that their loss is a significant aspect of the response to stress, amounting to an actual loss of 17% of all solids present. Possible factors responsible for the loss of solids may be loss of diffusible cellular proteins, loss of mineral ions to the blood, or excess secretion of pituitary hormones.

Summary. Effects of severe stress, immersion in water at 70°C for 5 seconds, were studied on the male albino rat in relation to response of the anterior pituitary. Using 5 different staining methods, no significant changes in *percentage* of acidophils, basophils or chromophobes were found 1, 3 or 12 hours after stress, although there were marked increases in *number of cells per field* in the 12 hour post-stress rats. Fresh weight of the pituitary showed no significant change in 1 and 3 hour post-stress animals, but the 12 hour group displayed a marked reduction, which was evident on both an absolute and a relative to body weight basis. Comparison of relative dry weight and amount of fluid lost in drying in unstressed and 12 hour stressed animals revealed that the weight decrease consisted of both fluid loss and reduction in solids.

1. Finerty, J. C., and Briseno-Castrejon, B., *Endocrinol.,* 1949, v44, 293.

2. Brokaw, R., Briseno-Castrejon, B., and Finerty, J. C., *Texas Reports on Biol. and Med.,* 1950, v8, 312.

3. Daughaday, W. H., Perry, W. E., and McBryde, C. M., *J. Clin. Endocrinol.,* 1950, v10, 410.

4. Finerty, J. C., Hess, M., and Binhammer, R., *Anat. Rec.,* 1952, v115, 15.

5. Knigge, K. M., *ibid.,* 1955, v122, 295.

6. Kraus, E. J., *Virchow's Arch.,* 1925, v247, 421.

7. Koneff, A. A., *Endocrinol.,* 1944, v34, 77.

8. D'Angelo, S. A., Gordon, A. L., and Charipper, H. A., *ibid.,* 1948, v48, 399.

9. Marshall, F. M., *J. Exp. Med.,* 1951, v94, 21.

10. Wilson, W. D., and Ezrin, C. F., *Am. J. Path.,* 1954, v30, 891.

11. Briseno-Castrejon, B., and Finerty, J. C., *Stain Technol.,* 1949, v24, 103.

12. Pearse, A. G. E., *Nature,* 1948, v162, 651.

13. Rennels, E. G., *Anat. Rec.,* 1951, v111, 462.

14. Halmi, N. S., *Stain Technol.,* 1952, v27, 61.

Received December 5, 1955. P.S.E.B.M., 1956, v91.

Hemolysis of Human Erythrocytes by a Sulfhydryl Inhibitor, p-Chloromercuribenzoic Acid.* (22283)

RAYMOND F. SHEETS, HENRY E. HAMILTON, AND ELMER L. DEGOWIN.
(With technical assistance of Richard H. King)
Department of Medicine, College of Medicine, State University of Iowa, Iowa City.

Although sulfhydryl groups have a function in the metabolism and integrity of erythrocytes, its nature is poorly defined. Ingbar (1) reported that cysteine caused sickling of erythrocytes from patients with sickle cell anemia. It was prevented by the addition of heavy metals or p-chloromercuribenzoic acid. Benesch and Benesch(2) have shown that either phenylmercuric hydroxide or mersalyl (salyrgan) caused hemolysis of sheep erythrocytes in a fairly consistent pattern; and that this hemolysis was blocked by sulfhydryl groups but not by sulfur in the disulfide linkage or other forms of sulfur. Dimant, *et al.* (3) more recently have been concerned with the question of *de novo* synthesis of glutathione by erythrocytes. In our previous studies(4) on the feeding of methionine, prothrombin and accelerator globulin decreased in all 3 human subjects. Increased erythrocyte destruction was demonstrated in only one subject.

This present study concerns the relation of certain factors of the red cell environment to hemolysis caused by p-chloromercuribenzoic acid.

Method. The sodium salt of p-chloromer-

* This work was supported in part by the Office of the Surgeon General, Department of the Army and by the Trust Fund of the Department of Medicine.

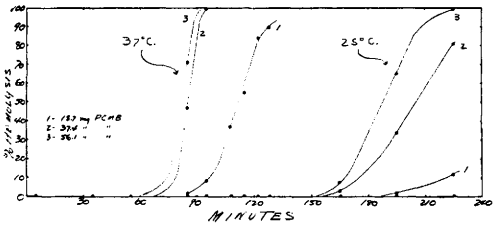

FIG. 1. Effect of concentration and temperature on hemolysis by the sodium salt of p-chloromercuribenzoic acid. Erythrocytes were added at 0 min. There was no hemolysis in the saline control indicated by hemispheres on the base line.

curibenzoic acid† (PCMB) was prepared in 5×10^{-4} molar concentration in 0.9% sodium chloride solution. Erythrocytes were prepared from heparinized blood of normal human donors. The cells were washed 3 times in cool 0.9% sodium chloride solution. After the last washing 0.5 ml of packed cells was added to 25 ml of 0.9% normal saline containing the chemicals studied in the particular experiment. Unless otherwise specified, the cells were incubated at 37°C during the period of observation. The degree of hemolysis was determined by measuring the amount of hemoglobin in the supernatant fluid of a suspension of cells at intervals throughout the study. By relating this figure to 100% hemolysis, obtained by completely hemolyzing a homogeneous suspension of the cells and measuring the optical density in a photometer, the per cent of hemolysis at any time could be calculated. Any existing hemolysis was measured. Correction was made by subtracting this value from the completely hemolyzed sample or the samples obtained at various times. The following equation shows this relationship:

$$\% \text{ hemolysis} = \frac{OD_t - OD_c}{OD_m - OD_c} \times 100.$$

OD_c = Optical density of supernatant immediately after adding red cells.

OD_m = Maximum optical density possible with this concentration of red cells (100% hemolysis).

OD_t = Optical density of supernatant at any time during test.

Zero time was the instant of adding the red cells. The samples of hemoglobin to be measured in the photometer were in a weak solution of ammonium hydroxide. No effort was made to control the pH. During hemolysis the pH of the supernatant fell from 7.6 to 7.3.

Results. 1. *Effect of PCMB concentration*

† Purchased from Sigma Chemical Co.

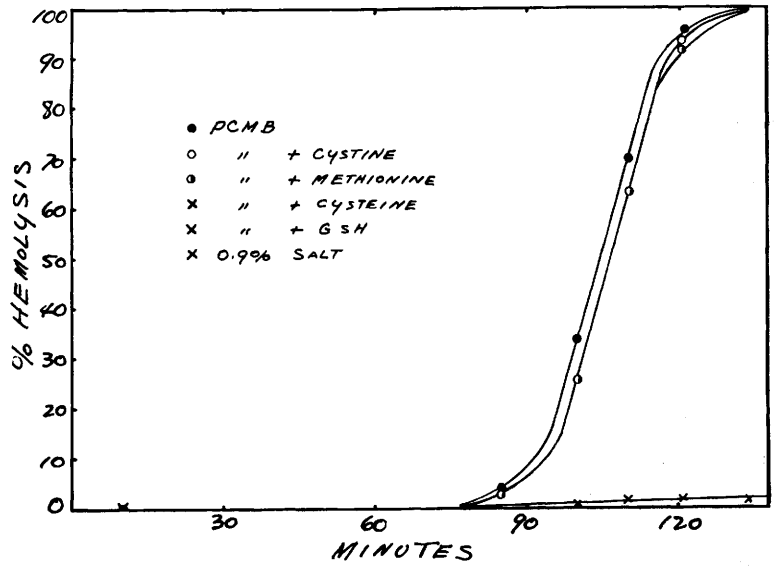

FIG. 2. Amino acids containing sulfhydryl groups inhibit the hemolytic effect of PCMB; those without sulfhydryl are ineffective. There is very little hemolysis of the control erythrocytes in 0.9% saline suspension in 4 hr. Erythrocytes were added at 0 min.

and temperature on hemolysis (Fig. 1). Increasing the concentration of PCMB 2 and 3 times the original $5x10^{-4}$ molar resulted in a slight increased rate of hemolysis; and the onset of hemolysis after initial contact with the reagent began at 75 instead of 90 minutes. Decreasing the concentrations of PCMB progressively prolonged the hemolytic process over a period of 3 to 4 hours. Under standard conditions with a concentration of PCMB of $5x10^{-4}$ molar at 37°C hemolysis began in about 90 minutes and was completed in 120 to 130 minutes. This has been a fairly consistent observation with different samples of PCMB and erythrocytes from different donors. Incubation at 25°C lengthened the time before the onset of hemolysis and, once hemolysis had begun, the rate was slower.

II. *Inhibition of hemolysis by amino acids containing –SH groups added to PCMB solution before contact with erythrocytes (Fig. 2).* Amino acids containing sulfhydryl groups in equimolar concentrations inhibited the hemolytic effect of PCMB if added to the solution before the erythrocytes. Cystine and methionine were completely ineffective in this inhibition.

III. *Effect of addition of –SH groups after contact of PCMB and erythrocytes (Fig. 3).* An equimolar concentration of glutathione was added immediately after the red cells were suspended in the PCMB solution and 30, 60 and 90 minutes later. At one minute and 30 minutes most hemolysis was prevented. There was considerable inhibition of the PCMB effect when glutathione was added as late as 60 minutes. Even at 90 minutes, just before hemolysis was about to begin, the addition of glutathione altered the curve remarkably although hemolysis went to completion.

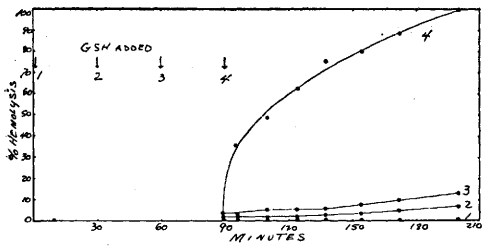

FIG. 3. Inhibition of hemolysis when glutathione added at intervals after contact between erythrocytes and PCMB. Erythrocytes were added at 0 min.

IV. *Effect on hemolysis of washing erythrocytes after contact with PCMB (Fig. 4).* Erythrocytes were washed 3 times in cool saline after one minute in contact with PCMB and after 30 and 60 minutes. The washed cells were suspended in 25 ml of fresh saline. Washing the erythrocytes one minute after contact with PCMB solution virtually eliminated the hemolytic effect. Washing after 30 minutes reduced the rate of hemolysis so that there was 20 per cent hemolysis at 210 minutes instead of the usual 90 minutes. Washing at 60 minutes had little influence on the onset and rate of hemolysis except that about 10% of the cells were resistant to hemolysis for over 4 hours. This finding was in contrast to that of the previous experiment when the addition of glutathione at 60 minutes prevented most hemolysis. The supernatant from the initial separation in this study was saved and tested for hemolytic activity by adding another 0.5 ml of red cells. These curves were reciprocals of those in Fig. 4, indicating a progressive loss of hemolytic activity from the solution the longer the erythrocytes had remained in it. The supernatant removed at 60 minutes was free of hemolytic effect. However, contact for 1 minute produced no detectable loss in hemolytic activity. The reaction between erythrocytes and PCMB is not instantaneous but requires a lapse of time.

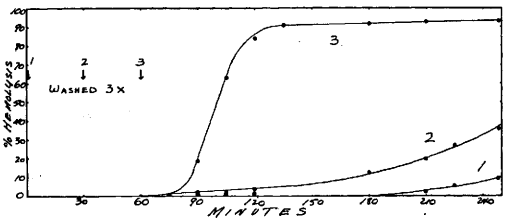

FIG. 4. Incomplete inhibition of hemolysis by washing PCMB from erythrocytes. Red cells were added to PCMB at 0 min.

V. *Exhaustion of PCMB hemolytic effect by exposure of PCMB to single aliquot of red cells (Fig. 5).* The usual suspension of red cells in PCMB solution was made and

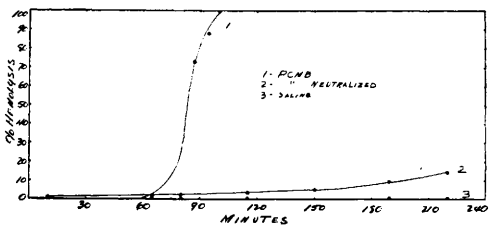

FIG. 5. Exhaustion of hemolytic effect of PCMB by one exposure to erythrocytes. In this chart only, time scale is measured from time when red cells were added to the PCMB in (1) and time when a second portion of red cells was added to the hemolyzed mixture in (2), time when the cell suspension was made in saline in (3).

hemolysis was allowed to proceed. As soon as hemolysis was complete another 0.5 ml of red cells was added to this hemolyzed mixture and more observations were made. There was very little hemolysis of the second portion of red cells, which indicates that the hemolytic effect of PCMB was exhausted by the first aliquot of red cells. This then raised the question as to whether the exhaustion of the hemolytic effect was the result of –SH groups in the cell stroma or inside the cell envelope.

VI. *Exhaustion of PCMB hemolytic effect by hemoglobin but not by stroma* (*Fig. 6*). A suspension of stroma and a hemoglobin solution were prepared by thrice rapidly thawing and freezing 0.5 ml of packed cells. A few milliliters of cold 0.9% sodium chloride solution were added to the hemolyzed mixture which was then centrifuged. The clear supernatant was transferred to a tube and the quantity was made up to 25 ml with 0.9% sodium chloride solution. A small

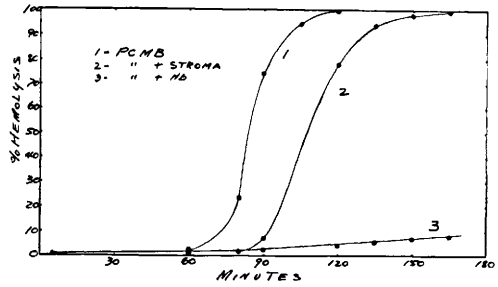

FIG. 6. Inhibition of PCMB hemolytic effect by incubation with hemoglobin solution and failure of inhibition by stroma suspension. Test erythrocytes were added at 0 min.

quantity of fluid containing the stroma was placed in another tube and the volume made up to 25 ml with 0.9% sodium chloride solution. Microscopic examination showed the hemoglobin portion to be virtually free of stroma and the stroma to be concentrated in the other tube. PCMB was added to the two tubes in the concentration of 5×10^{-4} molar followed by incubation for 90 minutes. Then 0.5 ml of erythrocytes was added to each tube and the usual procedure followed. The stroma fraction altered the shape of the curve but only to an insignificant degree. Hemolysis in the other tube was almost completely inhibited by the hemoglobin solution. Since the stroma was not washed the alteration in this curve may have been caused by the small amount of free hemoglobin in the stroma suspension.

VII. *Effect of glutathione plus glucose on the PCMB hemolytic system* (*Fig. 7*). Glu-

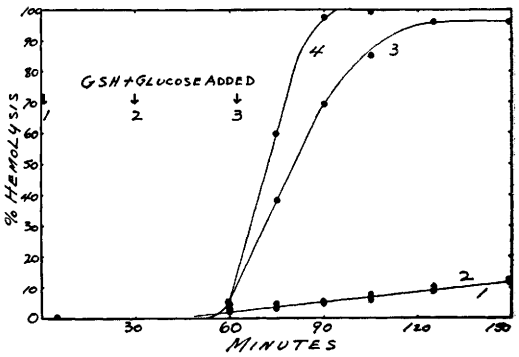

FIG. 7. Failure of glucose to modify inhibition of PCMB hemolysis by glutathione. Erythrocytes combined with PCMB at 0 min. In (4) red cells were suspended in solution of PCMB and NaCl only.

tathione in equimolar concentration with PCMB and glucose to produce a final concentration of 1 mg per 1 ml was added at intervals of 1 minute, 30 minutes and 60 minutes to the red cells suspended in PCMB solution. Glucose had no beneficial effect and was harmful when added at 60 minutes.

Discussion. Organic mercurials which combine with –SH groups cause hemolysis of human erythrocytes. Previous transformation of the organic mercury compound into the mercaptide with cysteine or glutathione

inhibits hemolytic activity. The rate of hemolysis is influenced by temperature(11) as well as concentration of the mercurial reagent. Benesch *et al.*(5) found a striking variation in the reactivity of the –SH groups of crystalline hemoglobin among different species. Those of dogs and man were less reactive than those of sheep. Racker(6) suggested that the difference in rate of reactivity between sulfhydryl groups and reagents may be influenced by properties of adjacent groups on the protein molecule.

That –SH groups are necessary for the integrity of the erythrocyte is a fact. The thiols responsible for this effect are not known nor is the mechanism known. A recent report (5) indicates there are 8 moles of –SH per mole of hemoglobin in man. Previous studies have demonstrated the presence of ergothioneine(7-9) and glutathione(10) within the erythrocyte. The glutathione is in a dynamic state(3) and may traverse the cell envelope. Less is known of the role of ergothioneine.

The necessity of –SH groups for the integrity of the erythrocyte suggests that control of the thiol system may be advantageous in the preservation of whole blood stored for transfusion.

Conclusions. 1. The sulfhydryl reagent, p-chloromercuribenzoic acid, causes hemolysis of human erythrocytes. This action can be prevented by prior reaction with sulfhydryl groups. 2. The hemolytic action of PCMB is reversible by washing cells for as long as 30 minutes after contact with this agent. Reversibility of hemolysis lasts up to 60 minutes after addition of free sulfhydryl group. 3. No protection from PCMB hemolysis was noted with stroma whereas hemoglobin solution was effective. 4. The reaction between PCMB and the –SH group of erythrocytes is relatively slow. 5. A physiologic concentration of glucose offered no protection from hemolysis in this system.

1. Ingbar, S., *Glutathione,* 1954, 228, Academic Press, Inc., New York.

2. Benesch, R. E., and Benesch, R., *Arch. Biochem. and Phys.,* 1954, v48, 38.

3. Dimant, E., Landsberg, E., and London, I. M., *J. Biol. Chem.,* 1955, v213, 769.

4. DeGowin, E. L., Sheets, R. F., Hamilton, H. E., and Carter, J. R., *Trans. Assn. Am. Physicians,* 1955, v68, 118

5. Benesch, R. E., Lardy, H. A., and Benesch, R., *J. Biol. Chem.,* 1955, v216, 663.

6. Racker, E., *Physiol. Rev.,* 1955, v35, 1.

7. Benedict, S. R., Newton, E. B., and Behre, J. A., *J. Biol. Chem.,* 1926, v67, 267.

8. Newton, E. B., Benedict, S. R., and Dakin, H. D., *ibid.,* 1927, v72, 367.

9. Hunter, G., and Eagles, B. A., *ibid.,* 1925, v65, 623.

10. Woodward, G. E., and Fry, E. G., *ibid.,* 1932, v97, 465.

11. Fegler, G., *Nature,* 1952, v170, 624.

Received January 9, 1956. P.S.E.B.M., 1956, v91.

Pyridoxine Responsive Anemia in the Human Adult.* (22284)

JOHN W. HARRIS,§ RICHARD M. WHITTINGTON, RUSSELL WEISMAN, JR., AND DANIEL L. HORRIGAN.

Departments of Medicine, Cleveland City Hospital, Crile Veterans' Administration Hospital, University Hospitals, and Western Reserve University School of Medicine, Cleveland.

Although pyridoxine is necessary for normal hematopoiesis in dogs(1,2), swine(3-5) and monkeys(6), no evidence is available that abnormal hematopoiesis occurs in humans secondary to a naturally occurring deficiency of pyridoxine or alteration in its metabolism(7,8). In general, the hematologic alterations resulting from pyridoxine deprivation in animals have been characterized by a hypochromic, microcytic anemia with

* Supported by research grants from the National Institute of Arthritis and Metabolic Diseases, National Heart Institute of National Institutes of Health, Public Health Services; and by grant supporting Hematology Research at Cleveland City Hospital by Eli Lilly and Co.

§ Markle Scholar in Medical Science, Western Reserve University School of Medicine.