urinary system, trunk, thyroid and thymus glands.

1. Nelson, M. M., *Pediat.*, 1957, v19, 764.
2. Kalter, H., Warkany, J., *Physiol. Rev.*, 1959, v39, 69.
3. Heidelberger, C., Gullberg, M. E., Morgan, A. F., Lepkovsky, S., *J. Biol. Chem.*, 1948, v175, 471.
4. Wilson, R. G., Henderson, L. M., *ibid.*, 1960, v235, 2099.
5. Ershoff, B. H., *Arch. Biochem.*, 1946, v9, 81.
6. Giroud, A., In: *World Reviews of nutrition and dietetics*, Pitman Co., London, 1959, pp 231-263.
7. Dann, W. J., Handler, P., *J. Biol. Chem.*, 1941, v140, 435.
8. Halliday, S. L., Sloboda, H., Will, L. W., Oleson, J. J., *Fed. Proc.*, 1957, v16, 191.
9. Chamberlain, J. G., Nelson, M. M., *Anat. Rec.*, 1962, v142, 337.
10. Landauer, W., *J. Exp. Zool.*, 1957, v136, 509.
11. Murphy, M. L., Dagg, C. P., Karnofsky, D. A., *Pediat.*, 1957, v19, 701.
12. Pinsky, L., Fraser, F. C., *Biol. Neonat.*, 1959, v1, 106.
13. ———, *Brit. Med. J.*, 1960, v2, 195.

Received February 18, 1963. P.S.E.B.M., 1963, v112.

Erythropoietic Effect of Red Blood Cell Components and Heme-Related Compounds. (28184)

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Several investigators(1-3) have suggested that products of hemolyzed blood are a stimulus to red blood cell production. Other investigators, Verzar(4), Zih(5), and Bomford (6), established the erythropoietic action of bilirubin using increased red blood cell counts as a quantitative indicator. Further studies have not been done with the more quantitative techniques now available. The present work was undertaken to determine the possible involvement of red blood cell moieties in a feedback mechanism. This report presents data on the erythropoietic activity of lysed red blood cells from various species and on compounds that are either metabolically or structurally related to the heme moiety(7).

Materials and methods. Albino female rats of the Sprague-Dawley (Holtzman) strain weighing 150-250 g were used. For bioassay of erythropoietic activity, fasting "dehydrated" rats were generally used. Rats on a complete nutritional diet were used for comparison. Subcutaneous injections of the test material or control solution were given daily, for 3 days, to rats which had been deprived of food and water for 48 hours prior to the first injection. Water was provided *ad libitum* for 24 hours between the second and third subcutaneous injections. Food was withheld during the bioassay. There was no apparent local inflammatory response to injection of lysed red blood cells, hemin, or related compounds. Immediately after the animals had received the third subcutaneous injection, they were anesthetized lightly with ether and injected intravenously (*via* the tail vein) with 0.5 μc of Fe^{59}-citrate in 0.25 ml of physiological saline solution. The specific activity of the Fe^{59} iron ranged from 23.6 to 30.8 μc/μg. The maximum amount of iron administered to an animal was 1.6×10^{-2} μg.

Twenty-four hours after Fe^{59} injection, the tip of the tail was cut and blood was taken by capillary tube for micro-hematocrit and reticulocyte determinations. There was a distinct advantage in obtaining blood from the tail for these procedures since large numbers of animals were sacrificed. The animals were next anesthetized, the abdomen opened, and blood drawn from the abdominal aorta with an oxalate-rinsed syringe. Two ml were then measured into a plastic counting tube for counting in a well scintillation counter to per-

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TABLE I. Effects of Lyophilized Red Blood Cells on Erythropoiesis in Rats.

Treatment	Days inj	No. rats	Het, mean %	Fe^{59} uptake, %	Retic, % of RBC
A. Fasted and "dehydrated" rats (wt at sacrifice: 170-180 g)					
Lyophilized RBC (190 mg)	3	6	48	37.2 ± 3.7*	2.4 ± .6*
Control	3	6	48	9.6 ± 2.4	.7 ± .4
B. Rats on complete nutritional diet (wt at sacrifice: 200-210 g)					
Lyophilized RBC (150 mg)	9	6	48	55.6 ± 4.8	4.6 ± .4
Control	9	6	49	39.1 ± 5.4	1.4 ± .4

* Standard deviation.

mit estimation of the percentage of the injected dose of Fe^{59} present in the peripheral red blood cells.

Total blood volume of rats was computed on the basis of 5.18 ml per 100 g of final body weight(8). Reticulocytes were stained by the brilliant cresyl blue method. One thousand red cells were counted.

Red blood cells were lysed by lyophilization after being washed with normal (0.9%) saline solution. The lyophilized cells were suspended in water to provide the required concentrations and to permit the volume for subcutaneous injection in all cases to be 1.5 ml.

Globin from rat red blood cells was prepared by the method of Anson and Mirsky (9). Stroma from the same source was prepared, with slight modifications, by the CO_2 precipitation method of Bernstein *et al.*(10). Glycine, biliverdin, bilirubin, delta-aminolevulinic acid (ALA), catalase, cytochrome c, succinic acid, cyanocobalamin, chlorophyllin (Na-K), recrystallized hemin, recrystallized hematoporphyrin, protoporphyrin type IX, bovine hemoglobin and pyrrole were also tested.† All compounds were dissolved or suspended in water and adjusted to pH 7.2 for injection, with the exception of bilirubin and cyanocobalamin. To each 10 ml of pH-adjusted bilirubin suspension (and its control) was then added one drop each of Triton x-35 and Triton x-100 (Rohm and Haas, Pa.). Cyanocobalamin was used directly from the vial. The solvents used to dissolve the test materials were injected into control animals. Analysis of results for statistical significance was made using the Mann and Whitney "U" test(11).

† Sources: Glycine and biliverdin, Eastman Org., N. Y.; bilirubin, Fisher Scientific, N. J.; ALA, catalase and cytochrome c, Sigma, Mo.; succinic acid, Krishell, Ore.; cyanocobalamin, Phila. Amp., Pa.; chlorophyllin, hemin, hematoporphyrin, protoporphyrin, hemoglobin and pyrrole, Mann, N. Y.

Results and discussion. Results of experiments which tested the erythropoietic response induced by lyophilized rat red blood cells in both fasting "dehydrated" rats and rats on a complete nutritional diet are shown in Table I. The 24 hour Fe^{59} uptake and % reticulocytes of red blood cells are both significantly increased ($p = <.002$) over controls regardless of diet conditions during the test.

Fe^{59} uptake in assay rats injected with donor cells lysed by the freeze-thaw technique showed no significant difference from that in assay rats injected with lyophilized cells ($p = >.08$). Lyophilization was the method of choice because it permitted the preparation of a stock of easily stored dry material for use in a series of experiments.

Further studies were made to ascertain whether the condition of the donor rat was important in the ability of its lysed cells to increase Fe^{59} uptake in the assay rats. The following comparisons were made: cells from 60 g females *vs* cells from 400 g females; cells from 160 g females *vs* cells from 160 g males; cells from phenylhydrazine-anemic females *vs* cells from non-anemic females. The differences found within the pairs were not statistically significant ($p = >.08$). Neither age nor sex differences nor the presence of phenylhydrazine anemia appear to influence the activity of lysed cells in increasing Fe^{59} uptake.

The results of experiments designed to test the erythropoietic effect of lyophilized red

TABLE II. Response of Fasting "Dehydrated" Rats to Lyophilized Red Blood Cells from Various Species.*

Donor†	No. rats	Hct, mean %	Fe^{59} uptake, %	Retic, % of RBC
Rat	12	48	27.9 ± 3.6‡	2.3 ± .7‡
Cat	12	49	15.0 ± 2.4	1.7 ± .4
Man	6	49	23.3 ± 6.5	2.5 ± .2
Rabbit	6	49	19.9 ± 3.9	2.0 ± .8
Dog	9	49	18.1 ± 4.3	1.9 ± .3
Control	12	50	8.1 ± 1.2	.9 ± .2

* Rat weights at sacrifice: 150-160 g.
† 3 Daily 200 mg injections of donor's lyophilized cells.
‡ Standard deviation.

TABLE III. Effects of Red Blood Cell Components on Radioiron Incorporation in Fasting "Dehydrated" Rats.*

Treatment†	No. rats	Hct, mean %	Fe^{59} uptake, %
Stroma (xt. from 150 mg RBC)	6	50	5.1 ± .9‡
Hemoglobin (150 mg)	6	49	34.0 ± 4.5
Globin (120 mg)	4	50	8.0 ± 2.2
Hemin (6 mg)	18	45	38.0 ± 3.4
Control	12	49	7.8 ± 2.0

* Rat weights at sacrifice: 170-180 g.
† 3 Daily injections.
‡ Standard deviation.

blood cells of various species are shown in Table II. Red blood cells of all species tested elicited a significant increase in Fe^{59} incorporation and reticulocytes in test animals when compared with the control group ($p = < .002$). Small species differences in red blood cell hemoglobin content do not explain the differences found.

To identify the part of the red cell responsible for the increased radioiron incorporation, various components were tested for their erythropoietic effects in fasting "dehydrated" rats. Stroma and globin from rat red blood cells, bovine hemoglobin and recrystallized hemin were tested. Table III shows that stroma and globin have little influence on radioiron incorporation in recipient rats, but that hemoglobin and hemin cause a definite erythropoietic response.

Experiments were next designed to test the erythropoietic effects of some compounds structurally or metabolically related to heme. Table IV gives data from these experiments carried out on fasting "dehydrated" animals. The compounds tested which are involved in synthesis of the porphyrin ring structure elicited no erythropoietic response; whereas, in every case where 4 pyrrole rings were linked together with methene bridges, significant activity was found when compared with controls ($p = < .002$). In addition, when the ring structure was opened, as in biliverdin and bilirubin, activity was still observed. It is interesting to note that cyanocobalamin with a corrinoid ring structure showed no activity. Variations in the side chain groups or metal moieties apparently do not affect the biological response.

The control of erythropoiesis may be dependent on many factors, one of which may

TABLE IV. Effect of Heme-Related Compounds on Radioiron Uptake in Fasting "Dehydrated" Rats.*

Treatment†	μMole/d injected	No. rats	Hct, mean %	Fe^{59} uptake, %	Retic, % of RBC
Glycine	40	6	50	6.6 ± 2.9‡	
Succinate	76	5	49	8.1 ± 1.8	
ALA§	68	6	50	4.6 ± .9	
Pyrrole	37	4	50	5.6 ± 1.5	
Hematoporphyrin	17	9	46	20.6 ± 4.5	2.2 ± .2‡‖
Protoporphyrin	18	9	46	21.4 ± 2.4	2.1 ± .6‖
Biliverdin	8	8	49	18.8 ± 3.0	1.6 ± .4
Bilirubin	8	4	48	18.0 ± 1.5	1.4 ± .1
Catalase	.6	9	45	32.5 ± 2.1	3.1 ± .6
Cytochrome c	7	9	47	20.2 ± 3.0	2.6 ± .5
Chlorophyllin	15	9	49	25.9 ± 3.9	2.4 ± .3‖
Cyanocobalamin	1.8	5	50	6.2 ± 1.4	
Control		25	50	6.0 ± 1.2	.6 ± .4

* Rat weights at sacrifice: 170-180 g. † 3 Daily injections. ‡ Standard deviation.
§ Delta amino-levulinic acid. ‖ Reticulocytes from only 5 rats.

be a feedback mechanism which involves a porphyrin or porphyrin-like compound. The effects could be direct or through stimulation of increased production of the plasma erythropoietic factor. Further studies are required to establish the mechanism by which hemin and related compounds affect erythropoiesis.

Summary. 1. Regardless of age, sex, or species of the donor animals, lysed mammalian red blood cells show erythropoietic stimulating activity. 2. Comparison of the stimulating effects of red blood cell components on erythropoiesis shows that hemoglobin and hemin are active; stroma and globin are not. 3. The following compounds related to heme appear to increase erythropoiesis: compounds containing 4 pyrrole rings connected by methene bridges; an open 4-pyrrole ring configuration, as in biliverdin and bilirubin. The nature of the side-chain groups is not critical for activity; a metal moiety is not essential to activity. 4. Several precursors involved in synthesis of the porphyrin ring, a single pyrrole ring, and cyanocobalamin which has a corrinoid ring structure, did not increase erythropoietic activity.

1. Itami, S., *Arch. f. Exp. Path. Pharmakol.*, 1908, v62, 93.
2. Kepinow, L., *Biochem. Z.*, 1910, v30, 160.
3. Isaac, S., *Therap. Halbmon.*, 1920, v34, 341.
4. Verzar, F., *Z. Gen. Exp. Med.*, 1929, v68, 475.
5. Zih, A., *ibid.*, 1939, v104, 675.
6. Bomford, R. R., *Brit. Med. J.*, 1940, v2, 549.
7. Altschuler, N., Brown, J. R., Cooper, J. A. D., *Fed. Proc.*, 1962, v21, 124.
8. Rambach, W. A., Alt, H. L., Cooper, J. A. D., *Blood*, 1957, v12, 1101.
9. Anson, M. L., Mirsky, A. E., *J. Gen. Physiol.*, 1930, v13, 469.
10. Bernstein, S. S., Jones, R. L., Erickson, B. N., Williams, H. H., Arvin, I., Macy, I. G., *J. Biol. Chem.*, 1937, v122, 507.
11. Mann, H. B., Whitney, D. R., *Annals Math. Stat.*, 1947, v18, 50.

Received November 2, 1962. P.S.E.B.M., 1963, v112.

Effect of Cigarette Smoke on Epinephrine Secretion in the Dog.* (28185)

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Various investigators have shown that large doses of nicotine, the active ingredient in tobacco smoke, when administered intravenously can cause an increase in secretion of epinephrine from the adrenal gland(1-4). It is controversial as to whether or not a similar effect can be produced as the result of inhalation of cigarette smoke(5). In an attempt to clear up this controversy the present experiments were carried out to determine whether or not inhalation of cigarette smoke could cause an increased secretion of epinephrine from the adrenal glands of dogs.

Methods. Twenty-two healthy mongrel dogs of either sex with weights ranging from 7.7 to 17.7 kg were used. The dogs were anesthetized with intravenous pentobarbital sodium (30 mg/kg) or chloralose (80 mg/kg). The dogs then inhaled cigarette smoke from a holder inserted into a tracheal cannula. The rate of smoking was regulated by means of a screw clamp on a 14 mm ID rubber tube which served as a bypass for the cigarette. The same standard brand of cigarette was used in all experiments. Blood samples were collected before, during and after inhalation periods. Blood pressure was recorded from the carotid artery by means of a polyethylene catheter connected to a Statham transducer and a Sanborn 150 M recorder. Heparin (3 mg/kg) was used as an anticoagulant in all experiments.

Blood samples were taken from the adrenal vein, inferior vena cava, the femoral artery and femoral vein. The adrenal vein was cannulated using the method of Satake(7), while

* This investigation was supported in part by a PHS traineeship from Division of General Medical Sciences and a research grant from Nat. Inst. of Mental Health. P.H.S.