Mechanisms of Methylene Blue Stimulation of the Hexose Monophosphate Shunt in Erythrocytes

EARL N. METZ, STANLEY P. BALCERZAK, and ARTHUR L. SAGONE, JR.

From the Department of Medicine, Ohio State University College of Medicine, Columbus, Ohio 43210

ABSTRACT The response of the hexose monophosphate shunt in erythrocytes was studied with the ionization chamber-electrometer apparatus to measure continuously $^{14}CO_2$ derived from ^{14}C-labeled substrates. The effect of methylene blue at high (0.1 mM) and low (1 μM) concentrations was evaluated under different gas mixtures; air, carbon monoxide, and 6% carbon monoxide in air. The latter gas mixture results in nearly 100% carboxyhemoglobin but provides a physiologic partial pressure of oxygen. The extent to which pentose is recycled through the shunt in response to methylene blue stimulation was examined with radioactive glucose substrates labeled on the first, second, and third carbon positions. Generation of hydrogen peroxide after stimulation of erythrocytes with methylene blue was evaluated by the catalase-aminotriazole trapping technique, [^{14}C]formate oxidation, and oxidation of reduced glutatione. Stimulation of the shunt with 1 μM methylene blue was markedly impaired in the absence of oxyhemoglobin, but stimulation with 0.1 mM methylene blue was only slightly impaired under the carbon monoxide-air mixture. The higher concentration of methylene blue produced evidence of hydrogen peroxide generation by all three techniques. Despite the evidence for the involvement of oxygen, oxyhemoglobin, and hydrogen peroxide in the response to methylene blue, cells containing methemoglobin induced by sodium nitrite or from a patient with congenital methemoglobinemia responded normally to methylene blue in the absence of oxygen. These experiments indicate that the reactions induced by methylene blue in erythrocytes are more complex than generally thought and that high concentrations are associated with production of peroxide.

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INTRODUCTION

The redox dye, methylene blue, is a standard agent for use in the study of the hexose monophosphate shunt (HMPS)[1] pathway (1–5). This compound functions as an intermediate in the transfer of electrons from pyridine nucleotides to a suitable electron acceptor, thereby stimulating the HMPS in a variety of cell systems. In the erythrocyte, methylene blue is reduced to leukomethylene blue primarily by an NADPH-dependent diaphorase. The diaphorase is reduced via oxidation of NADPH that, in turn, stimulates the HMPS, and leukomethylene blue transfers electrons to an acceptor such as methemoglobin. This series of reactions can be utilized clinically for the reduction of ferric to ferrous heme iron in patients with acquired methemoglobinemia. Leukomethylene blue may also react with oxyhemoglobin or molecular oxygen resulting in increased oxygen uptake by erythrocytes.

We have investigated further the mechanisms by which methylene blue stimulates the HMPS in intact human erythrocytes. By using a range of concentrations of methylene blue and different gas mixtures it is apparent that stimulation of the HMPS by methylene blue may occur by more than one mechanism and that the presence of oxygen is not required. On the other hand, methylene blue in high concentrations may react with oxyhemoglobin-producing oxygen radicals that react, in turn, with reduced glutathione and stimulate the HMPS via the glutathione peroxidase-reductase reaction. This mechanism might account for the toxicity of methylene blue when used in therapeutic excess (6).

The use of [^{14}C]glucose substrates labeled on different carbon atoms and the use of ionization chamber-electrometer apparatus to record continuously the production of $^{14}CO_2$ is also a convenient technique for quantitating the degree to which pentose is recycled through the

[1] *Abbreviations used in this paper:* GSH, reduced glutathione; HMPS, hexose monophosphate shunt; NEM, N-ethylmaleimide; RBC, erythrocytes.

HMPS at various levels of stimulation with methylene blue.

METHODS

Preparation of erythrocytes

Venous blood was collected in heparinized tubes from normal volunteers and a patient with congenital methemoglobinemia (NADH diaphorase deficiency) and was centrifuged at 1,000 *g* for 10 min. The plasma and buffy coat were removed and the erythrocytes were washed once in 5 vol of saline and resuspended in a pH 7.4 buffer containing 145 mM sodium, 5 mM potassium, 20 mM glycylglycine, 5 mM glucose, and 145 mM chloride. As reported previously, pH values in this incubation system were maintained at 7.3 or greater for more than 3 h (7). Leukocyte and platelet contamination was less than 1,000/mm^3 and 10,000/mm^3, respectively.

Metabolic studies

3–4 ml of packed erythrocytes (RBC) were resuspended in buffer to a final volume of 10 ml in a 50-ml triple-headed distilling flask to which was then added 5 μCi of radioactive substrate. All radioactive reagents were obtained from Amersham/Searle Corp., Arlington Heights, Ill.

The inlet of the flask was connected to a gas cylinder containing either compressed air, carbon monoxide (CO), or 6% CO in air. The latter gas mixture produces nearly 100% carboxyhemoglobin while maintaining a physiologic P_{O_2} (approximately 140 mm Hg). The outlet arm of the flask was connected to a 275-ml Cary-Tolbert ionization chamber and a Cary Model 401 vibrating reed electrometer (Cary Instruments, Monrovia, Calif.). The third arm of the flask was covered with a rubber stopper through which reagents could be added or samples withdrawn through a spinal needle. The use of the ionization chamber-electrometer apparatus for continuous monitoring of $^{14}CO_2$ produced by cell suspensions, and our modifications of this apparatus, have been described in detail elsewhere (7–9). A duplicate system was used so that $^{14}CO_2$ derived from [^{14}C]glucose could be measured simultaneously from both control and the experimental flasks. The incubation flasks were maintained at 37°C throughout these experiments and were stirred continuously. After base-line $^{14}CO_2$ production was established, agents (in buffer) were added through the center well to the experimental flasks and an equal volume of buffer alone (0.1–0.3 cm^3) to the control flasks. CO_2 production was calculated from the millivolt reading once steady-state conditions were reestablished in the experimental chambers and compared to the corresponding value from the control flasks. CO_2 production was calculated as previously described and expressed in micromoles of CO_2 per milliliter of RBC per hour (7).

In some experiments, the response of the HMPS was studied in RBC with methemoglobin. Methemoglobin was produced by incubation of 1 vol of packed cells with 1 vol of isotonic sodium nitrite for 30 min at room temperature. The packed cells were then washed three times with 8 vol of isotonic saline and resuspended in glycylglycine buffer. Controls for these experiments were RBCs incubated in saline at room temperature for 30 min and washed similarly to the nitrite-treated cells.

Studies to detect peroxide generation

Three methods were used to detect the generation of hydrogen peroxide in RBC incubated with methylene blue.

Glutathione stability. RBC were washed thoroughly and resuspended in glycylglycine buffer with or without glucose and with methylene blue. RBC reduced glutathione (GSH) concentrations were then determined before and after 2 or 4 h of incubation at 37°C. RBC GSH values were measured by the 5,5′-dithiobis-2-nitrobenzoic acid method of Beutler et al. (10).

Catalase inhibition technique. RBC were incubated for 30 min with N-ethylmaleimide (NEM) in amounts required to bind all intracellular GSH (2–3 μmol NEM/ml RBC). The cells were then washed with isotonic saline and incubated for an additional hour with or without methylene blue (0.1 mM) in the presence of 50 mM aminotriazole. In the presence of aminotriazole, hydrogen peroxide and catalase form an irreversible complex. Hydrogen peroxide generation can be detected by serial determinations of catalase (11). After the second incubation, stroma-free hemolysates were prepared in a concentration equal to a 1 : 1,500 dilution of packed RBC. Duplicate 1-ml aliquots of the hemolysate were incubated with sodium perborate substrate for 3 min and residual perborate was titrated with 1 N potassium permanganate according to the method of Tarlov and Kellermeyer (12). This system provides a qualitative estimate of peroxide generation and results are expressed as percent fall in catalase activity during a 60-min incubation with methylene blue.

[^{14}C]*Formate oxidation.* Since ^{14}C-formate is oxidized to $^{14}CO_2$ in the presence of H_2O_2 and catalase, this system was also used to detect H_2O_2 (13). RBC were incubated in metabolic flasks as previously described, except that [^{14}C]-formate was used as a radioactive substrate rather than [^{14}C]glucose. Since there is good evidence that H_2O_2 is preferentially reduced by glutathione peroxidase in RBC, [^{14}C]formate oxidation was also studied in cells treated with NEM to block cellular GSH (14).

RESULTS

Effect of CO in air on the HMPS of unstimulated and methylene blue-stimulated RBC. The rate of CO_2 production of RBC suspensions incubated with 6% CO in air compared with air or CO alone is illustrated in Fig. 1. As previously reported, unstimulated RBC suspensions incubated under CO have a rate of $^{14}CO_2$ production which is 40% of the value under air, suggesting that approximately 60% of baseline HMPS activity is related to reactions involving oxygen (15). In the presence of 6% CO in air, the rate of CO_2 production of 0.055 μmol/ml RBC per h was significantly lower than the value for RBC suspensions incubated under air ($P < 0.05$). These data indicate that a major portion of oxygen-dependent HMPS activity of unstimulated RBC suspensions is related to reactions involving oxyhemoglobin, and not molecular oxygen.

In RBC suspensions incubated under air, the addition of methylene blue in low concentrations (1 μM) resulted in a prompt increase in $^{14}CO_2$ production (Fig. 1). Under CO, a barely detectable increase in $^{14}CO_2$ production occurred confirming the anaerobic conditions of our experiments and an apparent requirement for oxygen in methylene blue stimulation of the HMPS. Under CO in air, methylene blue (1 μM) resulted in only a small in-

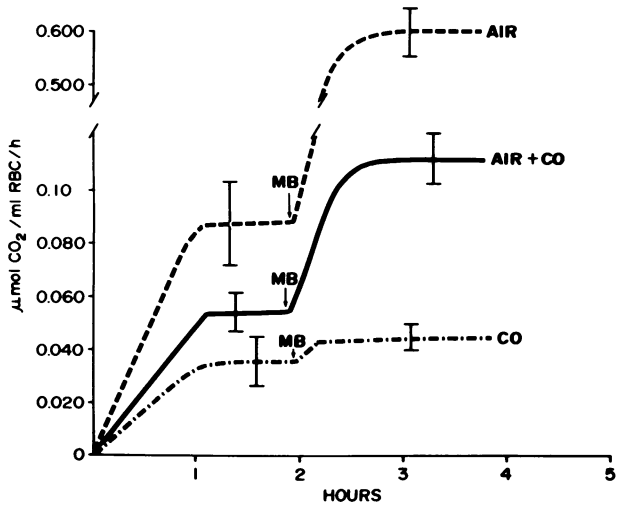

FIGURE 1 The effect of carbon monoxide on the oxidation of [1-^{14}C]glucose in RBC incubated with methylene blue (MB). CO_2 production was calculated for steady-state conditions at base line and after methylene blue stimulation (1 μM) under air (13 experiments), under 6% carbon monoxide in air (6 experiments), and under 100% carbon monoxide (7 experiments). The curves are constructed from the original continuous recordings using the mean values for the three experimental conditions. The brackets indicate ±1 SD.

crease in the CO_2 production to 0.110 μmol/ml RBC per h and was in contrast to the value of 0.580 μmol with air alone ($P < 0.01$). These data indicate that stimulation of the HMPS by low concentrations of methylene blue is primarily related to a reaction involving oxyhemoglobin. This requirement for oxyhemoglobin was less apparent when the HMPS was stimulated with high concentrations of methylene blue (0.1 mM). In contrast to experiments with lower concentrations of methylene blue, 0.1 mM methylene blue resulted in a marked stimulation of [1-^{14}C]glucose oxidation under air with CO and was 87% of the value for air alone (Table I). Stimulation of [1-^{14}C]glucose oxidation by an intermediate concentration of methylene blue (10 μM) was significantly impaired as a result of incubation under air with CO and was only 36% of the value of similar suspensions under air.

Effect of methemoglobin. Since methylene blue is effective in the transfer of electrons to methemoglobin, the rate of $^{14}CO_2$ production by sodium nitrite-treated cells was examined in the presence of methylene blue. The rate of $^{14}CO_2$ production was studied in the same three gas mixtures to determine if methylene blue stimulation of the HMPS could occur in the absence of oxygen. As seen in Fig. 2, the rate of $^{14}CO_2$ production by nitrite-treated cells incubated with 1 μM methylene blue under air was similar to untreated controls. In contrast to control RBC, however, the stimulation of $^{14}CO_2$ production of nitrite-treated cells by methylene blue was unaffected by the absence of oxygen. Likewise, methylene blue stimulation of the HMPS was unaffected in nitrite-treated cells which were incubated under the mixture of CO in air. These data indicate that methemoglobin can act as an electron acceptor for methylene blue in the absence of oxygen. It is of interest that incubation of nitrite-treated cells with methylene blue under CO resulted in complete reduction of methemoglobin to carboxyhemoglobin in 3–4 h and that completion of this reaction was accompanied by a prompt fall in HMPS activity.

TABLE I

[1-^{14}C]Glucose Oxidation with Low, Intermediate, and High Concentrations of Methylene Blue under Air Compared with 6% Carbon Monoxide in Air

	Methylene blue concentration			
	None	*1 μM*	*10 μM*	*0.1 mM*
Air				
Mean	0.087*	0.58	2.60	2.90
SD	±0.016	±0.09	±0.25	±0.38
	(13)	(13)	(3)	(8)
Air and CO				
Mean	0.055	0.110	0.95	2.50
SD	±0.007	±0.01	±0.11	±0.25
	(6)	(5)	(3)	(5)

* Results are expressed as micromoles of CO_2 produced per milliliter RBC per hour. Numbers in parentheses refer to number of experiments.

The HMPS activity of the RBC from a patient with congenital methemoglobinemia (approximately 33%)

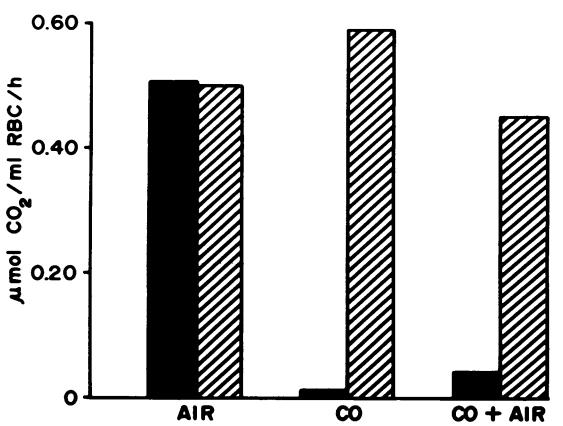

FIGURE 2 Oxidation of [1-^{14}C]glucose by nitrite-treated and normal RBC incubated with methylene blue (1 μM). The values represent the mean increase in $^{14}CO_2$ production after the addition of methylene blue. Three paired experiments were carried out under each gas mixture. Nitrite-treated cells are indicated by the hatched bars, control cells by the solid bars.

TABLE II

HMPS Stimulation with Methylene Blue under Air and Carbon Monoxide in RBC from a Patient with Congenital Methemoglobinemia

	Gas mixture	
	Air	Carbon monoxide
Base line	0.078*	0.046
Methylene blue (*1 μM*)	0.716	0.630

* Results are expressed as micromoles of CO_2 per milliliter RBC per hour.

were also studied to determine the similarity to nitrite-treated cells (Table II). The rate of $^{14}CO_2$ production of unstimulated suspensions incubated under air and the response to methylene blue were similar to normal RBC. The rate of $^{14}CO_2$ production of unstimulated RBC suspensions also was reduced by incubation under CO. The addition of methylene blue, however, resulted in a prompt increase in $^{14}CO_2$ production similar to nitrite-treated cells.

Peroxide generation in RBC incubated with methylene blue. Evidence for hydrogen peroxide generation in RBC incubated with methylene blue was demonstrated by the following experiments.

A significant fall in GSH occurred in RBC incubated without glucose in the presence of 0.1 mM methylene blue (Fig. 3). Methylene blue at a concentration of 1 μM did not affect GSH concentrations. GSH concentrations did not fall with either concentration of the drug when the incubations were supplemented with glucose.

A marked fall in catalase occurred in NEM-treated cells in the presence of the 0.1 mM methylene blue and aminotriazole (Fig. 4). No fall occurred in the presence of the 1μM concentration.

Oxidation of formate proved to be the most sensitive indicator of peroxide generation in response to methylene blue. $^{14}CO_2$ derived from [^{14}C]formate increased 3 times the base-line value after addition of methylene blue (1 μM) and 40 times the control value in the presence of the higher concentration (0.1 mM). Binding of GSH with NEM diverts peroxide to catalase and, as expected, preincubation of RBC with NEM (3.5 μmol/ml RBC) greatly augmented formate oxidation. Under these circumstances 1 μM methylene blue produced a 40-fold increase in formate oxidation which in-

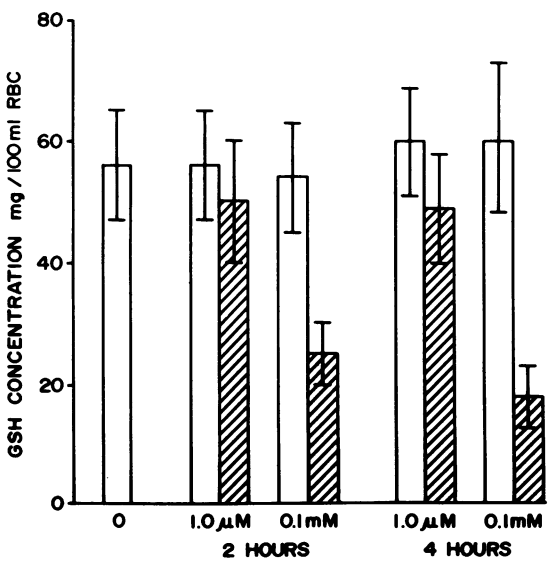

FIGURE 3 Effect of methylene blue on reduced glutathione. The bars represent RBC GSH concentration (±SD) in milligrams per 100 milliliters. The open bars indicate samples incubated with glucose and the hatched bars represent cells incubated without supplemental glucose. The methylene blue concentrations were 1 μM and 0.1 mM as indicated.

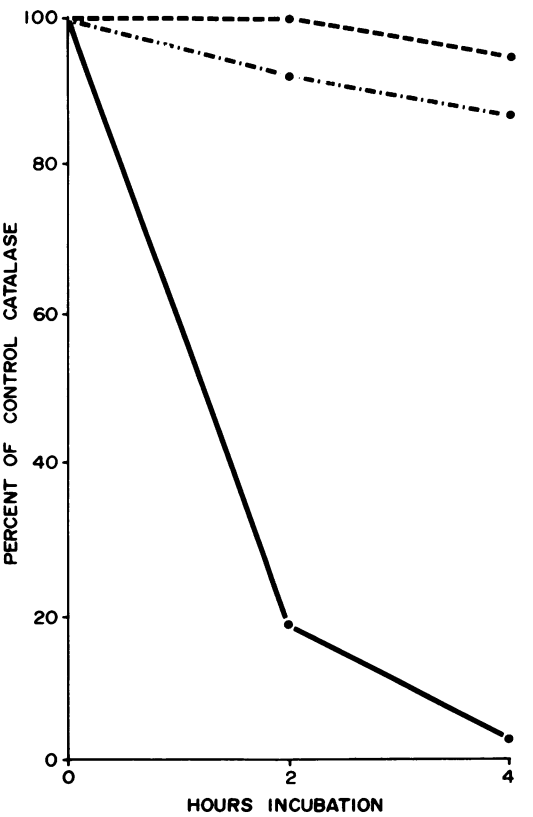

FIGURE 4 Hydrogen peroxide production by methylene blue (0.1 mM) determined by the catalase-aminotriazole trapping technique. The scale on the left represents the percent of initial catalase activity remaining after incubation. RBC were pretreated with NEM in a concentration sufficient to bind intracellular GSH (2–3 μmol/ml RBC). The solid line shows the effect of methylene blue, 0.1 mM, the uppermost dashed line shows the catalase content of control incubations, and the middle line shows the effect of NEM without methylene blue.

creased to approximately 250 times the base-line value after addition of 0.1 mM methylene blue.

Recycling of pentose with methylene blue stimulation. The use of [1-^{14}C]glucose as a substrate provides a semiquantitative estimate of HMPS activity in RBC and is a useful and convenient method for measuring relative changes in HMPS activity with various experimental manipulations (3, 7). In fact, however, oxidation of the first carbon atom yields quantitative data regarding the HMPS only under base-line conditions. It is well known that when activity of the HMPS is increased, pentose is recycled via transketolase and $^{14}CO_2$ can then be measured using glucose labeled in the second carbon position. The contribution of recycled pentose to total shunt activity increases progressively as the shunt is stimulated with methylene blue. These data are summarized in Fig. 5. Under base-line conditions, no $^{14}CO_2$ was detected from glucose labeled in the third position and oxidation of the second carbon was either absent or barely detectable. With addition of 1 μM methylene blue, CO_2 production, calculated from oxidation of [1-^{14}C]glucose, increased from 0.09 to 0.57 μmol/ml RBC per h. At this level of HMPS activity, $^{14}CO_2$ production from [2-^{14}C]glucose was readily detectable at a rate of 0.19 μmol/ml RBC per h, or 33% of the value from [1-^{14}C]glucose. Furthermore, oxidation of the third carbon atom of glucose was now 30% of the value from [2-^{14}C]glucose. With additional stimulation with 0.1 mM methylene blue, oxidation of [1-^{14}C]glucose grossly underestimated total HMPS activity. At this high level of shunt activity, oxidation of the second carbon was 74% of that from [1-^{14}C]glucose and oxidation of the third carbon atom was again increased proportionally and equaled 75% of the value from [2-^{14}C]-glucose.

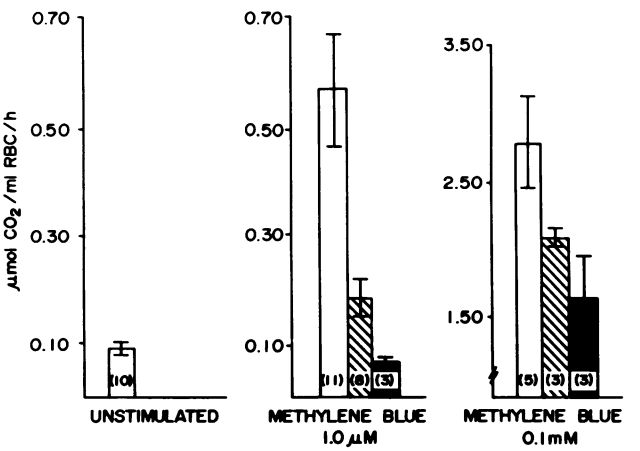

FIGURE 5 Recycling of pentose with HMPS stimulation. The bars indicate $^{14}CO_2$ production (±SD) from [1-^{14}C]glucose (open bars), [2-^{14}C]glucose (hatched bars), and [3-^{14}C]glucose (solid bars) after stimulation with methylene blue at the concentrations noted. Numbers in parentheses at the bottom of each bar note the number of experiments.

DISCUSSION

It is well established that addition of methylene blue to a suspension of RBC evokes a marked stimulation of HMPS activity. This stimulation increases progessively with increased concentrations of methylene blue and reaches a maximum at about 0.1 mM (4). It is clear from these data, however, that there are qualitative differences in the reactions stimulated by methylene blue at various concentrations. For example, stimulation of the HMPS by a low concentration of methylene blue (1 μM) is dependent upon the presence of oxyhemoglobin but hydrogen peroxide was not produced in amounts detectable by the aminotriazole trapping system nor was there any detectable oxidation of reduced glutathione. However, data derived from formate oxidation after NEM blockade suggest that even low levels of HMPS stimulation are associated with generation of small amounts of peroxide. High concentrations of methylene blue (0.1 mM) oxidize glutathione and produce enough peroxide to be detected by both formate oxidation and aminotriazole trapping. This production of hydrogen peroxide required, of course, the presence of oxygen, but in the presence of methemoglobin it was possible to dissociate shunt activity and oxygen utilization.

These observations help to clarify some of the apparent contradictions concerning the effect of methylene blue. For example, Jacob and Jandl found that methylene blue stimulation of the HMPS was not impaired by sulfhydryl blockade and concluded therefore that methylene blue stimulation of the shunt was not associated with hydrogen peroxide generation (5). Roth et al. arrived at a similar conclusion based upon the fact that cells depleted of GSH by incubation with nitrogen mustard responded normally to methylene blue while stimulation with ascorbate was impaired (16). In contrast, Tephly et al. showed that activation of the HMPS with methylene blue and glucose induced the oxidation of methanol and ethanol in intact cells by a reaction requiring catalase and concluded that this reaction must result in the generation of hydrogen peroxide (17). The differences in peroxide generation which we have demonstrated with high and low concentrations of methylene blue would seem to resolve this contradiction. Tephly made his observations utilizing a high concentration of methylene blue while Jacob and Jandl employed a much lower dose.

These experiments may also help clarify the interrelationship of HMPS stimulation with methylene blue, oxygen consumption, and methemoglobin reduction. Experiments in which RBC were exposed to a mixture of air and 6% carbon monoxide demonstrate clearly that oxyhemoglobin is a more effective electron acceptor in

the methylene blue reaction than molecular oxygen in intact RBC. This may relate to the altered electron spin state of oxygen liganded to hemoglobin (18). Further, the ability of methemoglobin to serve as an electron acceptor for the methylene blue reaction in the absence of oxygen suggests that the concept of oxygen consumption during stimulation of the HMPS is, in a sense, artifactual. We would suggest that the reaction between methylene blue and oxyhemoglobin results in the release of oxygen radicals and that the observed oxygen consumption is really related to the generation of these radicals or the reoxygenation of hemoglobin. Although a substantial portion of HMPS activity in RBC appears to be dependent upon oxygen, it also seems apparent that this activity represents a reaction to the presence of oxygen, or active radicals, rather than a requirement for oxygen.

The effectiveness of methylene blue in reducing methemoglobin is an established clinical fact. In addition, the experiments of Jacob and Jandl showed that stimulation of the HMPS with methylene blue in vitro resulted in accelerated methemoglobin reduction (5). In those experiments, however, the concentration of methemoglobin did not appear to have any influence on the degree of HMPS stimulation. Our data suggest that methemoglobin and oxyhemoglobin are equally effective as electron acceptors from methylene blue and that rate of activity of the HMPS is governed primarily by the concentration of methylene blue, $NADP^+$, and the HMPS enzymes rather than by the terminal acceptor. Smith and Thron reached a similar conclusion (19). They noted that reduction of methemoglobin by methylene blue was more rapid under anaerobic conditions than under air, an observation best explained by a competition between oxyhemoglobin and methemoglobin for electrons. This interpretation might also be a possible explanation for those instances of acute hemolytic anemia precipitated by toxic doses of methylene blue. When oxyhemoglobin is the only available electron acceptor, it is possible that there are sufficient oxygen radicals generated to result in RBC injury.

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