of equilibrium values in the methemoglobin-hemoglobin system prompted Taylor and Hastings (228) to employ electromotively active mediators. For example, they observed that in the presence of 3.5×10^{-5} M m-toluylenediamine indophenol, a stable potential was established within fifteen minutes and remained constant for an hour; in contrast, under the same conditions but in the absence of this mediator, the potential was still decreasing after two hours. Employing this method, Taylor and Hastings obtained a value of +139 millivolts for E_0 at pH 7 and at a temperature of 30°. This method has been utilized by Havemann (104) in an extensive study of the influence of pH and temperature on the oxidation-reduction potential. At 30° and pH 7, Havemann obtained a value of +150 millivolts for E_0.

III. BIOCHEMISTRY OF METHEMOGLOBIN

This section will be concerned with the *in-vivo* equilibrium between hemoglobin and methemoglobin, the effect of methemoglobin on the oxygen-carrying properties of the blood, the methods of determining the concentration of methemoglobin in the blood, and, finally, the enzymic mechanisms involved in the reduction of methemoglobin.

A. Methods for quantitative determination of methemoglobin in blood

It may be of value to describe briefly some of the principles and chief features of the quantitative estimation of methemoglobin in the blood. Most of the methods which have been described may be classified into three main groups: (a) gasometric methods, (b) those based on the spectrophotometry before and after treatment of blood with cyanide which converts the methemoglobin into cyanmethemoglobin, and (c) direct spectrophotometric methods.

1. *Gasometric methods.* It has been known for a number of years that normal human blood contains a hemoglobin moiety which contains trivalent iron and which lacks the power to combine reversibly with oxygen or carbon monoxide until the ferric ion is reduced to ferrous ion (5, 188, 197, 233, 234). This moiety has been termed "inactive hemoglobin." Nicloux and Fontes (188) introduced a method for determining this portion of the blood pigment by reducing it with $Na_2S_2O_4$ and noting the increase in the carbon monoxide capacity. The procedure has been refined by Van Slyke and his associates (233, 234). Ramsay (197) used essentially the same principle but reduced the inactive hemoglobin with titanous ion (Ti^{+++}) and utilized the ensuing increase in oxygen-combining power as a measure of the "inactive pigment" originally present.

2. *Spectrophotometric methods.* These methods depend upon the decrease of transmission of light of wave length of 635 mμ or 800 mμ, usually the former, when cyanide is added to a solution of methemoglobin. As pointed out earlier in this paper, the cyanide changes the brownish methemoglobin to the red cyanmethemoglobin but does not affect hemoglobin. Procedures based on this principle have been described by Evelyn and Malloy (73), Havemann, Jung and von Issekutz (107), Michel and Harris (178), and Horecker and Brackett (128). That of Evelyn and Malloy (73) may be described in order to illustrate more specifically the principle which is involved. One volume of whole blood is delivered into one hundred volumes of M/60 phosphate buffer of pH 6.6 in a colorimeter tube. The solution is allowed to stand for five minutes and the optical density is read at 635 mμ in an Evelyn photoelectric colorimeter or other suitable spectrophotometer. One drop of a neutralized sodium cyanide solution is then added to convert any methemoglobin to cyanmethemoglobin, and another reading is made at 635 mμ after several minutes. The difference between the two readings is proportional to the concentration of methemoglobin; if the Evelyn photoelectric colorimeter is used the concentration may be expressed directly in Gm. per 100 cc. as follows:

$$M = \frac{100(L_1 - L_2)}{2.77}$$

Other photoelectric colorimeters or spectrophotometers require, of course, different factors in the denominator.

If the concentration of methemoglobin is desired as a percentage of the total blood pigment, it is not necessary to note precisely the amount of blood that is added to the phosphate buffer. The solution of blood is divided into two portions.

In one portion, the difference between the readings at 635 mμ is noted before and after the addition of neutralized cyanide. The second portion is treated with ferricyanide to convert all the blood pigment into methemoglobin, and readings are taken at 635 mμ before and after the addition of neutralized cyanide. The difference in the readings of the first portion divided by the difference in the readings of the ferricyanide-treated sample yields the value for the percentage of methemoglobin.

The amount of methemoglobin in methemoglobin-hemoglobin mixtures has also been measured by determining the ratio of the absorption at different wave lengths as, for example, that at 575 or 540 mμ to that at 560 mμ (67, 198). However, such methods have now been largely abandoned in favor of those which depend upon the conversion of methemoglobin to cyanmethemoglobin.

TABLE I

Concentration of inactive hemoglobin in normal human blood as determined by the gasometric method

INVESTIGATOR	NO. OF SUBJECTS	NO. OF BLOOD SPECIMENS	PER CENT OF TOTAL Hb		
			Minimum	Maximum	Mean
Ammundsen (4)	53	63	0	12.0	3.0
Kallner (141)	20	20	−1.2	1.2	−0.2
Roughton, Darling and Root (203)	4	26	0.7	5.0	2.8
Kallner (142)	4	4	−1.5	0.5	−0.5
Ramsay (197)	38	38	−0.5	7.0	1.9
Van Slyke and associates (234)	19	19	0.6	1.8	1.28

The question concerning the extent to which values obtained by the gasometric method agree with those obtained by the cyanide-spectrophotometric method is discussed in the following section.

B. Concentration of methemoglobin present in normal blood

Table I shows the values recorded by various investigators for the concentration of "inactive hemoglobin" present in normal human blood. It may be noted that values as high as 12 per cent have been reported, but that the average values are usually about 1 to 2 per cent of the total blood pigment. In Table II are shown the concentrations of methemoglobin normally present in human blood, as determined by the cyanide-spectrophotometric method. With the exception of a high value of 8 per cent recorded by Havemann, most values range between 0.0 and 2.4 per cent of the total blood pigment.

A comparison of the values listed in Table I and II shows that the concentration of "inactive hemoglobin" appears greater than that of methemoglobin as determined by the cyanide-spectrophotometric method. This difference was made particularly clear by the study of Van Slyke and his associates (234). It would, therefore, seem that the "inactive hemoglobin" contains some other component

besides methemoglobin. Indeed, this component appears to be converted into active hemoglobin when blood is allowed to stand for several hours; in contrast, the concentration of methemoglobin does not decrease. Thus, in Van Slyke's work (234) the concentration of "inactive hemoglobin" as per cent of the total blood pigment was: 1.29 ± 0.35 per cent three minutes after being drawn from the vein; 0.90 ± 0.32 per cent, 0.5 to 1.5 hours later; 0.56 ± 0.23 per cent, two to four hours after venipuncture. In contrast, at these times the concentrations of methemoglobin, as determined by the cyanide-spectrophotometric method, were 0.41 ± 0.44 per cent, 0.44 ± 0.59 per cent and 0.33 ± 0.44 per cent, respectively.

It is of interest that methemoglobinemia is also normally present in animals. Indeed, in some species the extent of this normal methemoglobinemia appears to be greater than in normal man. Thus, Ramsay (197) found the "inactive hemoglobin" to range from 0 to 25.5 per cent and to average 7.0 of the total blood pigment in a series of seventeen horses. Issekutz (132), employing the more specific

TABLE II

Concentration of methemoglobin in normal human blood as determined by the cyanide-spectrophotometric method

INVESTIGATOR	NO. OF SUBJECTS	NO. OF BLOOD SPECIMENS	PER CENT OF TOTAL Hb		
			Minimum	Maximum	Mean
Havemann and associates (107)				8	
Schmidt-Burgh (208)	20	20	1.1	2.4	1.7
Paul and Kemp (191)	20	20	0.1	0.8	0.4
Van Slyke and associates (234)	19	19	0.0	1.1	0.4

cyanide-spectrophotometric method, found that in cats the concentration of methemoglobin ranged from 4 to 17 and averaged 8.6 per cent of the total blood pigment. Heubner and his associates, however, have failed to note such large differences between the methemoglobin content of the blood of man and various animal species. Employing spectrophotometric methods based on carboxyhemoglobin as well as cyanmethemoglobin formation, Heubner (114) obtained the average values for five to seven individuals in each species, as shown in Table III.

C. The hemoglobin-methemoglobin equilibrium

The presence of methemoglobin in normal blood as well as other phenomena which we shall presently note raises the definite possibility that there normally exists in blood the equilibrium, methemoglobin $\rightleftarrows$ hemoglobin, which is regulated by various oxidizing and reducing substances, but which under ordinary conditions is shifted far to the right. Such a concept helps to explain the differing extents of formation of methemoglobin by the same drug in different species, the differing rates of reduction of methemoglobin in various species and the occurrence of idiopathic methemoglobinemia.

1. *Rate of disappearance of methemoglobin.* An overall view of the rate with which methemoglobin is reduced to hemoglobin *in vivo* may be determined experimentally in either of two ways: (a) by following the rate of reduction of methemoglobin of intravenously injected methemoglobinemic erythrocytes; (b) by following the rate of disappearance of methemoglobin after the injection of a methemoglobin-forming substance.

The former method has been employed by Heubner and Moebus (117), Jung (139) and Gelinsky (80). Jung removed 30 to 40 cc. of blood from cats, converted the hemoglobin to methemoglobin by means of an isotonic sodium chloride-sodium nitrite mixture, washed the resulting methemoglobin-containing erythrocytes several times with saline, made them up to the original volume with saline and reinfused them into the cat. The rate of conversion varied with the initial concentration of methemoglobin. For example, when sufficient methemoglobin-containing blood was infused so as to yield an initial concentration of about 3.8 grams per 100 cc., the concentrations at the end of 1, 2, 4 and 6

TABLE III

Concentration of methemoglobin in normal animals

SPECIES	AVERAGE TOTAL HEMOGLOBIN CONC.	AVERAGE METHEMOGLOBIN CONC.	METHEMOGLOBIN AS FRACTION OF TOTAL BLOOD PIGMENT
	Gms. per 100 cc.	*Gms. per 100 cc.*	*per cent*
Man	14.3	0.10	0.7
Dog	14.9	0.14	0.9
Cat	12.2	0.13	1.1
Rat	12.3	0.10	0.8
Rabbit	12.8	0.02	<0.2
Guinea pig	12.7	0.03	<0.2

hours were, respectively, about 2.4, 2.1, 1.4 and 1.0 gram per 100 cc. When the initial concentration was about 1.3 grams per 100 cc., the corresponding concentrations at these intervals were about 0.9, 0.7, 0.5 and 0.2 gram per 100 cc. About eight hours were required to reduce an initial concentration of 5 grams of methemoglobin per 100 cc. of blood to negligible levels.

The comparison of the rates of conversion of methemoglobin to hemoglobin in different species is of interest. In contrast to Jung's results in cats, Gelinsky (80) observed that a period of about four hours was required in rats and only about one hour in guinea pigs for the disappearance of an initial concentration of 5 grams of methemoglobin per 100 cc.

The second method of studying the rate of conversion rests on the assumption that, although both methemoglobin-forming and methemoglobin-reducing reactions take place concurrently and the concentration of methemoglobin at any moment is the resultant of these two groups of reactions, the methemoglobin-forming reactions become negligible after a stated period in the case of certain compounds and that the apparent rate of disappearance of methemoglobin is practically identical with its true rate of disappearance.

Cox and Wendel (58) tested twenty-five compounds for their methemo-

globin-producing potency in dogs. After the attainment of the maximum concentration, the rate of disappearance was constant and amounted to about 11 per cent of the total blood pigment per hour. This value held whether the maximal concentration was 60 per cent of the total pigment, as with p-aminophenol and sodium nitrite, or about 25 per cent as was the case after injection of hydroxylamine. Such constancy of rate of conversion of methemoglobin had not been observed by Jung (139). In view of the paucity of data in this field, it is difficult to reconcile this conflict. It is possible that difference in technic or difference in species used may account for the discrepancy.

The comparative rates of reduction in infused and drug-induced methemoglobinemias have recently been considered in detail by Kiese (147). He injected intravenously 25 mg. of sodium nitrite per kg. into a dog and obtained a maximal methemoglobin concentration of about 5.2 grams per 100 cc., about 35 per cent of the total pigment. The rate of reduction was about 1.5 gram per 100 cc. per hour or about 10 per cent per hour, a value in essential agreement with that of Cox and Wendel (58). When blood was removed from the animal and an equal volume of methemoglobin-containing cells was then infused, the rate of reduction was 1.3 grams per 100 cc. or about 8.5 per cent per hour. However, since only about 40 per cent of the cells or about two million were methemoglobinemic, Kiese (147) assumed that the corrected rate of reduction should be $1.3 \times \frac{5}{2}$ or 3.2 grams of methemoglobin per hour.

It would thus appear that the rate of methemoglobin reduction is more rapid in methemoglobinemias induced by the infusion of methemoglobinic blood than in those that are drug induced. A possible explanation for this is that, even in a rapidly induced methemoglobinemia, the rate of reduction appears slower than is actually the case because of the continued presence of methemoglobin-forming material. This view is substantiated by the finding of Greenberg, Lester and Haggard (88) that in nitrite-induced methemoglobinemia in rats when the concentration of methemoglobin was maximal, nitrite was still present in appreciable amounts.

2. *Factors controlling rate of disappearance of methemoglobin.* The factors which may influence the rate of reconversion of methemoglobin to hemoglobin have received considerable attention. Brooks (32) reported that intravenous injection of glucose accelerated greatly the disappearance of nitrite-induced methemoglobinemia in rabbits; on the other hand, Cox and Wendel (58) found no such influence on nitrite-induced methemoglobinemias in the dog either when the blood glucose was elevated as the result of glucose infusion or decreased as the result of insulin injection. Cox and Wendel also observed that lowering the body temperature decreased the rate of methemoglobin disappearance. Of chief interest in this connection are those compounds which reduce the methemoglobin rapidly when they are injected or, in some instances, when they are fed. Among these are methylene blue and other oxidation-reduction dyes (49), ascorbic acid, 2–3 dimercaptopropanol, glutathione or cysteine (153). We shall discuss later the possible therapeutic implications of the substances which cause

the reversion of methemoglobin to hemoglobin. The elucidation of the mechanisms which are involved in the *in-vivo* reversion of methemoglobin to hemoglobin has gained much from *in-vitro* studies.

Warburg, Kubowitz and Christian (243) observed that, when methemoglobin-containing red cells were allowed to incubate in the presence of glucose, hexosemonophosphate, hexosediphosphate or lactic acid, methemoglobin was changed to hemoglobin and oxygen was absorbed. For example, the oxygen consumption of 2 cc. of normal rabbit erythrocytes in two hours in the presence of 0.1 cc. of 10 per cent glucose was 8 cmm. The oxygen consumption of 2 cc. of approximately 50 per cent methemoglobinized cells was 68 cmm. in the absence of glucose, and 238 cmm. in the presence of 0.1 cc. of 10 per cent glucose.

It may be stressed that no reduction of methemoglobin occurs when methemoglobinemic erythrocytes are washed free of glucose. Drabkin (66) showed that the reduction of methemoglobin in erythrocytes to hemoglobin was accompanied by a decrease in glucose. The reversion of methemoglobin to hemoglobin was inhibited by hemolysis, or by the addition of fluoride or iodoacetate. The inhibition by fluoride was overcome by means of the addition of pyruvate, but not of lactate.

These findings would indicate that one or more enzyme systems in the erythrocyte are involved in the reduction of methemoglobin to hemoglobin, that the existence of this enzyme system is dependent upon the integrity of the erythrocyte and is associated with the glycolytic process. Warburg and Christian (240) isolated a macromolecular "zwischenferment" and a coenzyme of low molecular weight which, together with hexosemonophosphate, was capable of reducing methemoglobin.

The nature of the enzyme systems which were capable of reducing methemoglobin prepared directly from crystallized horse hemoglobin has been studied by Kiese (148, 149). Employing the uptake of carbon monoxide as a measure of the formation of hemoglobin, he found that purified methemoglobin was reduced directly by glyceraldehyde, ascorbic acid or cysteine; the rate of reduction was dependent upon the concentrations of these substances and of methemoglobin and upon the pH. When the coenzyme, triphosphopyridine nucleotide (TPN), isolated by Warburg, Christian and Griese (241) from red blood cells, was incubated with methemoglobin and hexosemonophosphate, it was observed that the rate of reduction was much slower than in the intact erythrocyte. Kiese assumed that an additional enzyme factor in the erythrocyte was necessary for the reduction of methemoglobin and found that an extract of erythrocytes in sufficiently high concentration markedly accelerated the conversion to hemoglobin. The isolation of the enzyme factor was accomplished by treating horse erythrocytes with toluene and carbon monoxide. Considerable hemoglobin separated out at this step and again when the resulting solution was treated at 0° with potassium phosphate at a pH of 7.0. The precipitated hemoglobin was centrifuged and washed with M phosphate solution of pH 7.0. These washings and the supernatant solution were combined and precipitated fractionally with ammonium sulfate. The fraction, precipitated between 20 and 40 per cent am-

monium sulfate concentrations, was found capable of causing active reduction of methemoglobin in the presence of hexosemonophosphate (Robison ester). Apparently this precipitate contained both the coenzyme and an enzyme factor. These components could be separated. Kiese termed the enzyme constituent "hämiglobinreductase."

The role of the pyridine nucleotides in the reduction of methemoglobin was also stressed by Gutmann, Jandorf and Bodansky (89) who studied the well-known accelerative effect of methylene blue in the reduction of methemoglobin. The lack of reduction of methemoglobin in hemolyzed, methemoglobinemic blood raised the possibility that pyridine nucleotides were absent; there is evidence that pyridine nucleotides disappear rapidly upon the lysis of erythrocytes or the homogenization of animal tissues. Mann and Quastel (170) as well as Handler and Klein (91) had found that the addition of nicotinamide inhibits the disappearance of the pyridine nucleotides, presumably by inhibiting their enzymic hydrolysis. Applying this finding, Gutmann, Jandorf and Bodansky (89) observed that, whereas no reduction of methemoglobin occurred in hemolysates containing methylene blue and hexosediphosphate or lactate, appreciable reduction took place when nicotinamide was added. Much more direct proof of the role of the pyridine nucleotides was obtained when it was found that the reduced but not the oxidized form of diphosphopyridine nucleotide (DPN) reduced purified methemoglobin. This acceleration was increased greatly by the addition of methylene blue. It appeared from these experiments and the work of other investigators that the production of reduced pyridine nucleotides was an essential link in the reduction of methemoglobin. The removal of substrate or the inhibition of glycolysis by fluoride or iodoacetate interferes with the production of DPN-H_2 or TPN-H_2; hemolysis results in the destruction of these coenzymes in the red cells.

The study of the rare disease, familial idiopathic methemoglobinemia, has also contributed to our knowledge of the enzyme mechanisms involved in the conversion of methemoglobin to hemoglobin. In this disease, as the name indicates, methemoglobinemia is present in an individual from birth without apparent external cause. Lian and his coworkers (160) in 1939, as well as Barcroft and his associates (11) some years later, observed that in contrast to what occurs in the blood from normal individuals in whom methemoglobinemia has been induced the shed blood from persons with idiopathic methemoglobinemia shows little if any tendency for methemoglobin to disappear when the blood is allowed to stand. Sievers and Ryon (218) found that this stability of methemoglobin was not dependent in any way on the plasma, since plasma from a patient with the disease did not influence the rate of reduction of methemoglobin in normal human erythrocytes nor did normal plasma accelerate the reduction of methemoglobin in the erythrocytes of the patient.

As has already been noted, washed methemoglobinemic erythrocytes from normal persons show very little reversion of the methemoglobin to hemoglobin; such reversion takes place in the presence of added glucose or lactate (82, 84, 89). However, the presence of these substrates is not adequate for

the reduction of methemoglobin in the erythrocyte from the individual with idiopathic methemoglobinemia (84).

According to Gibson (84), methemoglobin reduction in normal erythrocytes takes place through the oxidation of triosephosphate and lactate. This reaction requires, in addition to the production of these substrates from glucose, dehydrogenase, coenzyme I and coenzyme factor I or diaphorase. It is diaphorase which appears to be deficient in the erythrocytes of patients with familial idiopathic methemoglobinemia. Gibson found that the amount of this factor in the bloods of five patients was lower than the amounts in a series of bloods from twelve normal persons.

However, there are other aspects which still remain to be explored in order to furnish a complete picture of the enzymic mechanisms involved in the reduction of methemoglobin. For example, Spicer and his associates (221) have recently tested a variety of substrates for their ability to reduce methemoglobinemic erythrocytes of the dog at neutral pH. Only four hexoses, namely, D-fructose, D-glucose, D-mannose and D-galactose were found effective. In addition to the established effect of lactate, fumarate and L-malate were also found capable of reducing methemoglobin. The effects varied with the concentration of the substrates. Phosphate ion was necessary for a maximal response with glucose, mannose or fructose but not with galactose. Oxalate and citrate inhibited the reduction of methemoglobin by glucose, but citrate ion accelerated the reduction by lactate.

D. Effect of methemoglobinemia on the oxygen dissociation curve

The quantitative aspects of the combination between hemoglobin and oxygen have been well explored (1, 64, 120, 130, 192). It is appreciated that when the oxygen tension is plotted against the degree of oxygenation (or per cent of oxyhemoglobin formed) a so-called dissociation or sigmoid curve is obtained. Mathematical expressions to describe the shape of this curve and hence to formulate a general expression for the affinity between oxygen and hemoglobin have been submitted by a number of workers such as Hüfner (130), Hill (120), Adair (1), and Pauling (192). The affinity of oxygen for hemoglobin is influenced by temperature, pH, ionic strength, concentration of hemoglobin and, what is of particular relevance for the subject of this review, by the presence of other blood pigments such as carboxyhemoglobin and methemoglobin. We shall first briefly present the studies concerning the effect of carboxyhemoglobin, since it illustrates the effect of methemoglobin and more data concerning it are available.

In 1912, Douglas and his coworkers (64) showed that the presence of carboxyhemoglobin shifted the dissociation curve of the residual hemoglobin to the left. This observation was confirmed by subsequent workers and it was also demonstrated that the form of the dissociation curve became more hyperbolic in the presence of carboxyhemoglobin (202, 223). A quantitative idea of these effects may be obtained from the following tabulation of the oxygen dissociation of the *residual* oxyhemoglobin in the presence of various amounts of car-

boxyhemoglobin (Table IV). The values have been interpolated from the curves presented by Roughton and Darling (202).

It must be recognized, however, that the effects of poisoning by carbon monoxide are twofold. Not only is some of the hemoglobin combined with carbon monoxide and therefore becomes unavailable for transport of oxygen, but as pointed out above, the residual oxyhemoglobin becomes less capable of dissociation. This again may be demonstrated by the tabulation of the following data obtained from curves submitted by Roughton and Darling (Table V). That the presence of carboxyhemoglobin makes the dissociation curve of the

TABLE IV

Oxyhemoglobin dissociation in presence of carboxyhemoglobin

OXYGEN TENSION	PER CENT OF HEMOGLOBIN AS OXYHEMOGLOBIN IN PRESENCE OF		
	No COHb	20% COHb	60% COHb
mm. Hg			
10	13	21	45
20	36	49	73
30	58	70	87
40	74	82	95
60	90	93	

TABLE V

Effect of carboxyhemoglobin and anemia on the form of the dissociation curve of oxyhemoglobin

OXYGEN TENSION	OXYGEN BOUND TO HEMOGLOBIN (VOLUMES PER CENT) IN PRESENCE OF		
	No COHb	60% COHb	Anemia 40% of normal
mm. Hg			
10	2.4	3.8	1.0
20	7.0	5.9	2.8
30	11.7	7.0	4.8
40	15	7.6	6.0
60	18	7.9	7.2
100	19.3	8.0	7.8

residual oxyhemoglobin less sigmoid and more hyperbolic may be seen from a comparison of the last two columns. Thus a person with 40 per cent of his hemoglobin left uncombined with carbon monoxide shows about the same absolute amount of oxyhemoglobin as an anemic person with 40 per cent hemoglobin at 100 mm. Hg oxygen tension, but about 25 per cent more at 40 mm. Hg, 100 per cent more at 20 mm., and 280 per cent more at 10 mm. oxygen tension. Again, Table V shows that the individual with a 60 per cent carboxyhemoglobinemia has only 40 per cent as much oxyhemoglobin as the normal individual at an oxygen tension of 100 mm., but 160 per cent as much at a tension of 10 mm.

That methemoglobin produces a similar effect on the dissociation of oxyhemoglobin was demonstrated by Darling and Roughton in 1942 (60). Quantitatively,

however, the effect of methemoglobin is less than that of carboxyhemoglobin. For example, a content of 23 per cent carboxyhemoglobin shifted the dissociation curve of the residual oxyhemoglobin in human blood about as far to the left as did a content of 43 per cent methemoglobin. The effect of methemoglobin on the oxygen dissociation curve is wholly reversible. Eder and his associates (69) have recently reported that the position and shape of the dissociation curve are not altered in familial idiopathic methemoglobinemia.

The alteration of the shape and position of the oxygen dissociation curve in carboxyhemoglobinemia or methemoglobinemia implies that the tissues are liable to anoxia, not only because of loss of the oxygen-carrying capacity of the blood, but also because the residual oxyhemoglobin is less capable of dissociating and therefore unloading oxygen in the tissues. We shall presently consider this as well as other physiological aspects of methemoglobinemia in greater detail.

The question naturally arises why carboxyhemoglobinemia or methemoglobinemia shifts the dissociation curve of oxyhemoglobin to the left and renders it less sigmoid and more hyperbolic. As we have already noted, a number of equations have been submitted for the dissociation of oxyhemoglobin itself. Although each of these does not offer a completely quantitiative explanation, the sigmoid shape of the curve is best understood by the intermediate compound hypothesis submitted by Adair (2) and later modified by Pauling and his associates (55, 192). According to this formulation, the blood hemoglobin molecule reacts with O_2 in four stages, one molecule combining stepwise with each of the four ferrous atoms, as follows:

$$Hb_4 + O_2 = Hb_4O_2 \; ; \; Hb_4O_2 + O_2 = Hb_4O_4 \; ;$$
$$Hb_4O_4 + O_2 = Hb_4O_6 \; ; \; Hb_4O_6 + O_2 = Hb_4O_8$$

At very low O_2 pressures the principal compound formed would be Hb_4O_2, and the form of the dissociation curve in this region is a straight line. The greater ease of formation of the dioxy-compound, Hb_4O_4, is reflected in the upward turn of the curve, and the tapering off of the curve is due to the formation of the trioxy- and tetraoxy-compounds. Each of the four reactions has its own equilibrium constant so that the final expression for the percentage of hemoglobin contains four constants. Pauling (192) submitted a simplified expression which contained two constants. It is beyond the scope of this review to discuss these expressions in further detail.

According to the hypothesis of intermediate compound formation, carbon monoxide could also be conceived to react stepwise with each of the four ferrous atoms. Indeed, according to Lemberg and Legge (153), twenty-one classes of intermediates are possible in which the four hemes are free, or are combined with oxygen, carbon monoxide, or with both. In the case of the hemoglobin-methemoglobin equilibrium, Darling and Roughton (60) postulated the existence of the following intermediates: $(Fe^{++})_3\,(Fe^{+++})_1\,X$; $(Fe^{++})_2\,(Fe^{+++})_2\,X$; $(Fe^{++})_1\,(Fe^{+++})_3\,X$; $(Fe^{+++})_4\,X$. Apparently, the formation of the intermediate compounds as the result of combination of ferrous ion with carbon monoxide as in carboxyhemoglobin, or as the result of oxidation of ferrous to ferric ions as in methemo-

globin, increases the affinity of the remaining ferrous ions for oxygen, and thus causes the shift of the dissociation curve of the residual oxyhemoglobin. The tendency for the dissociation curve to change from a sigmoid form towards a rectangular hyperbolic form as the concentration of methemoglobin is increased may be explained by the increasing concentrations of the molecules $(Fe^{++})(Fe^{+++})_3 X$, and $(Fe^{+++})_4 X$. The preponderant equilibrium involving oxygen at the higher methemoglobin concentrations would be

$$O_2 + (Fe^{++})(Fe^{+++})_3 X \rightleftarrows (O_2)(Fe^{++})(Fe^{+++})_3 X$$

IV. PHYSIOLOGY OF METHEMOGLOBINEMIA

In methemoglobinemia, as in carboxyhemoglobinemia, the decrease in hemoglobin available for oxygen transport together with the alteration in the shape and position of the dissociation curve constitutes a potential handicap to the organism in the unloading of oxygen in the tissues. We may now examine in somewhat greater detail the nature, extent, and consequences of this handicap.

A. Unloading of oxygen in the normal organism

Without entering into the vast details of the subject of respiration, we may mention briefly some of the salient features as a guide to our discussion of the effect of methemoglobinemia. The air in the alveolus of the lung contains oxygen at a tension or partial pressure of about 100 mm. Hg and carbon dioxide at a tension of about 40 mm. Hg. The alveolar air is practically in equilibrium with the arterial blood so that the tension of oxygen in the arterial blood is slightly lower and that of carbon dioxide slightly higher than in the alveolar air. Under normal conditions, the oxygen tension of the venous blood is about 40 mm. and that of carbon dioxide is about 45 mm. The oxygen tension in the tissues is less than 40 mm., perhaps even as low as 0 to 10 mm., depending upon the activity of the person. The carbon dioxide tension is higher than 46 mm. The arterial blood gives up oxygen to, and takes up carbon dioxide from, the peripheral tissues and it becomes venous blood in this process. The average oxygen dissociation curve for human blood, regardless of slight changes due to pH and CO_2 content, may be represented by figures, already given in Table V, which have been interpolated from a chart by Roughton and Darling (202). A change from an arterial oxygen tension of 100 mm. Hg to a venous oxygen tension of 40 mm. Hg implies a delivery of 19.4 minus 14.8 or 4.6 cc. of oxygen per 100 cc. of blood. This delivery occurs in a normal man at rest at sea level. A similar supply of oxygen can be furnished to the tissues under other conditions by virtue of the dissociability characteristics of oxyhemoglobin. For example, at high altitudes, as at an arterial oxygen pressure of only 50 mm. Hg, the delivery of 4.6 cc. of oxygen per 100 cc. of blood will be associated with a change to a venous tension of 30 mm. Hg. Again, in a normal man doing light work, the increased delivery of oxygen to the tissues may be reflected in a change from a normal arterial tension of 100 mm. of Hg to low venous tensions of 30 or even 20 mm. of Hg. In an anemia with a 40 per cent hemoglobin, a delivery of 4.6 cc.

oxygen per 100 cc. of blood will be accompanied by a change from an arterial oxygen tension of 100 mm. to a venous oxygen tension of 22 mm.

B. Unloading of oxygen in the carboxyhemoglobinemic or methemoglobinemic organism

The delivery of oxygen to the tissues can be accomplished not only by way of the mechanism just described, namely, the dissociation of oxyhemoglobin but also by calling into play such cardiovascular mechanisms as increased cardiac output and increased blood flow through the tissues. The presence of carboxyhemoglobinemia or methemoglobinemia limits the extent to which oxygen can be supplied to the tissues as a result of the dissociation of the residual oxyhemoglobin. The question therefore arises concerning the exact degree of carboxyhemoglobinemia or methemoglobinemia at which the cardiovascular mechanisms are invoked.

Asmussen and Chiodi (7) found that induced carboxyhemoglobinemias of 20 to 33 per cent in men were not extensive enough to affect the cardiac output. For example, in one individual with a carboxyhemoglobinemia of about 33 per cent, the changes in the arterial-venous oxygen content and tension were as follows:

	REST	LIGHT WORK	HEAVY WORK
A-V oxygen content (vols. per cent)	12.1 − 7.3 = 4.8	12.7 − 4.6 = 8.1	13.0 − 2.8 = 10.2
A-V tension (mm. of Hg)	99 − 16	99 − 11	100 − 7

The cardiac output did not increase in any of these states of activity after the induction of carboxyhemoglobinemia had been induced. However, there were definite increases when the carboxyhemoglobin content approached or exceeded 40 per cent of the total blood pigments (44). For example, in one subject at rest, the cardiac output per minute was 3.8 liters at 0 per cent HbCO, 4.1 liters at 16 per cent, 5.8 at 32 per cent and 5.6 at 45 per cent.

Similar data for methemoglobinemia in man are not available. Clark and his associates (45) found that in dogs the respiratory ventilation was not increased no matter what the degree of methemoglobinemia. The cardiac output did not increase except at degrees of methemoglobinemia greater than about 40 per cent of the total blood pigment. In other words, when the methemoglobinemia is less than this level, the oxygen supply to the tissues is maintained by virtue of the dissociation characteristics of the residual hemoglobin. For example, their dog No. 1 without any methemoglobinemia showed an A-V oxygen difference of 18.0 minus 13.4 or 4.6 cc. per 100 cc. of blood. At a methemoglobinemia of 56 per cent, the A-V oxygen difference was 7.5 minus 4.0 or 3.5 cc. per 100 cc. of blood. Evidently, not enough oxygen could be supplied by dissociation of the residual oxyhemoglobin, and this was evidenced by an increase in the cardiac output from a level of 1.8 liters per minute before induction of methemoglobinemia to a level of 2.5 liters per minute at a level of 58 per cent methemoglobin.

C. Effect of methemoglobinemia on oxygenation of working muscle

The data which we have just presented show that methemoglobinemia constitutes a potential impairment of the proper supply of oxygen to the tissues, first because less hemoglobin is available for oxygen transport and secondly because this residual hemoglobin has a greater affinity for oxygen and therefore unloads it less readily in the tissues. However, when the degree of methemoglobinemia is not excessive and the organism is at rest, an adequate oxygen supply is available. If the degree of methemoglobinemia exceeds about 40 per cent of the blood pigment, the cardiac output increases in order to aid in the supply of oxygen to the tissues.

Insufficient supply of oxygen to certain tissues results in a shift from the oxidative systems to the glycolytic systems in order to obtain the necessary energy (161). Such a shift is revealed by an increase in lactic acid production by the tissue with escape of this acid into the blood. The investigations of Bang (10) illustrate very forcibly this type of shift in man. During a prolonged period of mild work, the blood lactate rises at the beginning of the exercise. However, the cardiovascular and respiratory reflexes initiated by the exercise collaborate to deliver oxygen to the muscle at the rate required by the work; accordingly the liberation of energy switches back to the oxidative mechanisms and the blood lactate drops. If the work is strenuous, even though short, the oxygen supplied is not adequate; the muscle has to rely almost entirely on the anaerobic energy of glycolysis, and this is evidenced by a marked elevation of blood lactate and a slow return to normal levels. The extent of the rise in blood lactate after a measured amount of work may be used as a measure of the adequacy of oxygen delivery to the muscle tissue under non-resting conditions. The rate of delivery under these conditions may be inadequate at high altitudes, in physically untrained individuals or in other circumstances (229).

The extent to which methemoglobinemia interferes with the oxygenation of muscle in work was investigated by Tepperman, Bodansky and Jandorf (229). In four untrained subjects who were subjected to a work test on a cycle ergometer for three minutes at a load of about 4500 to 6000 ft. lbs. (621 to 828 kg. M.) per minute, the blood lactic acid curve was essentially the same before and after the induction of methemoglobinemias ranging from 7.5 to 16.8 per cent of the blood pigment. In two similarly untrained subjects, the blood lactate curve was higher and more prolonged in the presence of 21.7 and 27.1 per cent methemoglobin than in the absence of any methemoglobin. Trained subjects who worked at comparatively light work loads showed no upward displacement of the blood lactate curves with 7 to 17 per cent methemoglobinemia. However, at work loads higher than about 6500 ft. lbs. (897 kg. M.) per minute for three minutes, 10 to 20 per cent of methemoglobinemia resulted in impaired oxygenation of muscle as evidenced by elevated blood lactate curves.

D. Effect of methemoglobinemia on visual threshold

It has been noted by several groups of investigators that a rise in the threshold of the dark adapted eye occurs during anoxia induced by low oxygen tensions.

McFarland and his associates (168) found that carboxyhemoglobinemia raises the brightness discrimination threshold in cone vision as measured by the Crozier and Holway visual discriminometer (59) and that this effect and the increase in threshold at low oxygen tensions were additive. Bodansky and Hendley (25) observed that there was no significant increase in the visual threshold in individuals having concentrations up to 30 per cent methemoglobinemia either at sea level or at simulated high altitudes of 12,000 feet or 3660 meters (13 per cent oxygen) or 18,000 feet or 5490 meters (10 per cent oxygen). Short bouts of exercise lowered the rod threshold immediately after exercise in methemoglobinemic individuals, but not in normal individuals. However, five to ten minutes after exercise the threshold rose above the normal threshold to the same extent in both methemoglobinemic and nonmethemoglobinemic individuals.

It is somewhat difficult to account for the fact that methemoglobinemia does not affect the rod threshold whereas carboxyhemoglobinemia raises it. As was pointed out earlier in this review, methemoglobinemia usually shifts the position and alters the shape of the oxygen dissociation curve of the residual oxyhemoglobin much less than an equivalent degree of carboxyhemoglobinemia. Therefore, for a given amount of oxygen unloading, a lower venous and tissue oxygen tension would be reached in the case of carboxyhemoglobinemia than in the case of an equivalent degree of methemoglobinemia. This may explain why dark adaptation may be more readily impaired in the former instance than in the latter.

The rise in the rod threshold both in methemoglobinemic and nonmethemoglobinemic individuals, five to ten minutes after exercise, may be explained in terms of the acidosis of the post-exercise period, since Wald and his associates (238) have shown that acidosis causes a rise in the rod threshold whereas alkalosis causes a decrease. It is difficult to explain the decrease in threshold in methemoglobinemic individuals immediately following exercise.

E. Relation between degree of methemoglobinemia and symptomatology

The question naturally arises concerning the levels of methemoglobinemia at which the reserve of the oxygen dissociation curve and the circulatory adjustments, combined, prove inadequate in supplying oxygen to the tissues, as judged by the development of grave symptoms and the occurrence of death. The possibility exists that some symptoms and death may be due in certain cases to effects other than those of methemoglobin production *per se*. Vandenbelt, Pfeiffer and their associates (231) found that oral administration of p-aminoacetophenone and p-aminopropiophenone to dogs resulted in ataxia at 60 per cent methemoglobin, salivation and prostration at 75 per cent and loss of consciousness at 85 per cent. The lethal level of methemoglobinemia was between 87 and 95 per cent. Bodansky and Gutmann (24) injected dogs intravenously with varying doses of p-aminopropiophenone in propylene glycol. Of eight animals which developed maximal concentrations of methemoglobin ranging up to 81 per cent, all survived and were normal during the subsequent period

of observation of several days. Of twelve animals which developed maximal methemoglobin concentrations of from 83 to 92 per cent, only two survived. These results indicate a minimal lethal level of methemoglobinemia of about 80 to 85 per cent. The symptoms at the higher levels of methemoglobinemia were similar to those obtained by Vandenbelt, Pfeiffer and their coworkers. Essentially the same lethal level of methemoglobinemia with nitrite was obtained in the dog by Lester and Greenberg (157).

It may be readily appreciated that, at levels of 85 to 90 per cent methemoglobin, complete dissociation of the residual oxyhemoglobin would involve a fall of the venous oxygen tension to zero, yet would deliver to the tissues only 2 to 3 cc. of oxygen per 100 cc. of blood, an amount below the needs of the resting organism. It has already been pointed out that the cardiovascular mechanisms for increasing the oxygen supply to the tissues are called into play when the concentration of methemoglobin exceeds 40 per cent of the blood pigments. Hence it may be reasonably concluded that the combination of the reserve of the oxygen dissociation curve and the circulatory adjustments begin to prove inadequate in supplying oxygen to the tissues, as judged by the occurrence of ataxia, prostration and unconsciousness, when the methemoglobin concentration exceeds about 60 per cent. Failure of an oxygen supply necessary for sustaining life could be expected to result when the methemoglobin concentration reaches about 85 per cent. In this connection, it is interesting to note that the lethal level of carboxyhemoglobinemia is about 60 per cent of the total blood pigment (157). As has already been noted, this degree of carboxyhemoglobinemia causes as marked an alteration in the shape and position of the oxyhemoglobin dissociation curve as much higher concentrations of methemoglobin.

It should be stressed, on the other hand, that not all methemoglobin producing compounds are lethal by virtue of the methemoglobinemia which they induce. Lester and his associates (158) showed that doses of p-aminophenol and β-phenylhydroxylamine which produced maximal concentrations of methemoglobin were not sufficient to cause death. Thus, a maximum degree of methemoglobinemia, about 65 per cent of the total blood pigment, was produced in the rat by 5 mg. of β-phenylhydroxylamine per kg. Increasing the dose did not result in any increase in the concentration of methemoglobin, but death occurred at a dose of 35 mg. per kg. The amounts of methemoglobin formed by acetanilid in the cat are proportional to the concentration of total hemoglobin, but formation ceases at a residual concentration of hemoglobin that is adequate for supporting life.

F. Methemoglobinemia and hemolysis

A number of phenomena have been reported which indicate an association between methemoglobinemia and hemolysis or hemolytic anemia. The repeated administration of methemoglobin-producing drugs may cause a hemolytic anemia. For example, Van Loon and his associates (232) found that the chronic administration of acetanilid up to 36 mg. per kg. caused little or no abnormal hematological changes in dogs; larger doses produced a hemolytic anemia with-

out any evidence of bone marrow depression. When the drug was discontinued, the blood picture reverted to normal. Acetophenetidin produced similar effects but was not as active as acetanilid.

The exact mechanism of the drug-induced hemolytic effect is not known. These drugs are not directly hemolytic. The possibility exists that they or their metabolic products may render the cells more sensitive to hemolysis. However, Jung (136) has shown that, as judged by hemolysis in NaCl solutions of varying concentration, methemoglobinemic erythrocytes have the same resistance as normal erythrocytes.

G. Effect of methemoglobinuria on renal function

Bing (20) reported that the intravenous infusion of crystalline methemoglobin into acidotic dogs was followed by a fall in the effective renal plasma flow, the glomerular filtration rate and the tubular reabsorptive capacity for glucose. This was evidenced by anuria and rise in blood urea nitrogen. The animals usually became moribund by the third day. The renal lesions comprised hydropic degeneration of the proximal convoluted tubules, cellular necrosis in the distal segment and plugging of the collecting tubules. No such functional or structural changes developed in non-acidotic dogs which were infused with methemoglobin or hemoglobin, or in acidotic animals infused with hemoglobin or myoglobin. On the other hand, Harrison and his coworkers (95) were able to show impairment of renal function as the result of intravenous injections of hemoglobin as well as methemoglobin. Injection of methemoglobin into acidotic dogs appeared to produce a greater degree of renal impairment as judged by nitrogen retention, but the critical factor in the severity of the renal impairment, in these various instances, appeared to be the rate of urine flow.

V. PHARMACOLOGY OF METHEMOGLOBINEMIA

It is abundantly evident from the considerations we have so far presented that any compound will produce methemoglobinemia if it is capable of oxidizing hemoglobin, or of being metabolized to a compound which can oxidize hemoglobin. There is also the possibility that certain substances may activate the oxidation of hemoglobin to methemoglobin or may inhibit the cellular mechanisms of the erythrocyte which constantly tend to reduce methemoglobin to hemoglobin. The degree of methemoglobinemia formed by any particular substance will, therefore, depend upon the interplay of a number of factors: the mode of administration, whether oral or parenteral; the conversion in the intestinal tract by bacterial or other agents; the rate of absorption from the intestinal tract if the mode of administration is oral; the pattern of metabolism into oxidizing and non-oxidizing compounds; the rate of excretion of its metabolites; the level of the methemoglobin-reducing mechanisms in the organism, a level which may vary with species, age, and other influences not yet elucidated.

A. Direct oxidants of hemoglobin

A number of substances are capable of oxidizing hemoglobin directly to methemoglobin. This capacity may be revealed by adding such substances *in vitro* directly to blood, suspensions of erythrocytes, hemolyzed blood, or solutions of hemoglobin. This group includes the nitrites, chlorate, hydrogen peroxide, alloxan, the quinones, and dyes of high oxidation-reduction potential.

1. *Nitrites.* Perhaps the best known of these direct oxidants is nitrite. The interaction of nitrite with hemoglobin has been studied extensively. The early studies of Barcroft and Müller (12) indicated that the molar ratio of nitrite utilized to methemoglobin formed was 2, that is, two molecules of nitrite interacted with one molecule of hemoglobin to form one molecule of methemoglobin. However, ratios of about 1.0 have been reported by Van Slyke and Vollmund (235) and by Meier (176). More recently, lower values of 0.5 to 0.7 have been obtained by Austin and Drabkin (9), by Darling and Roughton (60), and by Greenberg and his associates (88). These results indicate that one molecule of nitrite reacts with two molecules of hemoglobin to form two molecules of methemoglobin. The reaction depends upon a number of factors, such as concentration of nitrite, temperature and pH. Greenberg (88) has pointed out that in some of the earlier work the conditions may not have been such as to permit the reaction to go to completion. Side reactions involving the formation of nitrosohemoglobin and nitrosomethemoglobin may also take place (146, 173).

Quantitative determinations of the *in-vivo* production of methemoglobin by nitrites appear to have been made first by Haldane and his coworkers (90) in 1897. A hypodermic dose of 300–330 mg. per kg. led to death in about thirty minutes, with a methemoglobin concentration of 74 per cent of the total blood pigment in one mouse and 91 per cent in another. The symptoms of poisoning were, progressively, motor incoordination, prostration, coma and death. In a rabbit injected with 130 mg. per kg., the methemoglobin rose to a maximum of about 55 per cent within approximately one hour, and began to decline at one and one half hours after injection and decreased to about 5 per cent in three and one half hours. Exposure to amyl nitrite also caused death with methemoglobinemias of about 80 per cent. Sakurai (205) observed in somewhat greater detail the relationship between toxicity and methemoglobin concentration. He found that the LD_{100} in cats was 35 mg. per kg. Death at this dose occurred in from fifty-five minutes to one hour and fifty-two minutes after subcutaneous injection; the concentration of methemoglobin at death ranged from 88 to 100 per cent. The LD_{100} in rabbits was 170 mg. per kg. Death occurred in periods similar to that in cats, and the methemoglobin concentrations at death ranged from 86 to 94 per cent. The rate of formation in cats was very rapid. Following an intravenous injection of 30 mg. per kg., concentrations of 22 to 37 per cent were apparent at fifteen minutes, and concentrations of 33 to 52 per cent at thirty minutes. Maximum concentrations began to be reached at one hour and thirty minutes.

More systematic investigations on the relationship in cats between the dose of intravenously administered nitrite and the maximal degree of methemo-

globin have been submitted by Issekutz (132). He found that the maximal concentration of methemoglobin formed was directly proportional to the dose. As will be pointed out later, for the aryl amino and nitro compounds, the dose-methemoglobin response curve is not, as for nitrite, a straight line but an S-shaped curve. Issekutz (132) found that the molecular ratio of the methemoglobin formed to the nitrite injected ranged from 0.7 to 1.4 and averaged 0.9. The significance and interpretation of molecular ratios of maximal methemoglobin concentration to dose of drug will be discussed more fully later. Issekutz also observed that the simultaneous injection of alcohol did not influence the formation of methemoglobin by nitrite.

Nitrites may be present in various preserved or cured meats. The original source of this nitrite is either a preserving solution which contains such nitrite or nitrate which has been added to the meat as the potassium salt and which is reduced in the course of time to nitrite either by bacterial organisms or by enzymes present in the meat. There exists a considerable literature concerning the detection and determination of nitrite in meat and meat products. Cases of poisoning due to the ingestion of such foods are reported from time to time. There is substantial evidence in some of the cases that the signs of toxicity or the occurrence of death is due largely to the methemoglobinemia induced by these nitrites. Thus, Schrader (212) has reported three deaths in young children from the presence of nitrite in meat-pickling salt or brine; cyanosis and gastroenteritis were noted in these cases. Bushoff (34) reported two instances of mass poisoning from the use of broth prepared from insufficiently clarified pickle-nitrite concentrate. Naider and Venkatrao (183) reported thirty cases of fatal nitrite poisoning in man. They estimated the lethal dose for man to be about 2 grams of sodium nitrite or about 2.6 grams of potassium nitrite. Severe cyanosis due to methemoglobin formation was reported by Barilari and Benedetto (13) from the ingestion of sausage containing sodium nitrite.

2. *Nitrates.* When nitrates are ingested, reduction to nitrite occurs in the intestinal tract, and absorption of the nitrite leads to methemoglobinemia. Three important sources of such methemoglobinemia in man are the administration of bismuth subnitrate to patients with gastrointestinal disorders, the use of ammonium nitrate as a diuretic and the inclusion of nitrate-containing well water in the milk formula of infants.

The first fatal case of methemoglobinemia due to the ingestion of bismuth subnitrate was reported by Bennecke and Hoffman (19) in 1906. Other cases of poisoning, some of them fatal, were subsequently reported by Böhme (26), Nowak and Gutig (189), Roe (201), R. C. Miller (180), G. E. Miller (179), Wallace (230), and more recently by Marcus and Joffe (172). In the older cases bismuth subnitrate had been ingested as a consequence of its use as a radiopaque medium in roentgenological technics. More recent cases were due to the use of this compound in order to control diarrhea, particularly in infants. Marcus and Joffe's (172) case was in an infant who had received 0.1 gram of bismuth subnitrate every three to four hours for two days.

The use of ammonium nitrate as a diuretic has also resulted in the produc-

tion of methemoglobinemia. In 1929, Eusterman and Keith (72) reported two nonfatal cases in adults. Tarr (226) later reported four new cases, and noted the existence of five others in the literature.

Of recent and considerable interest is the observation that young infants on milk formulas made with well water may develop methemoglobinemia. A number of such cases have been reported in this country within the past few years by Schwartz and Rector (215), Comly (50), Faucett and Miller (74), Chapin (42) and Stafford (224). Additional cases have been reported by Ferrant (75) from Belgium and by Medovy and his associates (174) from Canada. The etiology of this condition was established by Comly who found that well water used to make the milk formula in one of his cases contained 140 mg. nitrate nitrogen or 619 mg. of nitrate ion per liter. Chapin (42) found a concentration of 244 mg. nitrate nitrogen per liter. Medovy and his associates (174) reported a concentration of 250 mg. of nitrate per liter of well water. The nitrate ion comes, of course, from seepage of wastes into poorly constructed or located wells (243).

It has been established that the intestinal flora includes organisms which are capable of converting nitrate to nitrite ion. Apparently, if sufficient nitrate ion is introduced, this conversion is extensive enough so that large amounts of nitrite ion are absorbed into the blood stream. The subsequent oxidation of hemoglobin to methemoglobin has already been discussed in detail.

3. *Chlorates.* The capacity of potassium chlorate to form methemoglobin formerly attracted considerable attention because of the use of this compound in mouth-washes, gargles and dentifrices. When potassium chlorate is added to blood *in vitro*, methemoglobin formation is very slow or negligible at first but accelerates subsequently. Thus Richardson (200) found that the addition of potassium chlorate to human blood in a final concentration of 0.25 per cent did not lead to any methemoglobin formation in eight hours, but that complete conversion with some hemolysis occurred in twenty-four hours. When the blood was hemolyzed before the addition of chlorate, definite methemoglobin formation occurred in eight hours.

The *in-vivo* effects of potassium chlorate were first investigated by Marchand (171) in 1887; he observed that dogs given oral doses of about 1.8 to 2.7 gram of $KClO_3$ per kg. body weight showed methemoglobin formation in the blood. Such formation was not unequivocally demonstrated at lower doses. However, a number of other investigators were not able to demonstrate methemoglobin formation except after death. Richardson (200) did not find any significant methemoglobin formation in pigeons which received an average total oral dose of 26 grams in their drinking water over periods of thirteen to fifty-three days, even though death occurred. Average total intramuscular doses of 13.5 grams similarly failed to produce any methemoglobin formation. On the other hand, potassium chlorate was capable of forming methemoglobin in cats, provided that the dose was large enough. Thus, of three cats receiving 0.5 gram of $KClO_3$ per kg., two showed some methemoglobin formation although all three cats died.

The mechanism of methemoglobin formation by chlorate has been more

recently investigated by Jung and his associates (113, 134) who found that repeated subcutaneous doses of 1 Gm. per kg. produced no methemoglobin in rats whereas one such dose led to high concentrations of methemoglobin in cats, with death at levels ranging from about 50 to 80 per cent. Of interest was the finding that no methemoglobin was formed for two hours following the administration of potassium chlorate, and the rate then followed an S-shaped or autocatalytic type of curve. The existence of the latent period had been previously observed by Littardi and Zanichelli (164) and corresponds to that observed *in vitro* by Richardson (200). Becker and his coworkers (18) also noted that in rats, although chlorate ion produced no methemoglobinemia, the conjoint use of small doses of sodium nitrite and chlorate led to much higher degrees of methemoglobinemia than could be produced by nitrite alone. The use of methemoglobin-binding compounds also decreased the methemoglobin-forming power and toxicity of chlorates in the cat. These various observations have led to the formulation that the methemoglobin-forming action of chlorate *in vivo* is an autocatalytic phenomenon.

4. *Quinones.* That quinones can form methemoglobin *in vitro* has recently been demonstrated by Cannava and his associates (36–40), and by Hoffman-Ostenhof and his coworkers (121, 122). Thus the latter showed that a series of benzoquinone and naphthoquinone derivatives, including benzoquinone itself, 2-6-dichloroquinone, toluquinone and 1-2-napthoquinone, converted hemoglobin to methemoglobin directly in hemolyzed and nonhemolyzed dog blood at pH 6.8. However, this oxidation was not dependent upon the oxidation-reduction potential of the various quinones.

Cannava (39) found that the minimal dose of 2,3-dimethyl-1,4-napthoquinone necessary to produce methemoglobin in dogs was 2.5 mg. per kg. A dose of 20 mg. per kg. led to the development of about 30 per cent methemoglobin in twelve to twenty-four hours with a gradual diminution in two to four days. Similar degrees of formation of methemoglobin were found after the administration of 2-methyl-1,4-napthoquinone and of 2-methyl-1,4-napthohydroquinone (38).

An interesting insight into a possible physiopathological role of quinones in methemoglobin formation has been submitted by Fishberg (77, 78) who noted that urine in pathologic states brought about by ascorbic acid deficiency was capable of producing methemoglobin *in vitro*. It was found that a patient with "autotoxic enterogenous cyanosis" excreted large amounts of benzoquinone acetic acid, that this excretion could be suppressed by the administration of ascorbic acid, and that the amount of methemoglobin in the blood varied with the urinary excretion of benzoquinone acetic acid and hence with its formation as the result of a deranged metabolism of tyrosine.

5. *Other direct oxidants.* There are a number of other substances which convert hemoglobin to methemoglobin when added directly to intact or hemolyzed red cells or to hemoglobin itself. We have already considered the mechanisms which are involved in this conversion, and we shall now submit some data concerning the extent of methemoglobin formation *in vivo*.

Combemale (49) in 1891 showed that a dose of 500 mg. of methylene blue

per kg. induced methemoglobinemia in dogs. Nadler and his associates (182) found that the intravenous injection of 500 mg. of methylene blue in man or a dose of about 7 to 10 mg. per kg. produced methemoglobin concentrations of about 0.4 to 8.3 per cent, as determined manometrically. Bock (21) reported that lethal or near-lethal subcutaneous doses of 75 mg. per kg. in the dog and 100 mg. per kg. in the cat and the mouse produced methemoglobin concentrations of approximately 10 to 15 per cent about an hour after injection. The concentration of methemoglobin then decreased and subsequently, usually in the course of several days, rose to higher levels, about 30 to 50 per cent methemoglobin. As we shall note later, methylene blue is extremely effective in reducing methemoglobinemia. This apparently paradoxical capacity, namely, the capacity to produce methemoglobin in the normal organism and to cause its disappearance when it is already present in large amounts is discussed more fully in the section on Treatment.

We have already considered the role of hydrogen peroxide in the formation of methemoglobin. In the course of studies on the parenteral administration of hydrogen peroxide, Lorincz and associates (166) observed that methemoglobin was formed in those species with very low blood levels of catalase.

B. Indirect oxidants of hemoglobin

There is a large group of aromatic amino and nitro compounds which, as a whole, either do not form methemoglobin from hemoglobin *in vitro* or do so only very poorly. Yet these compounds are for the most part very active formers of methemoglobin *in vivo*. The possibility therefore naturally arises that such compounds are metabolized into some extremely active intermediate compounds.

It is manifestly burdensome to describe in detail the vast amount of work which has been done on the methemoglobin-forming capacity of these various aromatic amino and nitrogen compounds. In the hands of different investigators, the conditions of *in-vitro* experiments have varied greatly. Similarly, in *in-vivo* studies, there have been differences in the species of animal employed, the dose of the compound, the medium in which the compound was dissolved and the route by which it was administered, the times at which blood samples were taken for methemoglobin determination, and the precision of the methods by means of which the methemoglobin was determined. It appears preferable to review rather that work which illustrates certain generalizations which may be made concerning the methemoglobin-forming capacities of these compounds.

1. *In-vitro formation of methemoglobin by aryl amino and nitro compounds.* In order to evaluate the possibility that various aromatic amino and nitro compounds may exert their *in-vivo* action by being converted into compounds like aminophenols or hydroxylamines which are direct oxidants of hemoglobin, it is necessary to consider the available data on the *in-vitro* action of these compounds.

In 1913, Heubner (112) noted that the direct addition of the aminophenols to whole blood led to formation of methemoglobin; the order of effectiveness

of the different isomers was: ortho, para, meta. The addition of 0.25 mg. ortho-aminophenol to 2 cc. of hog's blood, equivalent to 12.5 mg. per 100 cc., produced a marked methemoglobin band in the spectrum in twenty-four minutes. Since the proportions in this conversion are about 0.1 mM of *o*-aminophenol and 1 mM of hemoglobin, Heubner calculated that 1 molecule of the former compound was capable of converting 10 molecules of hemoglobin. However, no methemoglobin was formed in the absence of atmospheric oxygen. Heubner also noted that aniline and metaxylidine but not dimethylaniline were capable of forming methemoglobin *in vitro*. These observations, as we shall later note, led Heubner to conclude that the aminophenols were a necessary intermediate in the formation of methemoglobin *in vivo*.

The hydroxylamines are also active formers of methemoglobin *in vitro*. Heubner and his associates (115) noted marked methemoglobin formation five minutes after mixing of 0.006 per cent of hydroxylamine with diluted cattle blood, and calculated that about 0.6 to 1 molecule of hydroxylamine was capable of converting 1 molecule of hemoglobin to methemoglobin. Lipschitz (163) also noted that hydroxylamine readily formed methemoglobin *in vitro*; the action of nitrophenylhydroxylamine was much weaker. Ellinger (71) observed that incubation of acetanilid with defibrinated cat's blood led to a gradual formation of methemoglobin as measured by reduction in the oxygen capacity; this amounted to about 35 per cent in the course of about forty-eight hours. However, no such decrease occurred with oxalated or citrated blood. As is well known, acetanilid forms methemoglobin *in vivo*. No methemoglobin was formed with acetyl-n-methylaniline or acetyl-p-toluidine either *in vivo* or *in vitro*. These observations led Ellinger (71) to conclude that acetanilid exerted its methemoglobin-forming action by being converted to acetylphenylhydroxylamine.

2. *Effect of animal species.* It was repeatedly noted in the older literature that different species of animals showed varying susceptibilities to methemoglobin formation by a stated compound. More recently, systematic quantitative studies have been made by Lester (156) and Spicer (220). The former worker studied the maximal amount of methemoglobin formed by acetanilid and acetophenetidin at various doses, expressed as millimols per kg. Using as a criterion of sensitivity the reciprocal of the dose required to produce a stated concentration of methemoglobin, Lester (156) found that the cat was most sensitive. If this species is listed as 100, the sensitivities of the other species are as follows for acetanilid: man, 56; dog, 29; rat, 5; rabbit, 0; monkey, 0. For acetophenetidin, the sensitivities were as follows: cat, 100; man, 63; dog, 35; rat, 5.

There is a considerable number of other studies in the literature which, although not as systematic as those of Lester, show the extreme sensitivity of the cat to methemoglobin formation by other compounds. For example, Reiter (199) found that in guinea pigs, a dose of 300 mg. of p-nitro-o-toluidine per kg. of body weight never produced more than 1 gram of methemoglobin per 100 cc. or about 15 per cent of the total blood pigment. In contrast, in the cat much smaller doses produced considerably higher degrees of methemoglobinemia.

Thus, 1 mg. per kg. yielded 1.3 gram of methemoglobin per 100 cc. or 12 per cent of the total blood pigment; 10 mg. per kg. yielded 5.9 grams or 56 per cent of the total pigment. Jung (138) found that about twice as much nitrosobenzol or phenylhydroxylamine was required in the rat as in the cat to yield similar ranges of maximal methemoglobin concentrations. According to Spicer (220), 15 mg. of aniline per kg. produced 28.3 per cent of methemoglobin in the dog, 56.4 per cent in the cat, and negligible amounts in the rat. The cat has been used almost entirely for the evaluation of methemoglobin-forming compounds because of the ease with which this pigment is formed in this species.

3. *Methemoglobin-forming capacity of aryl amino and nitro compounds.* Since aryl amino and nitro compounds do not readily oxidize hemoglobin *in vitro*, it is apparent that the formation of methemoglobin *in vivo* must be accomplished through some metabolic derivative. It has been held that such a derivative either oxidizes hemoglobin directly or interacts with oxyhemoglobin to form hydrogen peroxide which then oxidizes hemoglobin (153). We shall presently discuss these alternative hypotheses in detail. It is important to consider first, in quantitative terms, the methemoglobin-forming capacities of these various compounds.

As already pointed out in this paper, when a methemoglobin-forming compound is administered, methemoglobin begins to accumulate in the blood at a rate characteristic of the compound, attains a maximum concentration at which it remains for a variable period of time and then begins to decrease. The specific shape of the curve is the resultant chiefly of three processes: (a) metabolism of the compound to other substances which do not oxidize hemoglobin and which, with respect to our present interest, may be regarded as side reactions; (b) formation of an intermediate compound which reacts directly with hemoglobin to form methemoglobin; (c) back reduction of methemoglobin, as it is being formed, to hemoglobin by the enzyme systems of the blood. These three processes are continuous and interdependent. Although the kinetics of the back reduction of methemoglobin has received some study and, in general, appears to be independent of the particular methemoglobin-forming compound, the kinetics of the other two processes have only been very fragmentarily explored, and then only for a few compounds. It is therefore impossible at the present time to characterize with any precision the methemoglobin-forming capacity of various compounds by any general equation.

It may, however, be postulated that the maximum concentration of methemoglobin formed is dependent upon the total number of electrons accepted from hemoglobin by an intermediate oxidation agent, and that the concentration of the intermediate oxidation agent is proportional to the dose of the aryl amino or nitro compound administered to an animal. These assumptions would lead to an equation of the form, $\frac{[\text{Hb}]\,[\text{D}]}{[\text{MetHb}]} = K$, where [Hb] and [MetHb] are the concentrations of hemoglobin and methemoglobin, respectively, per 100 cc. of blood or per kg. of body weight, where D is the dose per kg. of body weight and where K is the methemoglobin-forming capacity of the particular com-

pound. Such an equation is of the same mathematical form as the Hüfner (130) equation for the hemoglobin-oxyhemoglobin equilibrium. Indeed, the data on certain compounds such as that of Reiter (199) on nitro-o-toluidine or of Petersen (196) on nitrosobenzol are sufficiently extensive to show that the concentration of methemoglobin increases with increasing doses of the compound until a point is reached beyond which relatively large increases in the dose of the drug produce small or negligible increases in the concentration of methemoglobin.

It is, however, of little value to attempt the application of a Hüfner-like equation or of more refined expressions to the data which are at present available in the literature. The value for the maximal concentration of methemoglobin at a stated dose may vary considerably in individuals of the same species; Bredow and Yung's (28) data for a dose of 70 mg. per kg. in the cat show the following values for four individuals in mM of methemoglobin per kg.: 0.066, 0.22, 0.30, 0.36. Such variability is probably an expression of the differing capacities of the individuals of a species in the metabolism of the drug or in the enzyme systems involved in the back reduction of methemoglobin. However, it is quite apparent that a dose-methemoglobin curve based, as it has been, on the use of one individual for each dose must inherently be subject to great error.

A review of the literature on the methemoglobin-forming capacity of various compounds shows that this has been formulated most frequently as the average molecular ratio of the methemoglobin formed to the dose of the compound; both quantities are expressed per kg. body weight. The cat has been the species most frequently used. The molecular value for the methemoglobin is obtained by multiplying the concentration in the blood, expressed as grams per 100 cc., by the value, 0.75 or 0.70, which represents the blood volume per kg. body weight. The values for the molecular ratio at different doses are then averaged, even though the individual values may differ greatly from each other.

We have already described the general nature of the relationship between doses of a methemoglobin-forming compound and the concentration of methemoglobin. It is apparent that the molecular ratio will decrease at high dosages. For example, Reiter's (199) data on p-nitro-o-toluidine show molecular ratios decreasing from 9.2 at a dose of 1 mg. per kg. to 1.6 at a dose of 30 mg. per kg.; averaging of these values for the molecular ratios at the different dosages can only yield a crude representation of the methemoglobin-forming capacity. Thus, Reiter (199) gives an average molecular ratio of 3.7. The standard error of this mean value is ± 0.78. In other words, this mean value of 3.7 cannot be said to differ significantly from molecular ratio values greater than about 1.5 or less than 5.9 and which have a similar standard error.

The possibility arises that a more precise estimate of the methemoglobin-forming capacity may be obtained if only a limited portion of the dose-methemoglobin concentration curve is employed for the calculation of the molecular ratios. Theoretical considerations as well as direct examination of data in the literature would appear to indicate that the dose-methemoglobin curve does

not begin to flatten out until methemoglobin concentrations in excess of 0.20 mM kg. are attained. However, as has already been pointed out, there is a great variability from individual to individual in the methemoglobin concentrations obtained with a stated dose. Moreover, even in concentrations of methemoglobin below 0.20 mM per kg., there is far from direct proportionality between the concentration and the dose. In a number of compounds, the values for the molecular ratios rise appreciably at very low concentrations of methemoglobin. Average values obtained from such data would therefore also possess considerable variance.

It is apparent, therefore, that the method which has been used so widely for calculating the average molecular ratio has not in general yielded precise values for methemoglobin-forming capacity. Nor can such data usually be treated or employed to yield more precise values. It is, of course, possible that the use of greater numbers of animals of a given species or the use of a more uniform strain of a given species may yield averages with less variance. For purposes of record, we list in Table VI the average molecular ratios which have been obtained by different investigators.

Although we cannot place too much quantitative emphasis on these results, the above tabulation reveals two findings which are qualitatively impressive. It may be noted, first, that the molecular ratio is greater than one for a considerable number of compounds. In other words, one molecule of each of these compounds forms an intermediate which can accept more than one electron. Since hemoglobin yields one electron when it is converted to methemoglobin and since probably only a small fraction of the original compound is metabolized to an oxidizing intermediary compound, then this intermediary compound must partake in a turnover reaction, being alternately reduced as it reacts with hemoglobin and converted by other systems back to an oxidizing intermediate.

The second point of interest in Table VI is that p-dinitrobenzene and phenylhydroxylamine are oustanding methemoglobin formers. Ortho-aminophenol, m-dinitrobenzene and nitrosobenzene, though not as distinctive in this respect, still appear to be much more active than the other compounds. In 1944, Vandenbelt, Pfeiffer and their associates (231) reported that p-aminoacetophenone and p-aminopropiophenone were extremely potent methemoglobin formers. Unfortunately, their determinations were done on dogs and do not permit direct comparison with other compounds for which the data has been obtained in cats. However, the data of Vandenbelt and his associates (231) and of Bodansky and Gutmann (24) permit calculation of a molecular ratio of about 30 for p-aminopropiophenone and of about 10 for p-aminoacetophenone when these agents are intravenously administered. If it can be assumed that the cat and the dog have the same relative sensitivities to these compounds as they have been shown to have to acetanilid and acetophenetidin, then the molecular ratios for p-aminopropiophenone and p-aminoacetophenone in the cat would be about 100 and 30, respectively, and therefore of about the same order of magnitude as that of m-dinitrobenzene.

The finding that certain compounds are potent methemoglobin formers natu-

rally raises the possibility that these compounds or compounds easily derived from them possess a chemical grouping which, either directly or in association with other systems, is of critical importance in the oxidation of hemoglobin. Thus, because of their relatively high methemoglobin-forming capacity, p-aminophenol, phenylhydroxylamine and nitrosobenzene have been implicated by various investigators as being the important intermediates in the metabolism

TABLE VI

Methemoglobin-forming capacity of aryl amino and nitro compounds in the cat

COMPOUND	MOLECULAR RATIO	REFERENCE
Aniline	2.5	Herken (110)
	2.7	Schwedtke (216)
Acetanilid	1.0+	Lester (156)
m-phenylenediamine	1.4	Jung (137)
Acetophenetidin	0.14+	Lester (156)
o-Aminophenol	6.8+	Petersen (196)
Nitrosobenzene	8.6+	Petersen (196)
p-Aminophenol	3.6	Schwedkte and Sing (217)
p-Aminophenol	1.3	Issekutz (122)
Phenylhydroxylamine	34.0	Issekutz (122)
Nitrobenzene	0.86	Bredow and Jung (28)
o-Dinitrobenzene	1.9	Heubner and Sing (118)
	3.7	Issekutz (122)
m-Dinitrobenzene	7.1	Issekutz (122)
	7.8	Bredow and Yung (28)
	6.4	Heubner and Sing (118)
p-Dinitrobenzene	55	Heubner and Sing (118)
	198	Issekutz (122)
Trinitrobenzene	4.8	Bredow and Jung (28)
o-Nitrotoluol	0.05	Bredow and Jung (28)
m-Nitrotoluol	0.04	Bredow and Jung (28)
p-Nitrotoluol	very slight	Bredow and Jung (28)
2,4-Dinitrotoluol	1.4	Bredow and Jung (28)
2,6-Dinitrotoluol	0.55	Bredow and Jung (28)
2,4,6-Trinitrotoluol	1.7	Bredow and Jung (28)
m-Chloronitrobenzene	2.3	Jung (137)
m-Aminonitrobenzene	3.0	Jung (137)
2,4-Dinitrochlorbenzene	0.6	Jung (137)
p-Nitro-*o*-toluidine	3.7	Reiter (199)

of other methemoglobin-forming compounds. We shall now consider the extent to which these implications are substantiated by data on the intermediary metabolism of various methemoglobin-forming compounds.

4. *Sulfonamides.* The introduction of sulfonamide drugs into medical practice about 1937 gave rise to the hazard of methemoglobinemia. The first cases were reported by Gibberd (81) and by Paton and Eaton (190). Hartmann and his associates (96) found that, in the great majority of patients receiving 0.1 gram or more daily of sulfanilamide per kg. of body weight, some cyanosis due to

methemoglobinemia was detectable. In general, there was an approximate correlation between the sulfanilamide concentration in the blood and the methemoglobin concentration; levels of 10 to 20 mg. of sulfanilamide per 100 cc. were usually associated with methemoglobinemia ranging from 20 to 30 per cent. Several other groups at about this time reported a high incidence of methemoglobinemia in patients receiving sulfonamides (79, 94, 237). With the replacement of sulfonamides by antibiotics in the treatment of various bacterial infections, the reported incidence has decreased greatly.

We shall presently discuss the intermediate metabolism of methemoglobin-forming compounds and the probable mechanisms and note that the formation of methemoglobin is due to the oxidizing action of some intermediate, the precise nature of which is not yet established. There appears to be some question whether a similar intermediate is involved in the action of sulfanilamide. Harris and Michel (94) and Fox and Cline (79) were inclined to the view that the action was indeed due to the oxidizing compounds formed in the metabolism of sulfanilamide. On the other hand, there has been some evidence that sulfanilamide inhibits the action of catalase. Such a finding would fit in with the theory that methemoglobin formation is due to the action of hydrogen peroxide (48).

In connection with cyanosis due to sulfanilamide administration, it has been pointed out by several investigators that such cyanosis may in part be due to colored intermediates. Indeed, Kallner (141) considers that the cyanosis is largely due to the combination of carbhemoglobin with sulfanilamide.

5. *Intermediary metabolism of methemoglobin-forming compounds and the oxidation of hemoglobin.* The metabolic fate of aniline derivatives has claimed much attention. In 1913, Heubner (112) observed that the direct *in-vitro* addition of various aminophenols to whole blood caused the formation of methemoglobin, in the proportion of several molecules of the latter per molecule of compound. He also observed that p-aminophenol, when allowed to stand in air, was readily converted to quinone imine. Heubner assumed, therefore, that aniline and its derivatives were metabolized to p-aminophenol which was then oxidized to quinone imine. The latter then oxidized hemoglobin to methemoglobin, being itself reduced back to p-aminophenol. The cycle could be repeated a number of times (112, 116).

Greenberg and Lester (86, 87), however, have recently submitted data which do not support Heubner's formulation. Heubner observed methemoglobin qualitatively on spectroscopic examination of cow's blood to which p-aminophenol in concentrations of at least 25 mg. per 100 cc. had been added. Greenberg and Lester found that no methemoglobin was formed *in vitro* at concentrations of 0.1 and 0.5 mg. of p-aminophenol per 100 cc., and occasionally and only to a negligible degree at 1.0 mg. per cc. Definite methemoglobin formation resulted at much higher concentrations, namely, 13.2 per cent methemoglobin at 5.0 mg. of p-aminophenol per 100 cc., and 21.2 per cent at 10 mg. of p-aminophenol per 100 cc. But Greenberg and Lester (87) found that methemoglobinemia could be produced in man without the appearances of any appreciable concentration of p-aminophenol in the blood. Thus, a human subject who received

0.975 gram of acetanilid showed a concentration of 4 per cent methemoglobin an hour after administration and 8 per cent methemoglobin at two hours; yet the concentration of p-aminophenol was less than 0.005 mg. per 100 cc., far below the amount required to produce methemoglobin *in vitro*. Moreover, the administration of 500 mg. of p-aminophenol resulted in the appearance of unconjugated p-aminophenol in the blood one half and one hour later; yet no methemoglobin was found. Thus, these experiments show that there is no necessary association between the presence of p-aminophenol and methemoglobin formation. It was also observed that the major metabolites of acetanilid, namely, N-acetyl p-aminophenol and its hydroxy conjugates with sulfuric and glycuronic acid, are not capable of forming methemoglobin either *in vivo* or *in vitro*.

It will be recalled that phenylhydroxylamine is also a potent methemoglobin former (132, 207). In 1920, Lipschitz (163) assumed that this compound was the essential intermediary step in the action of aniline and its derivatives. He considered that free aniline was formed and was then oxidized to phenylhydroxylamine and that the latter compound oxidized hemoglobin to methemoglobin, being itself reduced to aniline. Thus, there was set up a cyclic oxidation-reduction reaction in which several molecules of hemoglobin could be oxidized to methemoglobin by one molecule of aniline.

The existence of the oxidation-reduction reaction, phenylhydroxylamine + hemoglobin $\rightleftarrows$ nitrosobenzene + methemoglobin has, however, been disputed. Heubner and his associates (116) were unable to find that phenylhydroxylamine could oxidize reduced hemoglobin *in vitro*. Jung (138), however, observed that phenylhydroxylamine could be oxidized to nitrosobenzene by oxyhemoglobin, and that the nitrosobenzene in turn oxidized *reduced* hemoglobin to methemoglobin. Other formulations of the intermediary metabolism of acetanilid have been recently summarized by Greenberg and Lester (87).

The presence of phenylhydroxylamine or nitrosobenzene in blood after administration of acetanilid has not yet been established. Ellinger (71) isolated a small amount of N-acetyl-phenylhydroxylamine from the blood of cats given acetanilid. Jung (138) pointed out that if phenylhydroxylamine and nitrosobenzene were both present in the body they might condense to form azoxybenzene, and this compound might be transformed into other azo compounds. Greenberg and Lester (87) found that the amount of azo compounds appearing in the urine after administration of acetanilid corresponded to about 0.5 per cent of this compound.

The intermediary metabolism of the other potent methemoglobin formers, namely, p-dinitrobenzene, p-aminoacetophenone, and p-aminoacetopropiophenone, has not been investigated, and it is difficult, therefore, to venture any opinion whether intermediates through which they pass are similar to those postulated in the metabolism of acetanilid.

There is at present, therefore, no conclusive evidence concerning the nature of the intermediary compound which oxidizes hemoglobin to methemoglobin. That it is not an immediate derivative of p-aminophenol appears probable

from the work of Greenberg and Lester (87). However, the intermediate may be present in such small amounts that it has until now eluded chemical detection or analysis. It may be stressed that the minuteness of the quantity in which a metabolite of a methemoglobin former may be found to be present in the blood or urine does not militate against its role as an essential intermediate in the oxidation of hemoglobin to methemoglobin, for it is possible that such an intermediate may have a high turnover number and partake many times in a cyclic oxidation-reduction reaction.

The recent report of Spicer and Neal (222) is of interest in demonstrating the role of oxygen in the *in-vivo* formation of methemoglobin by an aryl amino compound, aniline. These investigators found that dogs exposed to simulated high altitudes (28,000 feet or 8540 meters) formed less methemoglobin in response to a given dose of aniline, 15 mg. per kg., than when they were exposed to normal atmospheric oxygen tension. There was, however, no difference in the response of methemoglobin formation to nitrite. It would appear, therefore, that low oxygen tension affects either the conversion of aniline to an oxidizing intermediate or the process of oxidation of hemoglobin to methemoglobin. The effect does not appear to be on the conversion of aniline to p-aminophenol or nitrosobenzene, for it was found that these latter substances also led to greater formation of methemoglobin at normal than at low tensions of oxygen.

In view of these results and those obtained on the intermediary metabolism of acetanilid, it would appear of value to investigate the mechanism of oxidation of hemoglobin to methemoglobin by aryl amino and nitro compounds in less complicated systems than the whole organism, such as in tissue slices, homogenates or extracts.

6. *Industrial hazards.* Various industrial processes involve exposure to methemoglobin-producing compounds. Thus, in electric arc welding, nitrous oxides are generated; as a rule, the results of such exposure are negligible for the concentration of methemoglobin is seldom more than 3 per cent (167). Although TNT is a methemoglobin producer, the effect of ingestion of small amounts, about 1 mg./kg., is negligible in man and there is no detectable change in the blood picture (129). A few years ago, Bass and his coworkers (17) reported the occurrence of methemoglobinemia in men who washed assembled roller bearings in mineral seal oil. This oil was found to contain 0.5 per cent of a methemoglobin former, 2-anilino-ethanol.

VI. TREATMENT OF METHEMOGLOBINEMIA

A. Symptomatology of methemoglobinemia in man

Except for cyanosis of the mucous membranes, dogs do not show any symptoms due to methemoglobinemia at concentrations of methemoglobin less than 60 per cent. The predominant symptoms are salivation, ataxia and vomiting at levels of 60 to 70 per cent, and ataxia and vomiting at levels of 71 to 80 per cent. At concentrations of 81 to 90 per cent, loss of consciousness is the outstanding feature. Lack of determinations of methemoglobin concentrations in the clinical literature make a similar correlation in man somewhat difficult.

Cyanosis does not become apparent until the concentration of methemoglobin reaches a level of about 15 per cent of the total pigment (23). Of course, the higher the concentration of methemoglobin the more striking is the cyanosis. Methemoglobinemias up to 30 per cent do not in general produce any symptoms, although occasionally dyspnea occurs upon exertion (76). At levels from 30 to 45 per cent, exertional dyspnea may be present if the methemoglobinemia has been rather acutely induced by the ingestion of various drugs. On the other hand, in familial idiopathic methemoglobinemia, which is usually present from birth, symptoms of fatigue, exertional headaches and reduced exercise tolerance may be mild or absent (76). This absence of symptoms may be explained by the secondary polycythemia compensating for inert pigment and by the lack of any shift in the position of the oxyhemoglobin dissociation curve (69). From the few available reports, it would seem that lethargy and semi-stupor do not become evident until levels of about 60 per cent are attained. The recorded existence of such levels without unconsciousness would seem to indicate, by analogy with the symptoms in animals, that the lethal level may well be above 70 per cent and approach the lethal level in dogs, namely, about 85 per cent (24).

B. Moderate drug-induced methemoglobinemias

It has been previously indicated that, following the administration of methemoglobin-forming drugs, the concentration of methemoglobin rises at a rate characteristic of the compound until it attains a maximum, persists there for some time, and then decreases again to negligible levels. Most cases of methemoglobinemia which are induced by the administration of a drug or other compound and in which the concentration does not exceed about 40 per cent of the total blood pigment can therefore be treated by "watchful waiting," provided the patient does not engage in strenuous physical activity which may tax his oxygen-unloading mechanisms. A knowledge of the particular compound which has been administered or inadvertently taken will aid in prognosis. For example, if one may judge from the results obtained in dogs, the methemoglobinemia due to nitrite may reach a maximum in about an hour and vanish within a few hours while that due to nitrobenzene may not produce its maximum effect for fifteen hours and then recede only slowly (58). There are few data on the rates of development and disappearance of methemoglobinemia induced by different drugs. Hartmann, Perley and Barnett (96) noted that the methemoglobinemia due to sulfanilamide administration decreased from a 20 per cent to zero level within twenty to seventy hours after withdrawal of the drug. The author observed that methemoglobinemias of 20 to 30 per cent due to the administration of a single oral dose of p-aminopropiophenone had decreased practically to zero level within twenty-four hours (23).

The treatment in such cases may consist simply in discontinuation of the drug, with an assurance to the patient that the methemoglobinemia will recede spontaneously within a day or two. However, the associated cyanosis may produce great anxiety in the patient or his family or may be disagreeable for cosmetic reasons. In cases where these conditions hold, it is advisable to institute more active treatment.

C. Familial idiopathic methemoglobinemia

As has already been pointed out in this review, familial iodiopathic or congenital methemoglobinemia is a condition in which cyanosis is present at birth to varying extents and which may become aggravated in childhood or early adulthood. Recent investigations have established that the cyanosis is due to methemoglobinemia as the result of derangement in the enzyme systems which are involved in the maintenance of the hemoglobin-methemoglobin equilibrium of the erythrocyte. We have previously considered this aspect in some detail. The outstanding symptom is breathlessness upon exertion. Secondary polycythemia develops. In 1948, Finch (76) noted that sixteen cases of the disease had been reported in the literature. In general, the concentrations in these cases range from about 30 to 45 per cent of the total blood pigment.

The two substances which have found the most widespread use in the treatment of congenital as well as other types of methemoglobinemia are methylene blue and ascorbic acid. Possibly because of its oxidation-reduction properties, methylene blue was regarded about twenty years ago as an antidote in the treatment of types of poisoning, such as those due to cyanide, carbon monoxide or methemoglobin formation, in which, through one way or another, cellular uptake of oxygen was denied (92). It is beyond the scope of this paper to consider the merits of this assumption insofar as carbon monoxide or cyanide poisoning is concerned. Hauschild (101–103) first demonstrated in animals that the toxicity of methemoglobin-forming compounds like sodium nitrite and phenylhydroxylamine could be counteracted by the administration of thionine or methylene blue and that these compounds could reduce methemoglobin to hemoglobin *in vivo* and, under certain conditions, also *in vitro*. The accelerating effect of methylene blue upon the rate of disappearance of methemoglobin *in vivo* has been confirmed by Wendel (244), and more recently by Bodansky and Gutmann (24). The latter also demonstrated that an intravenous dose of 1 mg. of methylene blue per kg. body weight is highly and rapidly effective in saving dogs from death at degrees of methemoglobinemia which would otherwise be fatal.

The use of ascorbic acid in the treatment of methemoglobinemia appears to have been introduced by Lian, Frumusan and Sassier (160) who, in observing two cases of congenital methemoglobinemia, reasoned that the concentration of methemoglobin represented a disturbance in the oxidation-reduction equilibrium of the blood pigment and that ascorbic acid should reduce the methemoglobin to hemoglobin. This surmise was confirmed in these two instances, and subsequent investigators have demonstrated this phenomenon and studied its quantitative aspects *in vitro*, both on crystallized methemoglobin and methemoglobinemic erythrocytes, and *in vivo* (24, 83, 155, 236). Bodansky and Gutmann (24) found that ascorbic acid was much less effective than methylene blue in accelerating the reduction of methemoglobin in dogs. At a dose of 200 mg. or 1.1 mM of ascorbic acid per kg., reduction of a maximal concentration of about 60 per cent to one-half value required 100 to 204 minutes. A much

smaller dose of methylene blue, namely, 1 mg. or about 0.003 mM per kg. accomplished the same degree of reduction in the much shorter period of twenty-two to forty-two minutes.

Lian and his associates (160) injected intravenously 100 mg. of ascorbic acid daily for three weeks. The concentration of methemoglobin decreased from 45 to 26 per cent; the cyanosis diminished and the dyspnea and headaches disappeared. Discontinuation of therapy led to a rise in the methemoglobin concentration. Deeny, Murdock and Rogan (61) treated two cases with oral administration of ascorbic acid at doses which began at 50 mg. twice daily and were increased to maintenance levels of 150 and 200 mg. twice daily. The blood methemoglobin concentration in the first case decreased from an initial level of about 34 per cent to 11 per cent at the end of sixty-three days of treatment. In the second case, the methemoglobin concentration decreased from about 43 per cent to 6 per cent in one month and remained at about that concentration on a maintenance level of 200 mg. of ascorbic acid twice daily. There are several other reports of treatment of this condition by oral or parenteral administration of ascorbic acid (41, 85, 218).

Methylene blue has also been found to be effective in the treatment of familial idiopathic methemoglobinemia. King and his associates (150) found the oral administration of about 260 mg. daily to a patient weighing 52 kg. decreased the methemoglobin concentration from an initial concentration of about 35 per cent to less than 10 per cent in approximately a week; a daily oral dose of 65 mg. was necessary to maintain the methemoglobin concentration at less than 10 per cent. Eder, Finch and McKee (69) found in their patient that an intravenous injection of 90 mg. of methylene blue reduced the concentration of methemoglobin from an initial value of about 45 per cent to half this value in ten minutes and to negligible levels in thirty minutes. Physical activity was then possible without headaches or dyspnea. An oral daily dose of 240 mg. of methylene blue in enteric-coated tablets was well tolerated and kept the patient free of cyanosis for a period of several years. The polycythemia disappeared during the treatment.

Since familial idiopathic methemoglobinemia is apparently one of the group of diseases classifiable as "inborn errors of metabolism," the methemoglobinemia requires constant attention during life. Treatment is usually not an emergency since the patient will have had the disease for some time and since the disease is not particularly incapacitating. Intravenous therapy is, therefore, not necessary. Oral administration of ascorbic acid in daily, divided doses of 400 to 500 mg. or of methylene blue in similar doses of about 250 mg. should be adequate in reducing the methemoglobin concentrations to levels of about 5 per cent within a few weeks and in maintaining the methemoglobin at this low level so long as the administration is continued. One may envision the necessity for more prompt intravenous medication with methylene blue in the event that a patient with familial, idiopathic methemoglobinemia succumbs to pulmonary or cardiac disease, but the probability of this coincidence is, of course, extremely low.

D. Enterogenous methemoglobinemia

The literature contains a number of clinically ill-defined cases of methemoglobinemia which have been grouped together and designated as enterogenous cyanosis. These patients usually have abdominal pain accompanied either by diarrhea or constipation (76). The degree of methemoglobinemia may fluctuate widely; pronounced degrees of methemoglobinemia are associated with cyanosis, headache, dyspnea and, if very pronounced, dizziness and collapse. The methemoglobinemia has been variously considered as being due to the excessive presence of nitrite-producing bacteria in the gastrointestinal tract, to the ingestion of large amounts of aniline derivatives for relief of headache, or to a metabolic derangement resulting in the formation of a methemoglobin-forming p-quinone (76–78). If the methemoglobinemia is of moderate degree, the treatment is that described in the previous section.

E. Severe and Lethal Methemoglobinemias

When the degree of methemoglobinemia is so pronounced that semi-stupor or unconsciousness is present, an emergency exists and treatment must be prompt. As we have already indicated, such dangerous degrees of methemoglobinemia may result from industrial contact with potent methemoglobin-forming agents or from ingestion of compounds, solutions or food products containing such agents.

The chief feature of treatment consists in the intravenous injection of methylene blue in a dose of 1 to 2 mg. per kg. body weight. This dose is far below the toxic level. Since methylene blue may be readily made up in a 1 per cent aqueous or saline solution, the necessary amount may be introduced from a 10 cc. syringe in less than one minute. It is, therefore, not necessary, and indeed may delay treatment, to employ infusions of methylene blue in more dilute solution over a longer period of time. In cases of severe methemoglobinemia such general supportive measures as inhalation of oxygen or intravenous infusions of glucose, of glucose-saline, or of whole blood are often considered and sometimes used. But the data which we reviewed in an earlier section show that there is no physiological justification for their use, and attempts to institute them may only delay effective treatment. On the other hand, as we have already noted, the intravenous injection of methylene blue in a dose of 1 mg. per kg. is highly and rapidly effective in saving dogs from death at degrees of methemoglobinemia which would otherwise be fatal. There is ample clinical evidence to show that a similar situation holds in man.

William and Challis (243) reported a case of methemoglobinemia induced by contact with p-bromanilin and p-bromorthosulfanilic acid. The patient was intensely cyanotic and semi-comatose when 100 cc. of a 1 per cent solution, equivalent to about 15 mg. of methylene blue per kg., were injected. Within one hour, the cyanosis had disappeared and the patient had revived. The dose used was unnecessarily high, as subsequent clinical reports indicate, but this case incidentally illustrates the relatively non-toxic nature of methylene blue. Steele and Spink (225) reported two patients with marked methemoglobinemia;

one was disoriented and the other was semi-stuporous. The intravenous injection of 50 cc. of a 0.5 per cent solution of methylene blue (about 3 to 4 mg. per kg.) resulted in dramatic improvement. Within fifteen minutes after the injection, the patients sat up and appeared well. No quantitative methemoglobin determinations were done in these cases.

That even smaller doses of methylene blue are highly effective is demonstrated by recent reports on the treatment of nitrite-induced methemoglobinemia, due to the inclusion in the milk formula of well water which contained nitrate. Faucett and Miller (74) reported two instances of poisoning in infants, with marked cyanosis, increased respiratory and heart rates, and marked lethargy. One half cc. of a 1 per cent solution of methylene blue was injected intravenously into one infant who had a 58 per cent concentration of methemoglobin and 0.6 cc. into the second infant who had a methemoglobin concentration of 71 per cent. These amounts correspond to doses of about 1 to 2 mg. per kg. Within thirty minutes after the injections, the infants appeared clinically well; the respiratory and heart rates had decreased to normal and the methemoglobin concentrations were 4 to 5 per cent of the total blood pigment.

It is occasionally considered paradoxical that methylene blue which is a methemoglobin former should be so effective in decreasing methemoglobinemia (70). However, the evidence we have presented earlier in this review shows that this situation is really not paradoxical (22). For although methylene blue is indeed a methemoglobin former, it is not, contrary to the usual impression, a good methemoglobin former *in vivo* in man (182). In contrast, doses much smaller than those which produce methemoglobin are capable of reducing methemoglobin. In other words, when methylene blue is injected into man, two reactions ensue: 1) a direct oxidation of hemoglobin to methemoglobin, and 2) an opposing reaction, the reduction of methemoglobin to hemoglobin, for which coenzyme and coenzyme factor are necessary. Apparently, the latter reaction is much more effective, so that the equlibrium state between the two reactions is pitched at a point of very low methemoglobin formation. This equilibrium is arrived at whether methylene blue is injected in a person with no methemoglobinemia or in one with a high concentration of methemoglobin.

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