Hemoglobin Concentration Overestimates Oxygen Carrying Capacity of Blood in Phenylhydrazine-Induced Hemolytic Anemia in Dogs[1] (37510)

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Acute hemolytic anemia in patients with red blood cells deficient in enzymes such as G-6-PD is characterized by excessive oxidation of hemoglobin (Hb) to a variety of soluble heme pigments and to precipitates, attached to the inner surface of the red cell membrane, known as Heinz bodies (1). These pigments and precipitates do not carry oxygen. The cyanmethemoglobin method, widely used to determine blood Hb concentration, detects not only Hb but methemoglobin and other non-oxygen carrying heme pigments as well; sulfhemoglobin is not detected by this method (2). The present study tested the hypothesis that non-oxygen carrying pigments might constitute a significant fraction of "hemoglobin," as measured by the cyanmethemoglobin method, during the course of acute hemolytic anemia induced in dogs by phenlyhydrazine, a potent oxidizing agent. Measured blood oxygen carrying capacity ($C_{max_{O_2}}$) was less than the $C_{max_{O_2}}$ expected from Hb in anemic dogs. The data thus confirm the hypothesis. Additional studies indicate that Heinz bodies constituted a major fraction of the non-O_2 carrying "hemoglobin."

Materials and Methods. Eight adult mongrel dogs of both sexes weighing from 10 to 21 kg were injected intravenously twice with phenylhydrazine hydrochloride (Fisher Scientific Co.), 20 mg/kg body weight, as a 38–44 mg/ml solution in 0.9 mEq/liter NaCl. Dogs 1 through 6 were injected on Days 0 and 2 and dogs 7 and 8 on Days 0 and 3 (Table I). Two other dogs, C_1 and C_2 in Table I, were injected with saline alone on Days 0 and 2. Venous blood was drawn before the first injection and then daily, if possible, for determination of Hb and $C_{max_{O_2}}$. The $C_{max_{O_2}}$ of Hb in aqueous solution was studied 7 days after the first of 2 injections in 6 additional dogs.

Oxygen content of whole blood and of Hb in aqueous solution was measured by the micromethod of Roughton and Scholander (3) following equilibration with room air for 10 min in a rotating flask tonometer. The oxygen content of the thus fully saturated hemoglobin, or oxygen carrying capacity, was expressed as dry gas at 0° and 760 mm Hg. Hemoglobin was measured by the cyanmethemoglobin method (2). Methemoglobin and sulfhemoglobin were estimated by the method of Evelyn and Malloy (4). Aqueous solutions of hemoglobin were prepared according to the method of Mann and Romney (5), with the single exception that dialysis was carried out in 0.10 *M* potassium phosphate buffer with a pH of 7.5 rather than 7.1. One aliquot of thrice-washed, lysed red cells was dialyzed without centrifugation, and is referred to as the *unspun* solution. A second aliquot was centrifuged at 45,000 rpm at 4° for 30 min. The supernatant was then dialyzed and centrifuged as before. The final supernatant is termed the *spun* solution.

Determinations of Hb and $C_{max_{O_2}}$ were done in duplicate. The average percentage variation of individual values from the mean was 0.51 ± 0.13 (SE) for Hb and 0.91 ±

[1] This study was supported by the Twenty-Five Club, a Woman's Auxiliary of the Magee-Womens Hospital.

[2] Recipient of National Institutes of Health Research Career Development Award K3 HD 18,321.

TABLE I. Maximal Decreases in Blood Hemoglobin Concentration and Oxygen Carrying Capacity Following Injection of Phenylhydrazine.

	Hemoglobin concn				Oxygen carrying capacity			
Dog no.	Control (Day 0) (g/100 ml)	Day lowest (no.)	Lowest (g/100 ml)	Lowest (% of control)	Control (Day 0) (ml/100 ml)	Day lowest (no.)	Lowest (ml/100 ml)	Lowest (% of control)
1	16.7	8, 13, 14	9.2	55	20.4	8	6.6	32
2	12.6	8	8.4	67	14.8	8	5.7	38
3	13.9	9	5.4	39	17.5	9	4.8	27
4	16.3	6	10.3	63	19.9	5	13.3	67
5	17.6	7	10.2	58	21.7	5	9.2	42
6	17.6	7	9.3	53	21.7	5, 7	9.2	42
7	17.6	9	13.3	76	24.0	9	14.9	62
8	14.0	8	7.0	50	17.4	8	7.0	40
Mean				58				44
C_1[a]	13.9	5	13.6	98	18.6	1	18.6	100
C_2[a]	14.2	14	14.8	104	17.7	14	18.6	105

[a] Injected with saline.

0.25 for $C_{max_{O_2}}$.

The mean oxygen binding capacity of hemoglobin ($C_{max_{O_2}} \div$ Hb) was 1.26 ± 0.02 ml/g prior to injection of the dogs with phenylhydrazine. We are not able to explain the discrepancy between this value and the traditional value of 1.34 ml/g.

Results. The extent of the dogs' reaction to phenylhydrazine was variable and unrelated to the severity of anemia. Three dogs remained in apparently good health; the other 5 were lethargic and anorectic for about 1 wk following injection of phenylhydrazine and then recovered coincident with increase in blood hemoglobin concentration. Four of these 5 dogs were severely ill with vomiting and weakness. Venous blood always had a brown hue during the period of declining Hb.

Each dog began to develop anemia, as indicated by decreases in both Hb and $C_{max_{O_2}}$, within 24–48 hr of the first injection of phenylhydrazine. Anemia was most profound 5–9 days after injection; many peripheral red blood cells contained Heinz bodies at that time. Lowest Hb values ranged from 39–76% of control (mean, 58%) and lowest $C_{max_{O_2}}$ values ranged from 27 to 67% of control (mean, 44%) (Table I). In 7 of the 8 dogs $C_{max_{O_2}}$ fell relatively more than Hb; in dog No. 4 (Table I) the relative fall in $C_{max_{O_2}}$ was slightly less than that of Hb. Both Hb and $C_{max_{O_2}}$ returned to control values 2.5–3 wk following injection. In the 2 dogs injected with saline alone Hb and $C_{max_{O_2}}$ varied insignificantly during 2 wk periods of study.

Figure 1 shows the time-course of the decreases in Hb and $C_{max_{O_2}}$ following injection of a 15 kg dog (No. 2 in Table I) with phenylhydrazine on Days 0 and 2. The lowest values of Hb and $C_{max_{O_2}}$ and the maximal discrepancy between measured $C_{max_{O_2}}$ and that expected from Hb coincided on Day 8. On that day $C_{max_{O_2}}$ (5.7 ml/100 ml) was but 54% of the expected value (10.6 ml/100 ml) calculated from Hb (8.4 g/100 ml) and Hb oxygen binding capacity (1.26 ml/g). These data are typical in that $C_{max_{O_2}}$ fell relatively more than Hb and in that this relative difference was most striking when the functional anemia (decreased $C_{max_{O_2}}$) and apparent anemia (decreased Hb) were most marked.

In Table II the lowest measured $C_{max_{O_2}}$ for each dog is expressed as a percentage of the $C_{max_{O_2}}$ expected that day from the Hb. The day of study on which lowest Hb

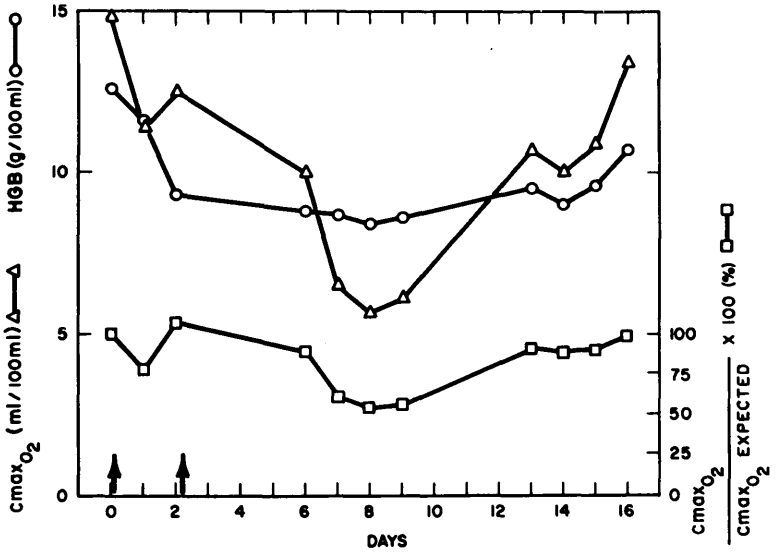

FIG. 1. $C_{max_{O_2}}$ decreased relatively more than Hb in a 15 kg dog (No. 2, Table I) injected with phenylhydrazine on Days 0 and 2 (arrows). Maximal discrepancy between measured $C_{max_{O_2}}$ and that expected from Hb coincided with lowest $C_{max_{O_2}}$ and Hb values on Day 8.

and $C_{max_{O_2}}$ values were noted coincided in 6 of the 8 dogs. Measured $C_{max_{O_2}}$ ranged from 51 to 93% (mean, 69%) of expected. In other words, from 7 to 49% (mean, 31%) of the pigments detected as Hb did not carry oxygen. Hb thus overestimated the actual $C_{max_{O_2}}$ by an average of 45% (range 8–96%).

The hypothesis that Heinz bodies might contribute substantially to the non-oxygen carrying pigments detected as Hb was tested by the following observations on 6 additional dogs. Seven days following the first of 2 injections of phenylhydrazine, mean values

TABLE II. Lowest Measured Blood Oxygen Carrying Capacity versus Oxygen Carrying Capacity Expected from Hemoglobin Concentration.

			Oxygen carrying capacity		
Dog no.	Day no.	Hb concn (g/100 ml)	Expected[a] (ml/100 ml)	Measured (ml/100 ml)	(Measured/expected) × 100 (%)
1	8	9.2	11.7	6.7	51
2	8	8.4	10.6	5.7	54
3	9	5.4	6.8	4.8	66
4	5	11.3	14.2	13.3	93
5	5	12.4	15.6	9.2	59
6	5	11.4	14.4	9.2	64
7	9	13.3	16.7	14.9	85
8	8	7.0	8.8	7.0	79
Mean					69
C_1[b]	1	14.6	18.4	18.6	101
C_2[b]	14	14.8	18.6	18.6	100

[a] Hemoglobin concentration × control hemoglobin oxygen binding capacity (1.26 ml/g).
[b] Injected with saline.

of whole blood Hb and $C_{max_{O_2}}$ were, respectively, 64 and 51% of control; $C_{max_{O_2}}$ values ranged from 75 to 92% of those expected from Hb, with a mean of 84%. Centrifuged and uncentrifuged aqueous solutions of hemoglobin were prepared from these samples of whole blood following lysis of the red cells. $C_{max_{O_2}}$ values for the *unspun* solutions ranged from 80 to 88% of those expected from Hb, with a mean of 85%, and thus agreed closely with the findings on whole blood. Heinz body-containing red cells ghosts were abundant on stained smears of the *unspun* solution. In the *spun* solution $C_{max_{O_2}}$, as percentage of $C_{max_{O_2}}$ expected from Hb, was greater than in the *unspun* solution for each dog (Fig. 2). The mean values for the $C_{max_{O_2}}$, as percentage of expected $C_{max_{O_2}}$, was 96% for the *spun* solutions, as compared to the mean value of 85% for the *unspun* solutions. Only very rare red cell ghosts could be detected in stained smears of the supernatant solution. Centrifugation, which removed most of the cellular debris and Heinz bodies from the Hb solution, thus also removed about 73% of the non-oxygen carrying pigments detected as Hb from the solution. In aqueous Hb solutions prepared from the blood of these dogs before they were injected with phenylhydrazine, measured $C_{max_{O_2}}$, as percentage of expected, increased but an average of 1.6% as compared to a mean increase of 13% noted when the dogs were anemic.

Discussion. It is clear from the present data that Hb, as measured by the cyanmethamoglobin method, underestimates the functional severity of the acute hemolytic anemia that occurs in dogs after administration of phenylhydrazine. The presence of non-oxygen carrying pigments detected as Hb by the cyanmethemoglobin method seems the most likely explanation for the discrepancy between measured $C_{max_{O_2}}$ and $C_{max_{O_2}}$ expected from Hb in whole blood. We also considered two unlikely mechanisms that might account for some of the discrepancy: (a) gross shift of the upper portion of the oxygen dissociation curve to the right, and (b) reduction of permeability of the red cell coat to oxygen secondary to injury by phenylhydrazine, either directly or by alterations in the red cell membrane at sites of attachments of Heinz bodies (6). Both mechanisms were ruled out by comparing the $C_{max_{O_2}}$ of blood of anemic dogs after equilibration with 50–90% O_2 with $C_{max_{O_2}}$ after equilibration with room air. The slightly greater $C_{max_{O_2}}$ after equilibration with 50–90% O_2 than with room air was entirely accounted for by oxygen dissolved in plasma. Further evidence against reduced oxygen passage across the red cell membrane during anemia is provided by the similar discrepancies between measured and expected $C_{max_{O_2}}$ of whole blood and hemoglobin solution in the second series of dogs.

The data strongly suggest that Heinz bodies constitute a large fraction of the non-oxygen carrying pigments detected as Hb. This interpretation is based on a 13% increase in the ratio of measured/expected $C_{max_{O_2}}$ of Hb in solution after removal of most of the cellular debris, including Heinz bodies, that remained after cell lysis. It is strengthened by the observation that removal of cellular debris by ultracentrifugation from lysed control blood of dogs used in this phase of the study was associated with an increase of but 1.6% in the ratio of measured/expected $C_{max_{O_2}}$ of the Hb solution, thereby excluding an important nonspecific effect of removal of cellular debris on Hb oxygen binding capacity.

Jacob (7) has reviewed current concepts of the formation of Heinz bodies in patients with congenital hemolytic anemia associated with unstable mutant hemoglobins. Most unstable hemoglobins (*e.g.*, Köln, Zurich and Genova) are associated with heme-deficient Heinz bodies. Loss of mutant β chains from the heme pocket of the globin molecule is thought to initiate hemoglobin denaturation, with subsequent decrease in heme binding, heme depletion and ultimate precipitation of insoluble heme-depleted β chains as Heinz bodies. On the other hand, Heinz bodies found in the blood of patients with the unstable hemoglobins Philly ($\beta^{35\ \text{tyrosine}\rightarrow\text{phenylalanine}}$) and Riverdale-Bronx contain much heme. In these cases loss of α and β monomers from a region on the globin molecule where α and β chains make contact is postulated to be the cause of hemoglobin instability and to

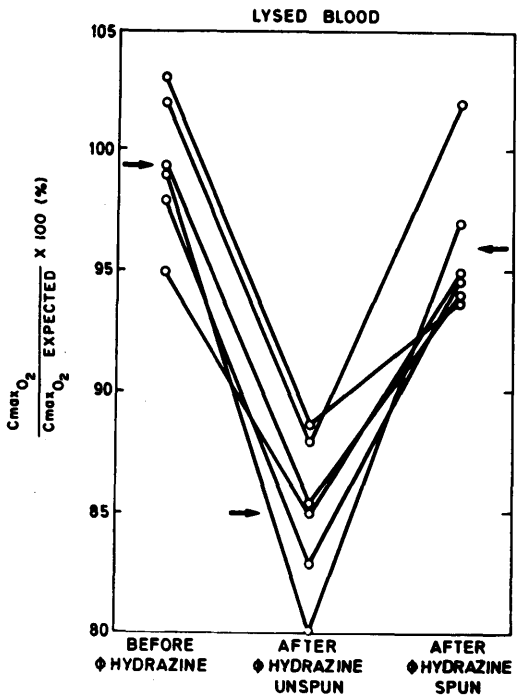

FIG. 2. Measured $C_{max_{O_2}}$ was always more nearly that expected from Hb in centrifuged than in uncentrifuged aqueous solutions of lysed blood of anemic dogs on the seventh day after the first of 2 injections of phenylhydrazine. Arrows indicate mean values.

result in denaturation of hemoglobin to heme-containing Heinz bodies. In the present study Heinz bodies contained about three-quarters of the oxidized (non-O_2 carrying) heme. We speculate therefore that phenylhydrazine disrupts stable canine hemoglobin at a site or sites other than the heme pocket of the globin molecule.

Concentrations of methemoglobin and sulfhemoglobin were measured in the venous blood of 2 dogs with marked anemia. Elevation of methemoglobin levels have been reported following injection of monomethylhydrazine into animals (8) and was anticipated here because of the brownish tinge regularly noted in venous blood as anemia developed. In one dog methemoglobin and sulfhemoglobin each represented 2% of total hemoglobin, and in the other each compound represented 4% of total hemoglobin. Concentrations of met- and sulfhemoglobin were $<0.25\%$ of total hemoglobin in the blood of these dogs prior to injection of phenylhydrazine. It will be recalled that sulfhemoglobin is not detected by the Hb method we used. Carbonmonoxyhemoglobin, which may constitute a few percent of total hemoglobin during severe hemolysis (9), was not measured. The methemoglobin levels were sufficient (perhaps fortuitously) to account for the non-oxygen carrying pigments remaining after removal of Heinz bodies by centrifugation. Our data on the concentration of soluble, non-oxygen carrying pigments in these dogs are too sparse to do other than point the way for future studies.

The acute experimental hemolytic anemia we studied is similar to that experienced by G-6-PD deficient patients exposed to an oxidant such as Primaquin both as to time-course and severity (10). The experimental and human hemolytic anemias are probably dissimilar at least to the extent that Heinz bodies, which our data indicate to constitute a major portion of the non-oxygen carrying apparent Hb, are more abundant in dogs than in man (11). We are currently studying the relevance of the present observations to the routine laboratory assessment of patients with acute hemolytic anemia secondary to red cell enzyme deficiencies.

1. Jandl, J. H., Engle, L. K., and Allen, D. W., J. Clin. Invest. **39**, 1818 (1960).

2. O'Brien, D., Ibbott, F. A., and Rodgerson, D. O., "Laboratory Manual of Pediatric Micro-Biochemical Techniques," p. 167. Harper & Row, New York (1968).

3. Roughton, F. J. W., and Scholander, P. F., J. Biol. Chem. **148**, 541 (1943).

4. Evelyn, K. A., and Malloy, H. T., J. Biol. Chem. **126**, 655 (1938).

5. Mann, L. I., and Romney, S. L., Amer. J. Obstet. Gynecol. **101**, 520 (1968).

6. Rifkind, R. A., and Danon, D., Blood **25**, 885 (1965).

7. Jacob, H. S., Seminars Hematol. **7**, 341 (1970).

8. Clark, D. A., and Fortney, S. R., Aerosp. Med. **38**, 1230 (1967).

9. Coburn, R. F., Williams, W. J., and Kahn, S. B., J. Clin. Invest. **45**, 460 (1966).

10. Hockwald, R. S., Arnold, J., and Clayman, C. B., J. Amer. Med. Ass. **149**, 1568 (1952).

11. Necheles, T. F., and Allen, D. M., N. Engl. J. Med. **280**, 203 (1969).

Received June 13, 1973. P.S.E.B.M., 1973, Vol. 143.