Methemoglobin Reduction

Studies of the Interaction between Cell Populations and of the Role of Methylene Blue

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In the course of studies of the rate of methemoglobin reduction in mixtures of normal and glucose-6-phosphate dehydrogenase (G-6-PD) deficient cells in the presence of methylene blue and glucose, we recently found unmistakable evidence of interaction between these two different types of erythrocytes. It became apparent that the normal erythrocytes in the mixture somehow facilitated the reduction of methemoglobin in the mutant cells. It is the purpose of this communication to describe the studies which have been made to better define this unusual phenomenon. These suggest that leukomethylene blue is the mediator of this intracellular metabolic interaction, and also shed some light upon the point of action of the TPN-linked methemoglobin reductase.

Materials and Methods

Normal red cells were obtained from normal Caucasian subjects or Negro subjects known to have normal levels of red cell G-6-PD, or from Caucasian subjects undergoing phlebotomy in the treatment of hemochromatosis or polycythemia vera. G-6-PD deficient red cells were obtained from Negro male volunteers known to have typical G-6-PD deficiency. Red cells from an individual with congenital methemoglobinemia due to DPNH diaphorase deficiency were obtained through the courtesy of Dr. Ernst Jaffé. ACD (acid-citrate-dextrose) solution was used as an anticoagulant, and the blood samples were stored at 4 C. for up to 2 weeks prior to use. The erythrocytes were sedimented by centrifugation and the plasma and buffy coat were discarded. The hemoglobin of the red cells was converted to methemoglobin by replacing the plasma with .145 M sodium nitrite solution. When control cells not treated with nitrite were used, the plasma was replaced with .154 M sodium chloride solution. After incubating with nitrite or saline at room temperature for 20 minutes, the cells were washed 5 times with 7 to 10 volumes of 0.9 per cent sodium chloride solution. Finally, the packed cells were reconstituted to a 40 per cent suspension by adding 1/2 volumes of .15 M potassium phosphate buffer, pH 7.4. Unless otherwise indicated, reaction mixtures were constituted as follows: 40 per cent red cell suspension in phosphate buffer, 3.9 ml.; 10 -4 M methylene blue in 0.154 M sodium chloride solution, .6 ml.; .167 M glucose in 0.154 M sodium chloride solution, .9 ml. In all instances incubation was carried out aerobically at 37 C.

Methemoglobin determinations were performed by the method of Evelyn and Malloy. Crystalline hemoglobin was prepared by the method of Drabkin. The material used in these studies had been prepared 4 years earlier and had been stored in dry form at 4 C. It was completely soluble and methemoglobin determination revealed that 96 per cent

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Fig. 1.—Methemoglobin reduction in nitrite-treated normal erythrocytes, G-6-PD deficient erythrocytes, and a mixture of the two. A 40 per cent red cell suspension, 3.9 ml., was mixed with .6 ml. of $10^{-4}$ M methylene blue and .9 ml. .167 M glucose in .154 M sodium chloride solution. Incubation was carried out aerobically at 37°C. It is apparent that after 2 hours' incubation, when the methemoglobin in normal erythrocytes had been reduced, the rate of reduction in the mixture was much more rapid than the rate computed on the assumption that there was no interaction between normal and G-6-PD deficient cells.

of it was in the form of methemoglobin. This material had no detectable TPN-linked methemoglobin reductase activity when aliquots containing 15 mg. hemoglobin were assayed by the method of Huennekens.⁶

Leukomethylene blue was assayed by the anaerobic transfer of 2 or 3 ml. of the solution to a cuvette containing 0.1 ml. of glacial acetic acid. Optical density readings were made at 655 mp before and after the addition of .01 ml. of .2 per cent potassium ferricyanide.

Leukomethylene blue was prepared either by reduction with hydrosulphite or by passing a stream of hydrogen through a .01 M solution of methylene blue in .3 per cent acetic acid in the presence of finely divided platinum dioxide.

RESULTS

A. Methemoglobin Levels in Normal Red Cells, G-6-PD Deficient Red Cells, and Mixtures

When normal nitrite-treated red cells were incubated in the presence of glucose and methylene blue, methemoglobin was reduced at a rapid rate. The rate of reduction was linear with respect to time until the methemoglobin
Methemoglobin reduction in nitrited normal erythrocytes, G-6-PD deficient erythrocytes, a 1:1 mixture, and in G-6-PD deficient cells separated from the 1:1 mixture. Conditions of the experiment are identical to those described for figure 1. After most of the methemoglobin in normal erythrocytes had been reduced, the rate of reduction of the methemoglobin in G-6-PD deficient cells separated from the mixture became more rapid and approximated that of normal erythrocytes. The level of the methemoglobin in the separated G-6-PD deficient cells is somewhat less, even at time zero, than the level in red cells which have not been treated with serum. This appears to be due to non-specific reducing substances in serum (see text).

The rate of methemoglobin reduction in G-6-PD deficient cells proceeded at a much slower rate, as has been reported previously by Dawson et al. The methemoglobin level which would be expected in a mixture of normal and G-6-PD deficient cells, if there were no interaction between these cell populations, was computed. As indicated in figure 1, the computed curve has two components, a rapid component representing the disappearance of hemoglobin from the normal cells, followed by a slow component representing the disappearance of methemoglobin from the G-6-PD deficient cells. Experimentally, however, quite a different curve was obtained. Although the rate of methemoglobin reduction closely followed the computed curve during its first component, it continued at the same slope until almost all the methemoglobin had been reduced. This suggested that methemoglobin
in the G-6-PD deficient cells in the mixture was being reduced by neighboring normal cells.

Further evidence that this was the case was obtained through differential agglutination studies. Normal cells were obtained from a type A donor while the G-6-PD deficient cells were obtained from a type B donor. Nitrite-treated normal type A cells, nitrite-treated G-6-PD deficient type B cells, and a mixture of the two were incubated with glucose and methylene blue. Methemoglobin determinations were carried out on the three incubation mixtures at appropriate intervals. In addition, at each point on the curve an aliquot of the mixture of red cells was added to a sufficient quantity of anti-A serum in an ice bath to agglutinate virtually all of the A cells, which were then removed from the mixture by centrifugation for one minute at 60 g. As shown in figure 2, the results of these studies agree well with the course of events as deduced in the experiment depicted in figure 1. Methemoglobin reduction proceeded at a slow rate in the G-6-PD deficient cells until most of the methemoglobin in the normal cells was reduced. At this point, the rate of methemoglobin reduction in the enzyme-deficient cells became accelerated and approximated that of normal cells. The finding that the methemoglobin content of the type B cells separated from the mixture is slightly lower at the beginning of the experiment and throughout the study than might be anticipated is explained by the fact that the normal plasma reduces approximately 5 per cent of the methemoglobin in nitrite-treated erythrocytes. Presumably, this occurs through the action of non-specific reducing substances such as ascorbic acid.

It could also be demonstrated that non-nitrite-treated red cells enhanced reduction of methemoglobin in erythrocytes from the following sources: normal man, G-6-PD deficient man, subject with congenital methemoglobinemia, rabbit, and horse. Red cells from the patient with congenital methemoglobinemia were able to enhance methemoglobin reduction in nitrite-treated normal erythrocytes.

Normal red blood cells were incubated in a solution of methemoglobin-reductase free crystalline methemoglobin which had been dialyzed against .15 M potassium phosphate buffer, pH 7.4. The final reaction mixture contained, in addition to the usual components, purified methemoglobin in a concentration of 3.18 Gm. per cent. At intervals, aliquots were removed from the suspension, centrifuged, and the methemoglobin content of the supernatant solution determined. Extraordinarily rapid reduction of methemoglobin occurred.

**B. Lack of Interaction between Cell Population when Nile Blue Sulfate is Substituted for Methylene Blue**

Nile blue sulfate, like methylene blue, has a capacity to greatly enhance methemoglobin reduction in erythrocytes in the presence of glucose. We have been able to show that Nile blue activates both the DPN- and the TPN-linked methemoglobin reducing systems, as does methylene blue. The rate of methemoglobin reduction was studied in normal red cells, G-6-PD deficient red cells, and mixtures of the two in the presence of glucose and
Nile blue sulfate. The reaction mixture was identical to that used with methylene blue, except that .01 per cent Nile blue sulfate (Indicator type, National Aniline Division, Allied Chemical Corp., Lot #480P) was substituted for methylene blue. In sharp contrast to the findings when methylene blue was used, there was little or no evidence of interaction between normal and G-6-PD deficient erythrocytes.2

C. The Effect of Lactate on Methemoglobin Reduction

It is well known that lactate may serve as a substrate for methemoglobin reduction, undergoing oxidation to pyruvate through the DPN-linked lactate dehydrogenase system of erythrocytes. Investigations of this pathway have, therefore, been carried out to determine whether lactate production may be the means by which non-nitrite-treated red cells enhance methemoglobin reduction in nitrite-treated red cells. The standard incubating system was employed, but .33 M sodium lactate was substituted for glucose. The method of preparing sodium lactate from lactic acid has been described previously.9 It was possible to show (fig. 3) that lactate in a concentration of 55 mM/L. was capable of serving as substrate for methemoglobin reduction in both normal and G-6-PD deficient cells. It was a less efficient substrate than glucose in normal cells, but more efficient than glucose in G-6-PD deficient cells. While methylene blue markedly enhanced the rate of methemoglobin reduction when lactate served as a substrate, Nile blue sulfate was found to have no effect. This was both in normal cells and G-6-PD deficient cells.

In view of these findings, it seemed possible that the means by which non-nitrite-treated red cells enhance methemoglobin reduction in methemoglobin-containing red cells might be by the production of lactate, its diffusion into the methemoglobin-containing cells, and its oxidation to pyruvate by these cells resulting in the reduction of methemoglobin. Indeed, in a preliminary communication, we suggested that this might be the mechanism of the effect.1 However, a more careful analysis of the biochemical course of events has shown that this is not the case. The accumulation of lactate and pyruvate in nitrite-treated normal and G-6-PD deficient red cells and a mixture of the two has been measured using highly specific enzymatic technics (C. F. Boehringer & Sons). In a mixture of normal and G-6-PD deficient cells, one would expect that the rate of accumulation of lactate would be less than computed from the unmixed cells and that the accumulation of pyruvate would be greater if lactate served as mediator of the effect. This was not found to be the case. Further, while the maximum concentration of lactate achieved even after 5 hours of incubation was less than 4.5 mM/L. and was less than 2.0 mM/L. at the time maximum interaction between the cell population was observed, it is clear from the studies illustrated in figure 4 that at these concentrations of lactate, little or no methemoglobin reduction would be expected.

D. The Role of Leukomethylene Blue

The possibility that leukomethylene blue diffused from non-nitrite-treated cells and subsequently reduced methemoglobin on the methemoglobin-con-
Fig. 3.—The rate of methemoglobin reduction in nitrited normal erythrocytes and G-6-PD deficient erythrocytes with lactate or glucose as substrate. Conditions of the experiment were identical with those described for figure 1, except that a .33 M sodium lactate solution was substituted for .167 M glucose where indicated. It is apparent that lactate can serve as the substrate for methemoglobin reduction in this system.

Taining erythrocytes has been considered. The enhancing effect of normal red cells on methemoglobin reduction of G-6-PD deficient cells could not be decreased by carrying out the incubation aerobically in a Dubnoff metabolic shaker operating at 110 cycles per minute. However, it was found that the rate of oxidation of leukomethylene blue formed by the addition of dithionite to methylene blue under these conditions was relatively slow.

Attempts to measure the steady-state concentration of leukomethylene blue in our system have been made by capping the system with oil, cooling it, and centrifuging. Two ml. of the supernatant solution was added to a cuvette containing 0.1 ml. glacial acetic acid. The resultant pH of 3.6 greatly retards the auto-oxidation of leukomethylene blue. Re-oxidation with ferricyanide produced no detectable change in optical density at 655 mμ, even after normal cells had been incubated for 2 hours in our assay system. Thus, it would appear that the steady-state concentration of leukomethylene blue in this system is very low.

However, it was demonstrated that leukomethylene blue had the capacity to rapidly reduce highly purified solutions of methemoglobin. A 5 Gm. per cent solution of methemoglobin was prepared from crystalline methemo-
Fig. 4.—The relationship between the concentration of lactate used as substrate and the rate of methemoglobin reduction in nitrited normal cells. Conditions of the experiment are identical to those described for figure 1 except that, where indicated, sodium lactate has been substituted for glucose. The concentrations of lactate given are the final concentrations in the reaction mixture. Only the higher concentrations of lactate employed resulted in appreciable rates of methemoglobin reduction.

...globin reductase-free hemoglobin which had been dialyzed against a .5 M potassium phosphate buffer, pH 7.4. Two μE of leukomethylene blue, formed from methylene blue by treatment with hydrogen in the presence of platinum dioxide in a volume of 0.1 ml., were added to 0.5 ml. of this solution, containing approximately 1½ μE of methemoglobin. The methemoglobin and leukomethylene blue were mixed briefly and methemoglobin determinations were carried out. Immediately after mixing leukomethylene blue and methemoglobin, approximately 90 per cent of the methemoglobin in the solution was found to have been reduced. Within a few minutes, the amount of methemoglobin again began to increase, presumably due to the large excess of methylene blue which catalyzed the gradual auto-oxidation of hemoglobin to methemoglobin.

**DISCUSSION**

These studies have demonstrated that non-methemoglobin-containing red cells can enhance the rate of methemoglobin reduction in other populations of erythrocytes mixed with them in the presence of methylene blue. In the
presence of Nile blue sulfate, the effect is not clearly discernible. The enhancing effect of non-nitrite-treated erythrocytes on methemoglobin reduction is so great that it actually appears to make no difference whether the methemoglobin which has been reduced by erythrocytes in the presence of methylene blue is contained within the erythrocyte which is providing the reducing power, whether it is contained in another cell, or whether it is in a solution surrounding the cell.

The mechanism of this effect is not yet entirely clear, but it seems probable that a diffusible reducing substance is produced by red cells incubated in the presence of methylene blue and that this substance actively reduces methemoglobin upon diffusing into other cells. Such a substance might be either a diffusible substrate which could be utilized by other cells for methemoglobin reduction, or the reduced form of the dye itself, leukomethylene blue. The usually accepted pathways of metabolism in the red cell do not provide any such diffusible substrate with the exception of lactate. Matthies et al. have shown that lactate produced by normal cells enhanced (in the absence of substrate or of dye) methemoglobin reduction in nitrited rabbit red cells. A negligible effect was reported in human red cells under the same conditions. The studies which we have described clearly demonstrate that lactate is not the material responsible for the effect which we have studied. The following findings indicate that lactate does not play this role: (1) our inability to detect a deficiency of lactate and surplus of pyruvate formation in mixtures, (2) the finding that the levels of lactate achieved in our system were insufficient to result in rapid methemoglobin reduction, (3) the fact that G-6-PD deficient cells exerted little enhancing effect, and (4) that congenital methemoglobinemic cells were susceptible to the effect, and (5) the fact that even purified methemoglobin solutions were reduced readily.

We must, therefore, consider the possibility that methylene blue, in its reduced form, may be the active agent. In an anaerobic system containing normal erythrocytes mixed either with iodoacetate-poisoned or hemolyzed methemoglobin-containing erythrocytes which was recently described by Banaschak and Schäfer, it was concluded that leukomethylene blue could mediate methemoglobin reduction in mixtures of cells. It seemed, at first, that the relative ease with which leukomethylene blue is auto-oxidized would mitigate against this possibility; the distance from cell to cell is short and the rate of auto-oxidation of leukomethylene blue in our system is gradual, requiring, under some circumstances, several minutes. The fact that the effect is observed only in the presence of methylene blue suggests strongly that leukomethylene blue is the mediator. Although the steady-state concentration of leukomethylene blue appears to be very low, this is not inconsistent with a role as the mediator of the effect. Such a low level of leukomethylene blue may be expected from the relatively large difference between the oxidation-reduction potential of the methylene blue-leukomethylene blue system on the one hand, and the methemoglobin-hemoglobin system on the other. At equilibrium, if only 1 per cent of the methylene blue
was in the leuko-form (a quantity too small to detect in our system), less than 3 per cent of the hemoglobin would be in the form of methemoglobin. If leukomethylene blue is the agent which reduces methemoglobin, Huennekens' interpretation that a diaphorase is interposed in electron transport between leukomethylene blue and methemoglobin would seem to be incorrect. In order to test this possibility, studies have been made to determine whether leukomethylene blue could, indeed, reduce methemoglobin in the absence of "methemoglobin reductase." Such reduction took place virtually completely and so rapidly that the rate could not be measured in our system. The fact that this occurred under aerobic conditions indicates that, even in the presence of oxygen, methemoglobin competes successfully for the electrons of leukomethylene blue. This might explain the fact that leukomethylene blue, quite an unstable compound under aerobic conditions, might still serve as a mediator for methemoglobin reduction.

We, therefore, favor the interpretation that leukomethylene blue diffusing from cells which no longer contain methemoglobin reduces methemoglobin, whether it is found in human or animal, G-6-PD deficient or normal cells, or even in purified solution. It is important for the investigator of red cell metabolism to recognize this type of cellular interaction. In addition, these findings suggest that the enzyme described by Huennekens as playing a role in TPNH-linked methemoglobin reduction probably exerts its effect before methylene blue is reduced. No mediator would seem to be required in the reduction of methemoglobin by leukomethylene blue.

**SUMMARY**

1. It has been shown that mixtures of normal and G-6-PD deficient nitrite-treated erythrocytes reduced methemoglobin at a rate considerably more rapid than that computed from their individual rates of methemoglobin reduction.

2. Differential agglutination studies demonstrated that when normal, methemoglobin-containing cells in such a mixture have reduced all of their methemoglobin, they facilitate methemoglobin reduction in G-6-PD deficient erythrocytes.

3. The same effect could be observed in other mixtures of cells, (e.g., normal with normal, G-6-PD deficient with G-6-PD deficient, etc.) and even with highly purified methemoglobin solutions.

4. This effect could be observed in the presence of methylene blue but not in the presence of another redox dye, Nile blue sulfate.

5. Lactate served as an effective substrate for methemoglobin reduction. Methemoglobin reduction by lactate was enhanced by methylene blue but not by Nile blue sulfate.

6. In mixtures of normal and G-6-PD deficient erythrocytes, no deficit in the rate of accumulation of lactate was found. This indicates that the mechanism of enhancement of methemoglobin reduction is not the diffusion of lactate from non-methemoglobin-containing cells to methemoglobin-containing cells.
7. It was demonstrated that leukomethylene blue could reduce highly purified solutions of methemoglobin in the absence of the enzyme "methemoglobin reductase."

8. The possible mechanism by which non-methemoglobin-containing cells may reduce methemoglobin in methemoglobin-containing cells is discussed. It seems most probable that leukomethylene blue is the mediator of the effect. This implies, contrary to earlier suggestions, that "methemoglobin reductase" acts prior to the reduction of methylene blue in the electron transport chain.

**SUMMARIO IN INTERLINGUA**

1. Esseva monstrate que mixturas de erythrocytos normal con erythrocytos a carentia de dishydrogenase de glucosa-6-phosphato (DG-6-P) tractate con nitrito reduce methemoglobina multo plus rapidemente que lo que deberea esser le facto secundo calculationes a base de lor rapiditates individual de reductio de methemoglobina.

2. Studios de agglutination differential ha demonstrate que quando le normal cellulas a contento de methemoglobina in tal mixturas ha reducite omne lor methemoglobina, illos promove le reduction del methemoglobina in erythrocytos a carentia de DG-6-P.

3. Le mesme effecto poteva esser observate in altere mixturas de cellulas (p.ex. normal con normal, deficiente in DG-6-P con deficiente in DG-6-P, etc.) e mesmo con altemente purificate solutiones de methemoglobina.

4. Iste effecto poteva esser observate in le presentia de blau methylenic sed non in le presentia de sulfato de blau del Nilo (que es etiam un colorante reducto-oxydatorij).

5. Lactato serviva como efficace substrato pro le reduction de methemoglobina. Le reduction de methemoglobina per lactato esseva promovite per blau methylenic sed non per sulfato de blau del Nilo.

6. In mixturas de erythrocytos normal con erythrocytos a carentia de DG-6-P, nulle deficit in le intensitate del accumulation de lactato esseva trovate. Isto indica que le mechanismo del promotion del reduction de methemoglobina non es le diffusion de lactato ab cellulas non continente methemoglobina ad cellulas continente methemoglobina.

7. Esseva demonstrate que blau leucomethylenic pote reducer altemente purificate solutiones de methemoglobina in le absentia del enzyma "reductase de methemoglobina."

8. Es discutite le mechanismo possibile per le qual cellulas non continente methemoglobina pote reducer methemoglobina in cellulas continente methemoglobina. II pare probabilissime que blau leucomethylenic es le mediator del effecto. Isto significa—per contrasto con previe suggestiones—que "reductase de methemoglobina" age ante le reduction de blau methylenic in le catena del transporto de electrones.

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8. Unpublished data.


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