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Autoxidation
Bohr effect
2,3-diphosphoglycerate
heme-heme interaction

hemoglobin, fetal and adult
methemoglobin
P₅₀

Oxygen Affinity of Hemoglobins F and A Partially Oxidized to Methemoglobin: Influence of 2,3-Diphosphoglycerate

H. T. VERSMOLD,⁽³⁹⁾ K. FRST, K. BETKE, AND K. P. RIEGEL

Division of Neonatology, Kinderklinik der Universitt, Munich, Federal Republic of Germany

Summary

Oxygen equilibrium curves of partially autoxidized adult (HbA) and fetal hemoglobins (HbF) were determined at 2-75% methemoglobin (met-Hb). Hemoglobin concentration of 5 mM, pH 7.15 (Tris 0.05 M), and temperature of 37 corresponded to intraerythrocytic *in vivo* conditions. With increasing met-Hb fractions the curves became shifted to the left and approached a hyperbolic form: P₅₀ of both HbA and HbF decreased in absence of 2,3-diphosphoglycerate (2,3-DPG) from about 13 to 7 Torr, and Hill's *n* from 2.7 to 1.25. Independent of the met-Hb fraction the alkaline Bohr effect was increased by 2,3-DPG. The allosteric effect of 2,3-DPG on P₅₀ decreased with increasing met-Hb. At all oxidation levels this effect was smaller on HbF than on HbA: At 50% met-Hb P₅₀ of HbF is increased by 0.7 Torr/mM 2,3-DPG increase, P₅₀ of HbA by 1.3 Torr, respectively. There was no evidence of preferential autoxidation of or non- chains of the hemoglobin molecule.

Oxidized hemoglobin does not take part in oxygen transport. In addition a left-shifted, more hyperbolic curve means restricted oxygen unloading from the remaining active hemoglobin. Thus, from application of our results to clinical conditions it must be concluded that sustained methemoglobinemia, despite partial compensation by 2,3-DPG, may interfere with oxygen supply to tissue, particularly in the perinatal period, when HbF prevails.

Speculation

Tissue oxygen supply, as calculated from the present data on O₂ capacity, P₅₀, 2,3-DPG reactivity, Hill's *n*, and Bohr factors, appears to be markedly lowered in methemoglobinemia, particularly in presence of fetal hemoglobin. Reduced tissue oxygen supply in the perinatal period, in addition to a generalized cytochrome *b*₅ reductase deficiency (22), might contribute to irreversible cerebral dysfunction in hereditary methemoglobinemia.

It is well known that the oxygen affinity of partially oxidized HbA is increased (8, 10). However, this pronounced left shift of the hemoglobin oxygen equilibrium curve with increasing fractions of met-Hb *in vitro* was not observed in patients with sustained methemoglobinemia (for references see the reviews of Jaff and Hsieh (14) and Kiese (17)). This discrepancy could have a methodologic cause. The *in vitro* experiments were performed on highly diluted solutions in presence of 0.6 M inorganic phosphate. Both dilution (12) and inorganic phosphate (5) affect hemoglobin oxygen affinity. Moreover, the role of organic phosphates, mainly 2,3-DPG, as important allosteric effectors of hemoglobin, was not taken in account at that time.

More recently partially oxidized hemoglobins have attracted new attention. For instance, the question arose of whether the altered hemoglobin oxygen affinity could be responsible for the

occasionally observed brain damage in hereditary methemoglobinemia (see review, Jaffé and Hsieh (14)). On the other hand, partially oxidized hemoglobins, particularly artificial mixed valency hybrids, served as experimental tools to study the properties of individual subunits within the tetrameric framework of the hemoglobin molecule.

The present study on concentrated hemoglobin solutions was performed to define the influence of 2,3-DPG on the oxygen affinity of partially oxidized solutions of fetal and adult hemoglobin. Red cell 2,3-DPG is known to increase in response to hypoxemia (see review (35)), and this also in methemoglobinemia (21, 32). The allosteric effect of 2,3-DPG is known to be smaller on HbF than on HbA (4, 6, 31), but the interaction of 2,3-DPG with partially autoxidized HbF has not yet been studied. Thus, data obtained under physiologic conditions (1) should provide estimations on the oxygen supply of tissues in newborn and older infants with methemoglobinemia; (2) might yield an information about the *in vivo* relevance of a nonequivalence of chains in hemoglobin oxidation.

PROCEDURES AND METHODS

Human adult and fetal hemoglobins were isolated from adult blood (with informed consent) and placental cord blood according to the method of Jonxis and Huisman (15) by DEAE-Sephadex A-50 chromatography (Tris 0.05 M). HbA₂, eluted at pH 8.1, was discarded. HbA was eluted at pH 7.8. The remaining fetal hemoglobin (HbF₀ and HbF₁) was washed from the resin at pH 6.5. The isolated HbA and HbF were reconcentrated to 30 g/100 ml in 0.05 M Tris, pH 7.15, by ultrafiltration using an Amicon (Oosterhout, Holland) membrane XM 50. The final 2,3-DPG concentration was $<10^{-5}$ M. To prevent met-Hb formation resin, buffers and hemoglobin solutions were equilibrated with oxygen throughout the procedure; temperature during chromatography did not exceed 10°, during ultrafiltration 4°; pH was kept above 7.5 until the final dialysis against 0.05 M Tris, pH 7.15.

Hemoglobins were autoxidized by keeping them aerobically at 37° and pH 7.15 until the desired level of met-Hb was reached. It took approximately 50 hr to reach 75% met-Hb. In random samples no bacterial contamination was found, and single control experiments in presence of 1 µg/ml gentamycin gave normal results. Gentamycin did not influence the rate of autoxidation.

After autoxidation 2,3-DPG was added to final concentrations of 1 mM and 5 mM to two of three aliquots. 2,3-DPG (Boehringer, Mannheim) had been freed of cyclohexyl ammonium ions by cation exchange chromatography (Dowex 50 W-X8, 200–400 mesh, H⁺ form), and titrated to pH 7.15 by NaOH.

The three specimens, containing either no 2,3-DPG, 1 mM 2,3-DPG, or 5 mM 2,3-DPG respectively, were equilibrated simultaneously in Laué tonometers at 37° with water vapor-saturated gas mixtures of known composition (O₂ in N₂). Oxygen content and oxygen capacity (hemoglobins equilibrated with 40% O₂) were measured in triplicate in 0.03 (0.02)-ml samples with a GCM 4 (Beckman Instruments, Munich) gas chromatograph combined with a Natelson microgasometer 600 (Scientific Industries, Springfield, MA) or a Lex-O₂-Con oxygen analyzer (Lexington Instruments Corp., Waltham, MA). Both methods yielded results in perfect agreement (Lex-O₂-Con (ml O₂/100 ml) = 1.0247 GCM (ml O₂/100 ml) - 0.313; *s_{y,x}* = 0.453; *r* = 0.999; range 3–46 ml O₂/100 ml; *n* = 55). O₂ tension and pH were determined by an IL pH/gas analyzer 113 S1 (Instrumentation Laboratories, Boston, MA). Oxygen hemoglobin equilibrium curves were constructed graphically according to the method of Bartels and Harms (1). Their position is expressed by log P₅₀ at pH 7.15 using Bohr factors ($\Delta \log P_{O_2}/\Delta pH$) - 0.39 for 2,3-DPG-free hemoglobin solutions, - 0.43 at 1 mM 2,3-DPG, and - 0.56 at 5 mM 2,3-DPG. These factors had been derived from experiments on specimens buffered with Tris 0.05

M, pH 7.1 and 7.4, at 50% O₂ saturation and were found not significantly influenced by met-Hb (in accordance with the data of Enoki *et al.* (10)) or the hemoglobin type. These data agree well with those of Bauer (2). Met-Hb was determined in duplicate spectrophotometrically according to the method of Evelyn and Malloy (11). Independently it was calculated from the "active" to total hemoglobin ratio. The active hemoglobin concentration was derived from the oxygen capacity divided by the Hüfner factor, which was 1.38 ± 0.02 (milliliters per g) in adult and cord blood hemoglobin.

One preparation of HbA (5 mM; pH 7.15) has been autoxidized to about 50% met-Hb at 37° under three different conditions: (I) aerobically, (II) at a pO₂ of about 10 Torr, and (III) aerobically in presence of 5 mM 2,3-DPG. The oxidized samples I and II were then examined in absence of 2,3-DPG and after addition of 5 mM 2,3-DPG, as compared to sample III which already contained 5 mM 2,3-DPG.

The effect of 0.6 M inorganic phosphate pH 7.15 on P₅₀ of HbA (*n* = 14) and HbF (*n* = 16) was determined by the same procedure at low and high met-Hb fractions. For these experiments an ultrafiltration of the isolated HbA and HbF against the phosphate buffer was performed before concentration of the hemoglobins.

RESULTS

OXYGEN AFFINITY

The effects of met-Hb and 2,3-DPG on P₅₀ of HbA and HbF at physiologic red cell hemoglobin concentration of 5 mM, pH 7.15, and 37° are illustrated in Figure 1 as semilogarithmic plots.

P₅₀ of the "stripped" (2,3-DPG-free) HbA in absence of met-Hb is approximately 12.5 Torr, at 50% met-Hb 10 Torr, and at 75% met-Hb only 7.5 Torr. When 2,3-DPG is added, the corresponding P₅₀ values are 15.0, 11.7, and 8.5 Torr at 1 mM 2,3-DPG, and 24.4, 16.5, and 11.5 Torr at 5 mM 2,3-DPG, respectively. It can be seen that: (1) hemoglobin autoxidation reduces P₅₀; this reduction is enhanced above 50% met-Hb, thus linear regressions relating log P₅₀ to met-Hb are flatter at 2–50% met-Hb than above 50% met-Hb (see Table 1 and Fig. 1) and that (2) increasing autoxidation of HbA reduces the allosteric effect of 2,3-DPG. In absence of met-Hb there is a $\Delta P_{50}/\Delta 2,3\text{-DPG}$ ratio of 2.4 Torr/mM, at 50% met-Hb of 1.3 Torr/mM, and at 75% met-Hb of 0.9 Torr/mM.

Compared to these results changes observed with HbF are as follows: (1) P₅₀ of "stripped" HbF is not significantly different from that of HbA; in the absence of met-Hb P₅₀ averages 13.2 Torr, at 50% met-Hb 9.8 Torr, and at 75% met-Hb 6.5 Torr. There are also different slopes below and above 50% met-Hb (Table 1). (2) The allosteric effect of 2,3-DPG on HbF is smaller than on HbA. The $\Delta P_{50}/\Delta 2,3\text{-DPG}$ ratio in HbF is similarly affected by autoxidation than in HbA: At low met-Hb levels this ratio is approximately 1.1, at 50% met-Hb 0.7, and 75% met-Hb 0.5 Torr/mM. These figures are 45, 54, and 56% of the respective HbA values.

Table 2 shows the results of HbA autoxidation to about 50 percent met-Hb at (I) pO₂ 140 Torr (Control), (II) pO₂ about 10 Torr, and (III) if autoxidation occurred in presence of 5 mM 2,3-DPG at pO₂ 140 Torr. No differences were observed in this series with regard to P₅₀ and $\Delta P_{50}/\Delta 2,3\text{-DPG}$ when oxidation had occurred at low pO₂, a condition which should enhance α chain autoxidation. Furthermore, no differences in P₅₀ were found when autoxidation had occurred in presence of 2,3-DPG, a condition which should enhance β chain autoxidation, as compared to autoxidation and subsequent addition of 2,3-DPG.

Oxygen affinity of HbA or HbF in 0.6 M phosphate buffer pH 7.15 was on the order of that found in 0.05 M Tris in the presence of 1 mM 2,3-DPG. At 9% met-Hb ($8.7 \pm 1.0\%$; *n* = 8), mean P₅₀ of HbA was $14.5 (\pm 0.8)$ Torr, and at 68% ($67.7 \pm 1.5\%$; *n* = 6) $9.9 (\pm 0.4)$ Torr. Corresponding values of HbF

Fig. 1. Dependence of $\log P_{50}$ (pH 7.15; 37°) of partially autoxidized HbA and HbF on the percentage of met-Hb in absence of 2,3-DPG ($<10^{-5}$ M) ($\blacktriangle$) and in presence of 1 mM (O) and 5 mM 2,3-DPG ($\bullet$). Regression lines were calculated for data below 50% met-Hb and above 50% met-Hb (see regression constants in Table 1). Hemoglobin concentration 5 mM (tetramer) in Tris 0.05 M, pH 7.15, corresponding to *in vivo* conditions.

Table 1. Oxygen affinity of HbA and HbF during autoxidation¹

Hb type	met-Hb range, %	2,3-DPG, mM	n	$b_{y \cdot x} \times 100$	a	r	$s_{y \cdot x}$	2P (b $\neq$ b)
A	0-55	0	27	-0.2045	1.1140	-0.813	0.0248	<0.001
		1	26	-0.2361	1.1925	-0.889	0.0200	
		5	30	-0.4017	1.4058	-0.921	0.0296	
	40-80	0	10	-0.4690	1.2334	-0.879	0.0330	<0.01
		1	8	-0.4668	1.3047	-0.967	0.0154	
		5	12	-0.4700	1.4293	-0.896	0.0303	
F	0-55	0	13	-0.2747	1.1286	-0.944	0.0176	<0.05
		1	13	-0.2863	1.1712	-0.922	0.0221	
		5	13	-0.2961	1.2825	-0.920	0.0232	
	40-80	0	13	-0.4935	1.2313	-0.597	0.0497	
		1	13	-0.4345	1.2336	-0.729	0.0305	
		5	12	-0.5065	1.3595	-0.902	0.0190	

¹ Regression constants of the correlation $\log P_{50}$ (Torr) vs. methemoglobin (percent) in presence of different concentrations of 2,3-DPG at 37°, pH 7.15; $\log P_{50}$ (Torr) = $b \times \text{met-Hb (percent)} + a$.

Table 2. P_{50} and 2,3-DPG reactivity of HbA after autoxidation at low O_2 tension and in presence of 2,3-DPG

Conditions during autoxidation ¹				Conditions at examination			Results	
Sample	pO_2 , Torr	2,3-DPG, mM	pH	Sample	met-Hb, %	2,3-DPG mM	P_{50} (pH 7.15; 37°), Torr	$P_{50}(\text{DPG})/P_{50}(\text{no DPG})$
I	140	0	7.15	Ia	55.0	0	8.0	1.89 (Ib/Ia)
				Ib	55.0	5	15.1	
II	10	0	7.15	IIa	46.2	0	8.3	1.92 (IIb/IIa)
				IIb	46.2	5	15.9	
III	140	5	7.14	III	46.3	5	15.5	1.94 (III/Ia)

¹ Further conditions as given in the text.

($n = 8$) were 15.0 ± 0.5 Torr (at $10.8 \pm 3.5\%$ met-Hb) and 9.6 ± 0.4 Torr (at $64.8 \pm 3.3\%$ met-Hb).

to detect differences between 2,3-DPG-free and 2,3-DPG-substituted hemoglobin solutions with regard to cooperativity.

HEME-HEME-INTERACTION

Besides the left shift of the hemoglobin oxygen equilibrium curve with increasing hemoglobin oxidation a transformation of the curve to a less sigmoid shape occurs (8, 10, 24). As is shown in Figure 2, changes of Hill's n with autoxidation in HbA and HbF are not significantly different. In unoxidized samples Hill's n is approximately 2.7, at 50% met-Hb 1.5, and at 75% met-Hb 1.25. Our methods were not sensitive enough

DISCUSSION

The results obtained on 2,3-DPG-free HbF are not significantly different from those on HbA. Upon autoxidation hemoglobin oxygen affinity increases and the heme-heme interaction decreases, both in a nonlinear fashion. P_{50} differences between HbA and HbF were seen in presence of 2,3-DPG. At all oxidation levels the allosteric effect of 2,3-DPG on HbF was smaller than that on HbA. The effect of 2,3-DPG on P_{50} of

Fig. 2. Dependence of cooperativity (Hill's n) of partially autooxidized HbA (●) and HbF (○) on the percentage of met-Hb. Conditions as in Figure 1.

HbA and of HbF decreased with increasing met-Hb. These findings shall be discussed with respect to a nonequivalence of chains in the hemoglobin tetramer and to the implications on oxygen transport.

STRUCTURE-FUNCTION RELATIONS IN HEMOGLOBIN OXIDATION

The functional properties of partially oxidized hemoglobins and their response to allosteric effectors should reflect the relation between structure and function of the hemoglobin molecule. Partially oxidized hemoglobin is a mixture of hemoglobin molecules oxidized to different degrees (13) with different functional properties (20). Fully oxidized molecules do not participate in reversible oxygen binding. At least one unoxidized heme is needed to react with oxygen, and at least two for cooperative binding of oxygen and 2,3-DPG.

In half-oxidized hemoglobins dissociation of ligand from the ferrous subunits causes a change from the quaternary oxy- or R structure to the deoxy- or T structure. That change, favored by 2,3-DPG, causes the tertiary r structure of the ferric subunits to alter (28–30). This concept would provide an explanation for the well preserved effect of 2,3-DPG on P_{50} of our partially autooxidized hemoglobins, irrespective of a functional heterogeneity of chains.

The functional properties of half-oxidized hemoglobins and their reactivity to 2,3-DPG depend, furthermore, on whether they exist mainly as α -met hybrids ($\alpha_2\beta_2$) or as β -met hybrids ($\alpha_2\beta_2$). A functional heterogeneity of chains in hemoglobin oxidation has been observed on artificial mixed valency hybrids at low hemoglobin concentrations. Those hybrids with oxidized α chains show a more pronounced 2,3-DPG binding than do the hybrids with oxidized β chains (3) at 5×10^{-5} M Hb (tetramer). Furthermore the 2,3-DPG effect on P_{50} of the α -met hybrid is stronger than on the P_{50} of the β -met hybrid (24) at a hemoglobin concentration of 5×10^{-5} M (heme equivalents).

A preferential autooxidation of α chains of oxyhemoglobin A has been demonstrated by Mc Quarry and Gibson (23), Mansouri and Winterhalter (25, 26), and Edelstein and Gibson (9). It has to be stated, however, that our observations on concentrated partially autooxidized hemoglobins do not allow a definite evaluation as to whether α or non- α chains (β or γ chains) are oxidized preferentially, because our data were obtained from a mixture of hemoglobin molecules oxidized to different degrees, and are therefore not directly comparable to pure mixed valency hybrids. Furthermore, under the conditions of our study, an exchange of hemes among hemoglobin molecules (7) will affect

the distribution of oxidized hemes on α and non- α chains. Therefore, our functional data should not reflect the preferential autooxidation of any chain type, but rather a final state of equilibrium.

An enhancement of preferential α chain autooxidation has been described in presence of 2,3-DPG (26), or inositol hexaphosphate (9) while autooxidation at low pO_2 is speeding up β chain oxidation (26). We therefore compared the functional properties of HbA after partial autooxidation in presence of 2,3-DPG to those after partial autooxidation at low pO_2 . Under these conditions, which should yield a relatively high fraction of oxidized α chains or β chains, respectively, no functional differences have been observed (Table 2), probably because of the redistribution of oxidized hemes mentioned above. These data indicate that *in vivo* the P_{50} of partially autooxidized hemoglobins is close to the presented values even when autooxidation occurs under the most extreme variations of red cell conditions.

OXYGEN TRANSPORT IN METHEMOGLOBINEMIA

The following parameters impair oxygen unloading from blood to the tissues in methemoglobinemia: (1) the diminished oxygen capacity, (2) the left shift of the oxygen equilibrium curve, and (3), to a certain extent, the reduced heme-heme interaction. In addition, otherwise reliable compensatory mechanisms must be considered insufficient. Cardiac output (other than in anemia) fails to respond substantially (27) to methemoglobinemia. Erythropoiesis, too, responds poorly to methemoglobinemia: the increase of total hemoglobin levels is smaller than in comparable arterial hypoxemia (16, 32).

Methemoglobin Distribution and Oxygen Affinity. A left shift of the blood oxygen dissociation curve in methemoglobinemia will occur only if met-Hb is not completely segregated on single red cells (27). In NADH met-Hb reductase deficiency an unequal distribution, although not an all-or-none distribution of met-Hb on red cells (16, 19) has been demonstrated by the elution technique of Kleihauer and Betke (18), while the toxic methemoglobinemia met-Hb is more equally distributed (19). An unequal distribution of partially oxidized hemoglobin on red cells should result in a wide range of P_{50} values among single red cells, with a tendency towards normal of the mean blood P_{50} (16).

2,3-DPG and Oxygen Affinity. According to the present data increased red cell 2,3-DPG concentrations as seen in methemoglobinemic patients (21, 32) are "correcting" the position of the left shifted blood oxygen equilibrium curve back towards normal. Indeed, in spite of 47% met-Hb in a 1-year-old patient with NADH met-Hb reductase deficiency, we observed (32) an only moderately decreased blood P_{50} value of 24.8 Torr (pH 7.4, 37°; normal children of comparable age 28.4 ± 1.1 ($n = 11$)), which was well explained by a 2,3-DPG level increased to 7.9 mM (normal children 4.4 ± 0.9 ($n = 17$)). Different red cell 2,3-DPG concentrations and possibly an unequal distribution of met-Hb may explain the conflicting reports on decreased or even normal P_{50} values in this disease.

These changes, however, do not outweigh the reduced O_2 capacity; all O_2 equilibrium curves determined as yet in hereditary methemoglobinemia—with a low or a "normal" P_{50} —have in common a shift far to the left in comparison to reference curves of equally anemic subjects. The "correction" of P_{50} in methemoglobinemia is less pronounced in the presence of HbF, i.e., in early infancy.

2,3-DPG and Methemoglobin Formation. Excess 2,3-DPG may be a disadvantage in hereditary methemoglobinemia: there is evidence that met-Hb formation is enhanced by 2,3-DPG (26, 32–34). This is irrelevant under normal conditions, since the met-Hb reducing capacity exceeds the demands some 150-fold. In hereditary NADH met-Hb reductase deficiency, however, an increased rate of met-Hb formation will result in an increase of the steady state met-Hb level in the red blood cell.

Hemoglobin Function and Tissue Oxygenation. Oxidized hemoglobin does not take part in oxygen transport. In addition the left-shifted, more hyperbolic curve means restricted oxygen unloading from the remaining active hemoglobin. Both factors together interfere with oxygen supply to tissues, the more since cardiocirculatory adaptations are poor in methemoglobinemia (27). A quantitative estimate of the deficit of tissue oxygenation in methemoglobinemia may be derived from Figure 3. It shows the dependency of a fictive arterial venous oxygen difference ($AVDO_2$) upon the degree of hemoglobin autooxidation at different levels of RBC 2,3-DPG, taking into account the quantitative and qualitative changes of hemoglobin function. As can be seen, these fictive $AVDO_2$ values are considerably lowered in methemoglobinemia, particularly in the presence of fetal hemoglobin (Fig. 3b). Obviously, such impairment could interfere with growth and development of the brain and other organs.

Whether altered functional properties of hemoglobin are the predominant factor leading to brain damage in hereditary methemoglobinemia, cannot be derived from these figures which neglect the—although limited—*in vivo* adaptive mechanisms mentioned above. Recently Leroux *et al.* (22) demonstrated a generalized cytochrome b_5 reductase deficiency in non erythroid tissues in a patient with hereditary methemoglobinemia with mental retardation. In addition to reduced tissue oxygen supply such a defect may be the biochemical basis for irreversible cerebral dysfunction.

CONCLUSION

In solutions of hemoglobin, at 5 mM concentration, temperature of 37° and pH 7.15, corresponding to *in vivo* conditions, quantitative data on the oxygen transport function of blood in methemoglobinemia were determined for HbA and HbF, respectively. The effects of 2,3-DPG were evaluated with respect to a possible influence on the oxidation pattern of α and non- α (β and γ) chains within the hemoglobin tetramer, and to its influence on the O_2 -carrying characteristics of partially oxidized HbA and HbF.

Fig. 3. Oxygen unloading from adult hemoglobin and from fetal hemoglobin at different 2,3-DPG concentrations depending upon the level of methemoglobin (abscissa). $AVDO_2$ (ordinate) is the fictive arteriovenous O_2 difference. Constants: total hemoglobin concentration 16 g/100 ml blood, red cell pH 7.15, temperature 37°, arterial PO_2 80 Torr, mixed venous PO_2 35 Torr.

No evidence was obtained regarding a preferential autooxidation of α or non- α chains. P_{50} , Hill's n , and 2,3-DPG reactivity of HbA and HbF decreased with increasing fractions of met-Hb. The Bohr effect remained unchanged. Evidently the reduced oxygen capacity in methemoglobinemia is of particular significance regarding the O_2 unloading capacity of blood. In contrast to the adaptive P_{50} increase in a comparable anemia, P_{50} in methemoglobinemia is considerably lower; elevated 2,3-DPG levels do not favor blood oxygen release to the same extent as in anemia.

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39. Requests for reprints should be addressed to: H. T. Versmold, M.D. University of California, San Francisco, Cardiovascular Research Institute and Department of Pediatrics, San Francisco, CA 94143 (USA).
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Corrigendum

In the article titled "Methylenetetrahydrofolate Reductase in Cultured Human Cells. I. Growth and Metabolic Studies" by David S. Rosenblatt and Richard W. Erbe in the November 1977 issue of *Pediatric Research*, formula 2, which appeared on p. 1137, was published in incomplete form. It should have appeared as follows:

5-Methyl-H₄PteGlu + L-homocysteine

H₄PteGlu + L-methionine (2)