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MECHANISM OF METHEMOGLOBIN FORMATION BY DIPHENYLSULFONES

EFFECT OF 4-AMINO-4′-HYDROXYAMINODIPHENYLSULFONE AND OTHER *p*-SUBSTITUTED DERIVATIVES

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Abstract—4,4′-Diaminodiphenylsulfone (DDS) can produce methemoglobinemia *in vivo*, apparently by the action of an unidentified metabolic product. To discern the chemical nature of such a compound, 13 *p*-substituted diphenylsulfones have been examined for their ability to oxidize hemoglobin (Hb) to methemoglobin (MHb) in human erythrocytes *in vitro*. 4-Amino-4′-hydroxyaminodiphenylsulfone (DDS-NOH) is most effective, $1{\cdot}9 \times 10^{-5}$ M of drug oxidizing as much as 60 per cent of the Hb, or 100 heme equivalents/mole of DDS-NOH in 2 hr. Oxygen is required for this process, and glucose, while not required, enhances MHb formation. With dialyzed hemolysates and solutions of purified Hb, the heme equivalents oxidized/mole of drug becomes less than 10, but a ratio of 80 : 100 can be restored by adding reduced glutathione (GSH) or NADPH. Apparently DDS-NOH produces MHb by coupled oxidation with Hb and O_2 and DDS-NOH can be regenerated by reducing compounds to yield the high heme oxidation ratio. In intact cells, DDS-NOH causes a 7-fold increase in the activity of the hexose monophosphate shunt as measured by $^{14}CO_2$ production from glucose 1-^{14}C. Incubation with galactose, which is minimally oxidized by the shunt pathway, results in decreased formation of MHb, and the GSH concentration drops by 75 per cent. Depletion of intracellular GSH by a sulfhydryl oxidizing compound also reduces MHb formation by DDS-NOH. These observations implicate GSH or NADPH or both in the recycling of DDS-NOH in erythrocytes. Consistent with these interpretations, DDS-NOH produced only one-half as much MHb in erythrocytes from three individuals with glucose 6-phosphate dehydrogenase deficiency as it did in normal erythrocytes.

PRIMARY aromatic amines such as aniline often produce methemoglobin (MHb)† both *in vivo* and *in vitro*, but only after undergoing a biochemical transformation. Kiese[1] found that the *N*-hydroxyl derivatives of primary aromatic amines play a more significant role in MHb formation than the ring-hydroxylated metabolites such as the amino-phenols, although the latter are often active. It has been reported that these same *N*-hydroxyl functions are involved in the carcinogenic activity of many of the aromatic amines, but there is apparently no direct relationship between carcinogenic and MHb-forming activities.[2]

4,4′-Diaminodiphenylsulfone (Dapsone) is an antimalarial drug which can produce abnormal amounts of MHb *in vivo*, apparently through the action of an unidentified

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† Abbreviations used: MHb, methemoglobin; Hb, hemoglobin; DDS, 4,4′-diaminodiphenylsulfone; DDS-NOH, 4-amino-4′-hydroxyaminodiphenylsulfone; GSH, reduced glutathione; NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide respectively; NADP and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate respectively; HMP, shunt, hexose monophosphate shunt; G-6-PD, glucose 6-phosphate dehydrogenase; azoester methylphenylazoformate.

metabolite. Hjelm and DeVerdier[3] have reported that the serum of Dapsone users is effective in producing MHb and activating the hexose monophosphate (HMP) shunt of human erythrocytes *in vitro*. They suggested that an *N*-hydroxyl or nitroso metabolite might be responsible. Wind[4] has found up to 27% MHb in dogs treated with bis-4,4′-dihydroxyaminodiphenylsulfone, an *N*-hydroxyl derivative of 4,4′-diaminodiphenylsulfone (DDS).

This report describes the effectiveness with which a number of *p*-substituted diphenylsulfones oxidize hemoglobin (Hb) *in vitro*. Among the derivatives examined, only 4-amino-4′-hydroxyaminodiphenylsulfone (DDS-NOH) was a potent MHb-producing agent. The biochemical requirements for this activity and its mechanism of action were explored.

MATERIALS AND METHODS

4-Amino-4′-hydroxyaminodiphenylsulfone was prepared by the method of Jackson[5] and recrystallized from acetone–water, m.p. 191–2°. *Anal.* Calc. for $C_{12}H_{12}N_2O_3S$: C, 54·53; H, 4·58; N, 10·60; S, 12·13. Found: C, 54·79; H, 4·84; N, 10·47; S, 12·15. Nuclear magnetic resonance and infrared spectroscopy confirmed qualitatively that the correct compound had been prepared. All other diphenylsulfones were obtained from the Division of Medicinal Chemistry, Walter Reed Army Institute of Research, and were used without further purification, with the exception of DDS which was recrystallized from methanol–water, m.p. 179°. D(+) Galactose, β-D(−) fructose, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (G-6-PD) (Torula yeast) and oxidized glutathione (Sigma); reduced glutathione (GSH) (Nutritional Biochemicals Corp.); 5,5′-dithiobis (2-nitrobenzoic acid) (Aldrich); methylphenylazoformate (azoester) (Calbiochem); Triton X-100 (Rohm & Haas); and NADP, NAD (Pabst) were used without further purification. Crystalline horse liver alcohol dehydrogenase was purchased from Calbiochem (A grade) and dialyzed four times against a 100-vol. excess of 0·1 M sodium phosphate, pH 7·4, 4°, and the solution clarified by centrifugation. All other chemicals were reagent grade. Deionized, glass-distilled water was used throughout. Sodium phosphate, 0·01 M, pH 7·4, was the buffer used throughout. It was adjusted to isotonicity with NaCl when intact cells were used.

Methemoglobin was measured by the method of Evelyn and Malloy as modified by Hainline[6] and GSH by the method of Beutler.[7] Hemoglobin was determined by the method of Drabkin.[8]

Hexose monophosphate shunt activity was measured by observing $^{14}CO_2$ production from glucose 1-^{14}C. Suspensions of erythrocytes (4 ml) with a hematocrit of 20 per cent were incubated in stoppered 25-ml flasks for 3 hr at 37° in a shaking water bath. The reaction was stopped and $^{14}CO_2$ was liberated by the addition of 4 ml of 3·5 per cent perchloric acid. Released $^{14}CO_2$ was trapped in plastic wells containing Hyamine hydroxide. One hr after adding the acid, the Hyamine hydroxide well was added to 10 ml of scintillation fluid [toluene; methanol; 2,5-diphenyloxazole; 1,4-bis-2-(4-methyl 5-phenyloxazolyl) benzene] and counted for 10 min in a Packard Tri-Carb model 3375 spectrophotometer.

To measure MHb formation in intact erythrocytes, cells from healthy human donors were washed three to five times with isotonic phosphate buffer, pH 7·4, and the hematocrit was adjusted to 20 per cent with buffer. Metabolic inhibitors or sugar

substrates or both were added to the red cell suspension. Methylphenylazoformate, when used, was added to a dry glass tube with a 1-μl syringe, an aliquot of blood suspension was pipetted into the tube with constant agitation, and the suspension was allowed to remain at room temperature for 7 min to permit depletion of GSH. Aliquots of cell suspension were equilibrated to 37° in a Dubnoff incubator. Drugs dissolved in 95 per cent ethanol were added to the samples of suspended cells such that the final concentration of ethanol was 1 per cent. Ethanol had no effect on the MHb levels produced, but was included in controls. Methemoglobin formation at 37° was measured after designated incubation times.

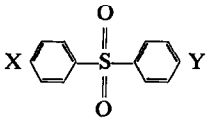
Studies on hemolysates were done in an identical fashion, except that the washed packed cells were disrupted by hypotonic lysis in four times their volume of 0·01 M phosphate buffer, pH 7·4, followed by centrifugation at 12,500 *g* for 30 min to remove stroma. Some samples were dialyzed three times against a 10-vol. excess of phosphate buffer. Hemoglobin was prepared as described by Li and Johnson[9] and dialyzed four times against a 10 to 20-fold excess of phosphate buffer. It was free of NADPH-MHb reductase activity.

Unless otherwise indicated, all values given are the average of duplicate determinations made in each of two or three separate experiments.

RESULTS

Human erythrocytes were incubated at 37° *in vitro* with a variety of *p*-substituted diphenylsulfones. Only the compound with the *N*-hydroxyl function produced a substantial increase in the percentage of MHb found relative to untreated erythrocytes (Table 1). 4-Amino-4′-hydroxyaminodiphenylsulfone produced rapid and extensive

TABLE 1. EFFECT OF VARIOUS *p*-SUBSTITUTED DIPHENYLSULFONES ON METHEMOGLOBIN FORMATION IN INTACT ERYTHROCYTES *in vitro**



X	Y	$\%\ \text{MHb} = \frac{(\text{MHb})}{(\text{Hb}_{tot})} \times 100$
Control (no drug)		< 1·0
H	H	< 1·0
NH_2	NH_2	< 1·0
HCO—N—	HCO—N—	< 1·0
HCO—N—	H	< 1·0
NO_2	H	< 1·0
OH	OH	< 1·0
OH	Cl	< 1·0
Cl	Cl	< 1·0
Cl	NH_2	< 1·0
NO_2	NO_2	2·2
NO_2	NH_2	2·4
CH_3CO—N—	NO_2	2·9
—NOH	NH_2	48 (11)†

* Red blood cell suspension (6·8–7·9 g/100 ml) in 10 mM glucose, pH 7·4, 37°; 2 hr incubation. Drug concentration, $1{\cdot}9 \times 10^{-5}$ M.

† Number of determinations.

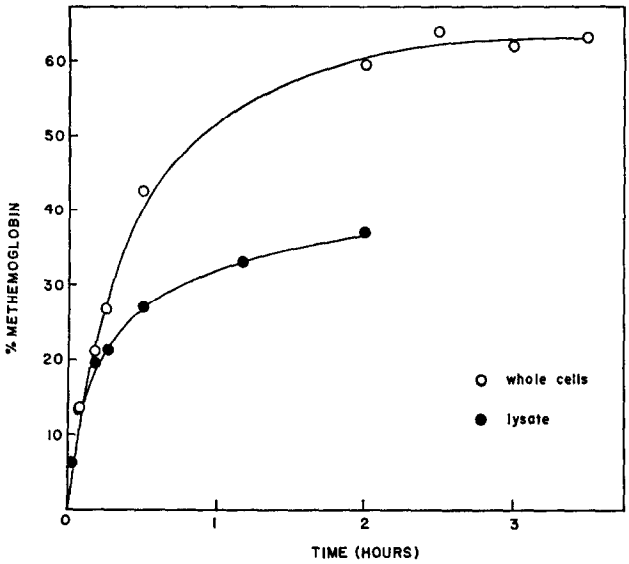


FIG. 1. Time dependence of methemoglobin formation by $1{\cdot}9 \times 10^{-5}$ M DDS-NOH in a 20 per cent suspension of intact erythrocytes and a 6·8 g/100 ml red cell hemolysate, pH 7·4, 37°.

MHb formation (Fig. 1, Table 2), $1{\cdot}9 \times 10^{-5}$ M drug oxidizing as much at 60 per cent of the Hb present or about 100 heme equivalents/mole of DDS-NOH in 2 hr. Such stoichiometry indicates that the active functional group was being regenerated. Glucose, while not required for MHb formation, enhanced the effect of the drug.

4-Amino-4′-hydroxyaminodiphenylsulfone also produced MHb when the red blood cells were hemolyzed, but the equilibrium level of MHb attained was lower (Fig. 1). Flushing the hemolysate with CO converted the oxyhemoglobin to carboxyhemoglobin, which did not produce MHb when incubated with the drug (Table 2).

TABLE 2. EFFECT OF DDS-NOH ON METHEMOGLOBIN FORMATION IN INTACT ERYTHROCYTES AND HEMOLYSATES *in vitro**

	Erythrocytes		Hemolysate	
Additions†	% MHb‡	No. determ.	% MHb	No. determ.
None	0·8 (0·4–1·2)	9	1·4 (0·6–1·9)	4
DDS-NOH	48 (38–63)	11	32 (29–42)	5
DDS-NOH + CO treatment			3·0	3
DDS-NOH (no glucose)	31	2		

* Red cell suspensions (6·3–8·3 g/100 ml of Hb) and hemolysates (5·3–7·5 g/100 ml of Hb) contain 10 mM glucose. Two-hr incubation at pH 7·4, 37°.

† All drug concentrations at $1{\cdot}9 \times 10^{-5}$ M.

‡ Per cent MHb = $(\text{MHb})/(\text{Hb}_{\text{tot}}) \times 100$.

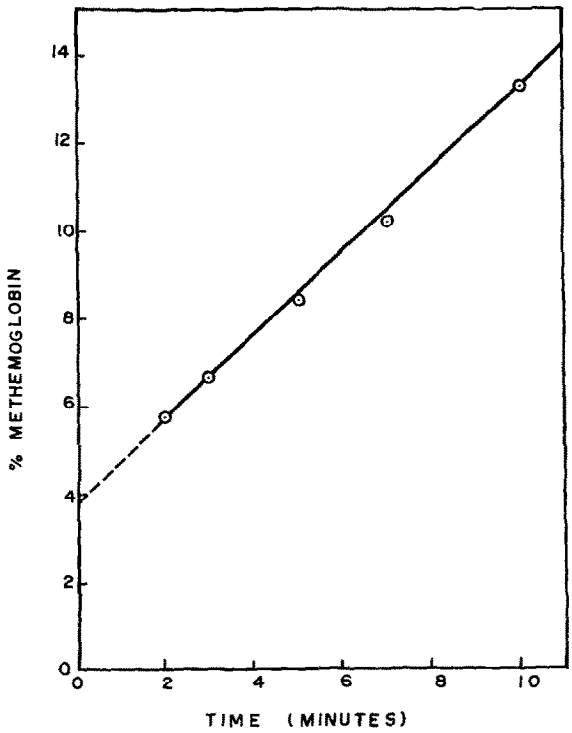


FIG. 2. Initial rate of methemoglobin formation by $1{\cdot}9 \times 10^{-5}$ M DDS-NOH in a 20 per cent suspension of intact erythrocytes incubated in 10 mM glucose at pH 7·4, 25°.

Examination of the kinetics of MHb production in intact erythrocytes showed an initial, very rapid appearance of 3–4 per cent MHb, perhaps formed directly by DDS-NOH and rate-limited only by the time required for diffusion into the erythrocyte (Fig. 2). This was followed by a zero-order production of MHb. The energy of activation for this latter reaction was calculated from the slope of a semilogarithmic plot of rate constant versus reciprocal temperature to be 16·2 Kcal/mole, indicating that the rate-limiting step in this portion of the reaction could well be the enzymatic regeneration of DDS-NOH or reducing compounds.

To discern further the mechanism of this regenerating process, the effect of DDS-NOH on dialyzed hemolysates was examined. A comparison of the first and fourth entries in Table 3 reveals that only about 4 per cent MHb was formed when $1{\cdot}9 \times 10^{-5}$ M DDS-NOH was added to dialyzed hemolysates, an amount which is an order of magnitude less than that observed with undialyzed hemolysates. This represents less than 10 heme equivalents oxidized/mole of DDS-NOH. In the presence of either GSH or an NADPH-regenerating system, the formation of about 30 per cent MHb, representing a high ratio of 60 hemes oxidized/mole of drug, could be restored. NADH was only about one-third as effective as the other compounds.

The effect of DDS-NOH on purified Hb was also examined (Table 4). Incubation of solutions of crystallized Hb at 37° for 2 hr produced 8 per cent MHb, and addition of DDS-NOH alone caused an increase of only 4 per cent MHb. The addition of

TABLE 3. EFFECT OF REDUCING COMPOUNDS ON METHEMOGLOBIN FORMATION BY DDS-NOH IN DIALYZED RED BLOOD CELL HEMOLYSATES*

Additions	% MHb = (MHb)/(Hb_{tot}) × 100
None	2·5
NADH-regenerating system†	2·5
NADPH-regenerating system‡	2·5
DDS-NOH§	6·1
DDS-NOH + GSH (3 mM)	31
DDS-NOH + NADPH-regenerating system	30
DDS-NOH + NADH-regenerating system	11

* Hemolysates (5·3–7·5 g/100 ml of Hb) contained 10 mM glucose. Two-hr incubation at pH 7·4, 37°.

† NADH-regenerating system: horse liver alcohol dehydrogenase, 2×10^{-7} M; ethanol, 174 mM; NAD, 0·4 mM.

‡ NADPH-regenerating system: glucose 6-phosphate, 4 mM; $MgSO_4$, 7 mM; NADP, 0·4 mM; G-6-PD, 1·5 units.

§ DDS-NOH concentration, $1·9 \times 10^{-5}$ M.

TABLE 4. EFFECT OF DDS-NOH ON METHEMOGLOBIN FORMATION IN SOLUTIONS OF PURIFIED HEMOGLOBIN*

Additions	% MHb = (MHb)/(Hb_{tot}) × 100
None	8·0
DDS-NOH†	12
DDS-NOH + GSH (1 mM)	34
DDS-NOH + GSH (2 mM)	39
DDS-NOH + GSH (3 mM)	41
DDS-NOH + GSH (9 mM)	46
DDS-NOH + NADPH (3·6 mM)	37

* Hemoglobin concentration, 3·0–3·7 g/100 ml. Two-hr incubation at pH 7·4, 37°.

† DDS-NOH concentration, $1·9 \times 10^{-5}$ M.

either GSH (3 mM) or NADPH (3·6 mM) alone produced only 2 and 1 per cent increases in MHb respectively. Addition of increasing amounts of GSH to solutions of both Hb and DDS-NOH progressively increased the amount of MHb formed and, with 9 mM GSH, as much as 46 per cent of the Hb was oxidized. The reduced coenzyme NADPH was similarly effective.

These data imply that the HMP shunt may play an important part in the MHb-forming process. Hence, HMP shunt activity was measured in intact erythrocytes in the presence and absence of DDS-NOH by measuring the amount of $^{14}CO_2$ produced from glucose 1-^{14}C. Radiolabeled CO_2 production increased from 889 ± 149 counts/min (three determinations) in the absence of drug to 6200 ± 1555 counts/min in the presence of 3×10^{-4} M DDS-NOH, a relative increase of about 700 per cent.

Hexose monophosphate shunt activity can be reduced by substituting galactose for glucose in the incubation medium, since galactose undergoes considerably less metabolism via the HMP shunt than does glucose under comparable conditions.[10,11] When erythrocytes, which had been freed of detectable glucose by extensive washing

TABLE 5. ERYTHROCYTE METHEMOGLOBIN AND GLUTATHIONE LEVELS AFTER DDS-NOH TREATMENT IN THE PRESENCE OF EITHER GLUCOSE OR GALACTOSE*

Sugar	Additions	% MHb = (MHb)/(Hb_{tot}) × 100	GSH† (mg/100 ml cells)
Glucose (10 mM)			
	No drug	0·4	85·6
	Drug‡	44	85·6
Galactose (50 mM)			
	No drug	0·8	83·3
	Drug	28	16·7

* Red blood cell suspension (7·5 g/100 ml) was incubated for 2 hr at pH 7·4, 37°.
† The GSH concentration of the erythrocytes incubated 2 hr at 37° in the absence of sugars is 82·8 mg/100 ml cells.
‡ Drug (DDS-NOH) concentration, $1·9 \times 10^{-5}$ M.

with normal saline, were incubated with galactose and DDS-NOH, MHb production was reduced substantially when compared with cells incubated with glucose and DDS-NOH. Concomitantly, intracellular GSH levels, while unaffected by the drug in the presence of glucose, decreased to 20 per cent of the control in the presence of galactose (Table 5). A galactose concentration of 50 mM was compared with a glucose concentration of 10 mM because galactose is poorly metabolized by the red cell and these concentrations have been reported to produce similar amounts of ATP and lactate.[10]

"Azoester" has been reported by Kosower *et al.*[12] to be a sulfhydryl-oxidizing reagent that is permeable to intact erythrocytes and specific for GSH. It produces MHb in intact erythrocytes only to the extent of 3–6 per cent when the ratio of azoester to GSH is kept near one.[13] Depletion of GSH by azoester is rapid, requiring only a few minutes, but GSH is also very rapidly generated by glucose-containing cells. If GSH is being utilized to recycle DDS-NOH, the depletion of GSH by azoester would be expected to decrease the MHb production by DDS-NOH.

The usual 2-hr incubation at 37° with DDS-NOH was not feasible in studies utilizing azoester because of the rapid regeneration of GSH by the cells. Glucose-depleted cells in 10 mM galactose were used to further minimize the rate of GSH regeneration. A 15-min incubation was found to be sufficient to permit considerable MHb formation by the drug, but too short to permit regeneration of substantial amounts of GSH (Table 6). Under these conditions, the difference in MHb production between DDS-NOH-treated erythrocytes and drug-free control cells was about 6 per cent MHb when azoester was present and almost 19 per cent MHb in its absence. These data seem to indicate that erythrocytes were less susceptible to MHb formation after depletion of intracellular GSH by azoester.

Hydroxylamines form nucleophilic addition products with the aldehyde carbon of sugars. 4-Amino-4′-hydroxyaminodiphenylsulfone apparently interacts with glucose in this manner, as evidenced by the alteration of the ultraviolet spectrum of DDS-NOH in the presence of glucose. The additional product formed is probably 4-D-glucosylamino-4′-*N*-D-glucosylhydroxyaminodiphenylsulfone.[5] In order to ascertain the effect of this reaction on the production of MHb, DDS-NOH was preincubated with solutions of glucose at 37° for 1 hr. Intact erythrocytes were then added

TABLE 6. EFFECT OF AZOESTER ON THE FORMATION OF METHEMOGLOBIN BY DDS-NOH IN INTACT ERYTHROCYTES*

Azoester	Additions	% MHb = (MHb)/(Hb_{tot}) × 100	GSH (mg/100 ml cells)
No azoester			
	No drug	0·6	78·0
	Drug†	19·4	51·0
Azoester‡			
	No drug	6·6	38·0
	Drug	12·6	22·5

* Red cell suspension (7·8–9·5 g/100 ml of Hb) in 10 mM galactose, pH 7·4, 37°; 15-min incubation.
† DDS-NOH concentration, $1·9 \times 10^{-5}$ M.
‡ Azoester, 2·4 μmoles methylphenylazoformate/ml cells.

and the MHb production was measured. No difference in the extent of MHb formation was observed between unincubated controls and preincubated drug solution. Thus, whether or not DDS-NOH reacted with glucose does not alter the MHb-forming properties of the compound.

If, as the results above seem to implicate, the HMP shunt does play a significant role in the recycling of DDS-NOH, erythrocytes from G-6-PD-deficient individuals should produce less MHb than normal cells. Sixty-min incubations of erythrocytes from normal individuals with $1·9 \times 10^{-5}$ M DDS-NOH produced 48 ± 3·0 per cent MHb (four determinations), while those from individuals with documented G-6-PD deficiency produced only 24 ± 2·1 per cent MHb (three determinations). G-6-PD-deficient blood was obtained from two Negro males (A—variant) and one Caucasian male (B—variant).

DISCUSSION

The mechanism by which primary aromatic amines, represented most simply by aniline, produce MHb has recently been reviewed by Kiese.[1] The *N*-hydroxyl derivatives of these compounds have been shown to be the most important contributors of MHb formation *in vivo*. Heubner[14] showed that phenylhydroxylamine, Hb and O_2 react in a "coupled oxidation," and there is indirect evidence for a free radical mechanism involving the hydroxyl radical, hydrogen peroxide, or both. However, this mechanism is not sufficiently catalytic to product the many equivalents of MHb per mole of phenylhydroxylamine observed with intact red blood cells, and Kiese[1] concluded that phenylhydroxylamine must be regenerated.

When glucose was washed from erythrocytes *in vitro*, phenylhydroxylamine produced only a small amount of MHb. Kiese *et al.*[15] surmised from this requirement for glucose that Hb was being oxidized as part of a cyclic process requiring hydrogen-transferring coenzymes which were produced by the metabolism of glucose in the erythrocyte. Figure 3 summarizes his conclusions, indicating that the nitrosobenzene produced in the coupled oxidation of phenylhydroxylamine and Hb is recycled by an NADPH-dependent reductase, thought to be NADPH-MHb reductase.

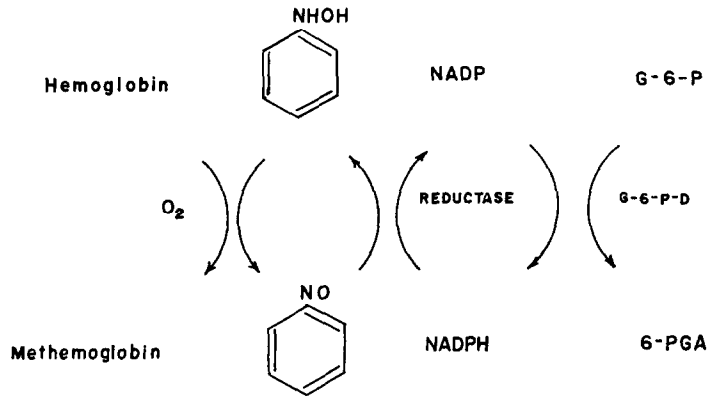


FIG. 3. Proposed scheme for the cyclic regeneration of phenylhydroxylamine after "coupled oxidation" with hemoglobin and oxygen (according to Kiese[1]).

The derivative of the aromatic amine DDS that is analogous to phenylhydroxylamine is DDS-NOH. The fact that the ratio of heme equivalents oxidized/mole of DDS-NOH can approach 100 in intact cells, but is less than 10 in solutions of purified Hb indicates that like phenylhydroxylamine, DDS-NOH is cyclically regenerated in the cell. Similarly, O_2 is required for the process. However, the appearance of these high ratios in purified Hb solutions, devoid of NADPH-MHb reductase activity, when reducing compounds such as GSH or NADPH are added, indicated that the reducing compounds *per se* can regenerate DDS-NOH and the regeneration process does not require an enzyme catalyst.

Unlike phenylhydroxylamine, DDS-NOH produces substantial amounts of MHb (up to 30 per cent) even in cells with no measurable glucose content. Although glucose does enhance MHb formation, it appears that glycolysis plays only a secondary role in the reduction of oxidized DDS-NOH, perhaps by maintaining the intracellular concentration of GSH/or NADPH or of both, which provide the actual reducing equivalents. The concomitant decrease in the GSH levels observed when erythrocytes incubated with galactose or azoester are exposed to DDS-NOH suggests that GSH may be the agent responsible for recycling the oxidized DDS-NOH. However, NADPH could also be a primary source of reducing equivalents. The decrease in GSH levels observed with galactose or azoester-treated cells may be accountable on the basis of competition for NADPH between oxidized DDS-NOH and the regeneration of GSH, catalyzed by the enzyme, NADPH-GSH reductase. While it is not possible to differentiate specifically GSH from NADPH as the compound responsible for recycling oxidized DDS-NOH, these data and the marked stimulation of the HMP shunt produced by DDS-NOH indicate that the enzymatic regeneration of NADPH (HMP shunt) or GSH (through NADPH-GSH reductase) or of both in critically involved in the production of MHb by DDS-NOH, a process which may itself be nonenzymatic. The decreased MHb formation observed in erythrocytes from G-6-PD-deficient individuals is in accord with this viewpoint. It is of interest that the enzyme system, which has generally been considered to be protective against the production of MHb, in this instance enhances its production.

4-Amino-4'-hydroxyaminodiphenylsulfone could produce an increase in MHb

levels by inhibiting the enzymatic reduction of MHb to Hb, but examination of the effect of DDS-NOH on enzyme activities in red blood cell hemolysates showed no inhibition of NADH-MHb reductase (quantitatively the most important enzyme for reducing MHb *in vivo*), making such a mechanism unlikely.

Israili *et al.*[16] have recently isolated a metabolite of Dapsone from a system containing DDS and rat liver microsomes which is capable of producing MHb in intact erythrocytes. It has been identified as DDS-NOH, showing that this compound is indeed a microsomal metabolite of DDS *in vitro*. Hjelm and DeVerdier[3] also have observed that the MHb-forming metabolite in the serum of DDS users is a stimulator of the HMP shunt. The present observation that DDS-NOH is a potent producer of MHb and also a stimulator of HMP shunt activity reinforces several-fold the likelihood that DDS-NOH is indeed produced as a quantitatively minor metabolite of DDS *in vivo*, and that it plays a significant role in the methemoglobinemia observed in persons using the drug Dapsone. The data presented for purified Hb systems and GSH-depleted cells indicate that mechanisms different from those previously published for other phenylhydroxylamines may be operative.

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REFERENCES

1. M. KIESE, *Pharmac. Rev.* **18,** 1091 (1966).
2. W. J. P. NEISH, *Naturwissenschaften* **46,** 535 (1959).
3. M. HJELM and C. H. DEVERDIER, *Biochem. Pharmac.* **14,** 1119 (1965).
4. C. A. WIND, *Pharmacologist* **11,** 240 (1969).
5. E. L. JACKSON, *J. Am. chem. Soc.* **68,** 1438 (1946).
6. A. HAINLINE, *Standard Methods of Clinical Chemistry*, Vol. 5, p. 143. Academic Press, New York (1965).
7. E. BEUTLER, *J. Lab. clin. Med.* **61,** 882 (1963).
8. D. L. DRABKIN, in *The Practical Manual for Clinical Laboratory Procedures*, p. 22. The Chemical Rubber Company, Cleveland (1965).
9. T. K. LI and B. P. JOHNSON, *Biochemistry, N.Y.* **8,** 2083 (1969).
10. D. H. BERRY and P. HOCHSTEIN, *Biochem. Med.* **4,** 317 (1970).
11. E. BEUTLER and C. K. MATHAI, in *Hereditary Disorders of Erythrocyte Metabolism* (Ed. E. BEUTLER), p. 67, Grune & Stratton, New York (1968).
12. N. S. KOSOWER, K. SONG and E. M. KOSOWER, *Biochim. biophys. Acta* **192,** 1 (1969).
13. N. S. KOSOWER, K. SONG and E. M. KOSOWER, *Biochim. biophys. Acta* **192,** 15 (1969).
14. W. HEUBNER, R. MEIER and H. RHODE, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **160,** 149 (1923).
15. M. KIESE, D. REINWEIN and H. D. WALLEL, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **210,** 393 (1950).
16. Z. H. ISRAILI, P. G. DAYTON, J. M. READ and S. A. CUCINELL, *Pharmacologist* **13,** 194 (1971).