Chlorpromazine sulfoxide is reported to have minimal sedation and potentiation effects in mice, rats and rabbits and only one-eighth the action of chlorpromazine in dogs. In addition its hypotensive properties in dogs are of a low order of magnitude(11). Effects of sulfoxide on mental state have not been reported. Thus it is evident that the sulfoxide derivative does not possess clinical properties of chlorpromazine, promazine and promethazine in areas which have been tested, and this parallels the findings on inhibition of cytochrome oxidase and ATP-ase *in vitro*. Since sulfoxide is a major metabolite of chlorpromazine(11), it seems that chlorpromazine is the active principle and does not act through its conversion to sulfoxide.

The prerequisite of a divalent sulfur in the ring might imply that other phenothiazine derivatives with an unsubstituted sulfur atom may also have the inhibitory properties reported. Collier and Allenby(12), have reported that neither phenothiazine, phenothiazine sulfoxide, phenothiazone nor thional were inhibitory to rat-liver cytochrome oxidase. Thus it would appear that substitution on the N atom of the ring might also be necessary to produce inhibition of this enzyme system.

Summary. 1. Inhibitory properties of 3 chlorpromazine analogues show that oxidation at the sulfur atom, abolishes inhibition of ATP-ase and cytochrome oxidase systems by chlorpromazine. 2. Removal of chlorine atom from the ring *viz*: promazine, and alteration of the N-substituted sidechain *viz*: promethazine, result only in a small diminution of the inhibitory properties. 3. Inhibitory findings are correlated with known pharmacological effects of the drugs.

1. Abood, L. G., PROC. SOC. EXP. BIOL. AND MED, 1955, v88, 688.

2. Bernsohn, J., Namajuska, I., and Cochrane, L. S. G., *Arch. Biochem. Biophys.*, in press.

3. Brody, T. M., and Bain, J. A., *J. Biol. Chem.*, 1952, v195, 685.

4. Schneider, W. C., and Potter, V. R., *ibid.*, 1945, v149, 217.

5. DuBois, K. P., and Potter, V. R., *ibid.*, 1943, v150, 185.

6. Gomori, C., *J. Lab. Clin. Med.*, 1942, v27, 955.

7. Rea, E. L., Shea, J., and Fazekas, J. F., *J.A.M.A.*, 1954, v156, 1249.

8. Kopera, J., and Armitage, A. K., *Brit. J. Pharm.*, 1954, v9, 392.

9. Baxter, R. W., Bolster, J. A., and McKechnie, S., *Anesthesia*, 1954, v9, 79.

10. Wyeth Laboratories, personal communication.

11. Salzman, N. P., Moran, N. C., and Brodie, B. B., *Nature*, 1955, v178, 1122.

12. Collier, H. B., and Allenby, G. M., *Can. J. Med. Sci.*, 1952, v30, 443.

Received April 12, 1956. P.S.E.B.M., 1956, v92.

Erythropoiesis. II. Assay of Erythropoietin in Hypophysectomized Rats.* (22428)

W. FRIED, L. PLZAK, L. O. JACOBSON, AND E. GOLDWASSER.

Argonne Cancer Resesarch Hospital and the Departments of Medicine and Biochemistry, University of Chicago, Ill..

Experimental evidence supporting the theory that a humoral factor in the blood plasma mediates red cell production has been reviewed by several investigators(1,2). This factor, which has been referred to as erythropoietin by Bonsdorff and Jalavisto(3) and others(4), has not been isolated nor is it known whether it is a single substance or several. The method utilized to determine the presence of erythropoietin in the plasma of anemic animals has been confined largely to evidence of increased erythrocyte production in normal animals that have received injections of plasma from animals made anemic by repeated phlebotomy or injections of phenylhydrazine. The hematologic technics for determining increased

* Number I in this series of papers appears in *J. Lab. Clin. Med.*, 1955, v46, 671.

red cell production consist largely of reticulocyte and red cell counts, hemoglobin or hematocrit determinations on the peripheral blood, and histologic study of erythroblastic activity of the blood-forming tissue. As previously reported(5,6), Fe^{59} uptake in newly-formed red cells is probably the most reliable single method for studying the effect of anemic plasma on erythropoiesis. Under ideal experimental conditions, the findings obtained by all these methods correlate well with one another, but an amount of plasma equivalent to one-half the blood volume is necessary to elicit a response. A sensitive method of assaying plasma from normal or anemic subjects is described in this communication.

Materials and methods. (a) Preparation of animals. Young adult male Sprague-Dawley rats, approximately 3 months of age and ranging in weight from 175 to 200 g, were used throughout these experiments. Rats were hypophysectomized by a standard technic† and delivered to us on the day following the operation or at later intervals thereafter. They were maintained on a diet consisting of milk, fresh vegetables, and Rockland mouse diet *ad libitum*. The control animals were of the same sex, age, and weight. *(b) Preparation of plasma.* Rats were bled daily for 3 days by cardiac puncture until their hematocrit was reduced to 25% or less. On the fourth day, the animals were anesthetized lightly with ether, and as much blood as possible was withdrawn by cardiac puncture. To this blood was added sufficient heparin to prevent clotting (circa 20 units per cc of blood). The blood was immediately centrifuged at 3000 rpm.‡ The plasma was separated, pooled with other plasma drawn at the same time, and stored at −10°C. This routine was followed for preparation of the plasma that we shall hereafter refer to as "anemic plasma." Plasma, which was prepared from the blood of normal, previously unbled rats, served as the control. *(c) Test of plasma for erythropoietin.* The hypophysectomized and normal rats were weighed just prior to and at the conclusion of each experiment. The plasma to be tested was administered intravenously to each animal in 2-cc doses on 3 consecutive days. Two hours following the last injection of plasma, 1 cc of Fe^{59} citrate solution, diluted with normal saline to contain 2 to 3 μc of radioactive Fe^{59}, was introduced into the tail vein. Sixteen hours following injection of Fe^{59}, a 1-cc sample of blood was withdrawn from each rat by cardiac puncture. The radioactivity in this sample was measured in a Nancy Wood well-type scintillation counter. The radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Using the blood volume of the rats (estimated to be 6% of body weight, verified by Cr^{51} blood volume determinations after the method of Gray and Sterling(7), the amount of radioactivity injected into the rats, and the radioactivity in the 16-hour sample, it is possible to calculate the percent of the injected dose of Fe^{59} appearing in the peripheral blood cells at this time interval. In each experiment, the percent of Fe^{59} taken up in a control group of rats (5 animals per group) was compared with the uptake in groups of rats receiving anemic plasma or normal plasma.

Results. (a) Effect of anemic plasma on uptake of Fe^{59} in hypophysectomized animals. Our first experiments were conducted on rats that were hypophysectomized 10 to 13 days prior to the injection of plasma. The uptake of Fe^{59} in hypophysectomized animals that were given daily injections of 2 cc each of normal plasma for 3 successive days was 5% or less; while in the hypophysectomized animals that were given anemic plasma, the uptake was 16% or more (Table I). The uptake

TABLE I. Effect of Anemic Plasma on Rate of Erythropoiesis in the Hypophysectomized Rat, 6 Experiments.*

No. rats per group	Avg % uptake of Fe^{59} into RBC			
	Anemic plasma	Normal plasma	Saline	No inj.
5	27.4	3.2	5.0	4.0
5	16.3	3.3		
4	17.1	5.0		
5	18	2.7	3.8	
3	19.3	5.0		
3	21.8	5.0		

* 10 to 15 days post-hypophysectomy.

† Obtained from Hormone Assay Laboratories, Chicago, Ill.

‡ International Centrifuge, size 1.

TABLE II. Effect of the Interval after Hypophysectomy on Apparent Sensitivity of Rat to Anemic Plasma.

Plasma inj.	Avg % Fe^{59} uptake into RBC of rats*: Prior to hyp.†	4 days post-hyp.	8 days post-hyp.	13 days post-hyp.
Normal	37.5	9.1	4.0	5.0
Anemic	44.6	18.5	18.1	18.0

* 16-hr sample. † hyp. = hypophysectomy.

of Fe^{59} in normal rats following the injection of normal or anemic plasma according to the same regimen averaged respectively 37 ± 4% and 48 ± 4%. The data given below are from experiments in which hypophysectomized animals were used as recipients.

(b) Effect of interval post-hypophysectomy on senstivity to anemic plasma. A series of experiments was undertaken to determine at what time after hypophysectomy the animal is most sensitive to anemic plasma. Normal or anemic plasma was given to rats at various periods after hypophysectomy, and the rate of erythropoiesis was observed. The results are summarized in Table II. An appreciable reduction in Fe^{59} uptake occurred by 4 days after hypophysectomy and reached a minimum by 8 days. We found it advisable, therefore, to wait at least 8 days after surgery before using the animals for assay purposes.

(c) Effect of various amounts of anemic plasma on the uptake of Fe^{59}. Because of the greater sensitivity noted in the hypophysectomized rat, it was thought desirable to investigate the possibility of using a single injection of plasma, testing its efficacy when administered at various intervals prior to the injection of Fe^{59}. The data in Table III indicate that the erythropoietic effect induced by a single 2-ml injection of anemic plasma, given 4 hours prior to the Fe^{59}, is not manifest in the 16-hour sample, but is clearly seen when administered 24 hours prior to the administration of Fe^{59}. The stimulus is still detected 2 or 3 days after administration of plasma. These points were further substantiated by the observations that 2 doses of 2 cc each given 4 hours and 24 hours prior to Fe^{59} injection produced no greater effect on Fe^{59} uptake than did one injection given 24 hours previously. However, 2 injections, given 24 hours and 48 hours before Fe^{59}, gave a greater response. On the basis of these results, we elected to use 2 injections of 2 cc each in future assays unless otherwise noted.

TABLE III. Relationship of Interval between Single 2-ml Injection of Anemic Plasma and Administration of Fe^{59} to Elicitation of the Erythropoietic Stimulus; 5 Experiments.

Rats (No.)	Avg % uptake of Fe^{59} in RBC of hypophysectomized rats: Plasma given: 4 hr prior to Fe^{59}	24 hr prior to Fe^{59}	48 hr prior to Fe^{59}	Normal plasma
4		12.1		5.0
5		11.3	16.8	6.4
4		18.5	18.0	4.6
3		12.0	12.0	
10	4.5		15.5	4.0

(d) Production of anemic plasma factor by the hypophysectomized animal. Hypophysectomized animals were bled on 3 successive days until their hematocrit was 25% or less. Anemic plasma was obtained from these animals by the method that is described above. This anemic plasma, collected from hypophysectomized animals and injected into hypophysectomized animals, produced a significant increase in the uptake of Fe^{59} (Table IV). In other experiments it was found that plasma from normal animals subjected to splenectomy, adrenalectomy, thyroidectomy, gonadectomy, or combinations thereof and then bled increased the uptake of Fe^{59} in the hypophysectomized assay animal as well as did that from normal, bled animals.

Discussion. The data show that the anemic plasma factor(s) (erythropoietin) can be assayed in the hypophysectomized recipient using the technic of Fe^{59} incorporation into newly-formed red cells. The mechanism of the exaggerated response to anemic plasma observed in hypophysectomized animals as compared with the response of hypophysectomized animals given normal plasma is probably a relatively simple one. Our data indicate that by 4 days after hypophysectomy, erythropoiesis is already reduced but not to the maximum reduction, which occurs at 8 to 14 days. The reduction in erythropoiesis that we have shown to occur, using Fe^{59} uptake by the newly formed red cell as the criterion, has been corroborated by the finding of a reticulo-

TABLE IV. Effect of Hypophysectomy on Capacity of Animal to Elaborate the Anemic Plasma Factor.

Rats (No.)	Total vol of plasma, 2 cc/inj.	Avg % uptake of Fe^{59} in RBC of hypophysectomized rats after administration of various plasmas — Source of plasma: Unoperated, nubled animal	Hypophysectomized, nubled animal	Unoperated, anemic animal	Hypophysectomized, anemic animal
5	4	2.7	5.9	14.8	12.0
5	4	5.0	5.8	16.7	16.3
5	2			11.2	14.2
13	2			12.4	12.3

cyte reduction that coincides with the decreased uptake of Fe^{59}(8). It seems likely that an overall reduction in the metabolic requirements of the animal occurs very soon after hypophysectomy. Several workers(9, 10) have shown that a new equilibrium level of red cell mass is established within 2 or 3 months after hypophysectomy. Following the operation, for example, at day 10, the metabolic requirement of the animal is reduced but the new red cell mass equilibrium has not yet been achieved. The animal is therefore essentially comparable to an animal made polycythemic by transfusion. Consequently, red cell production falls to a minimum since a plethora of red cells already exists.

The production of erythropoietin probably also falls to a minimum under these circumstances, and, therefore, the administration of anemic plasma to the hypophysectomized animals produces an exaggerated response.

It is obvious from our data that the hypophysis is not directly concerned in the production of erythropoietin since plasma from an anemic hypophysectomized animal produces an excellent response in the hypophysectomized assay preparation. We need not enter into a discussion of whether a specific pituitary factor as suggested by Van Dyke *et al.*(10) and Contopoulos *et al.*(11) is involved directly in red cell production; but on the basis of the data reported in this paper and other data that have not yet been published, it seems likely that the effect of the pituitary on erythropoiesis is only indirect.

The great sensitivity of the hypophysectomized assay preparation to anemic plasma has made it possible for us to explore the presence of the factor(s) (erythropoietin) mediating red cell production in the plasma of normal rats and in other species with various types of anemia. These studies are being extended, and many other obvious facets of the problem, which are being investigated, will be reported at a later date.

Summary and conclusions. Using incorporation of Fe^{59} into newly-formed red cells as index of red cell production, it has been shown that erythropoiesis is gradually reduced, reaching a minimum in rats at 8 to 13 days after hypophysectomy. A factor of 10 exists between incorporation of Fe^{59} into red cells of normal control (37 ± 4) and the hypophysectomized rat (4 ± 2) at this interval. We have also found that the hypophysectomized rat is an extremely sensitive preparation for assay of factor(s) in anemic plasma that stimulates or mediates erythropoiesis. Our observations may be summarized as follows: 1. Administration of anemic plasma to hypophysectomized assay animal increases incorporation of Fe^{59} 3- to 7-fold. 2. Plasma from hypophysectomized animals and unoperated controls made anemic by repeated phlebotomy increases Fe^{59} red cell incorporation to the same extent when administered to the hypophysectomized assay animal. 3. A single injection of 2 ml of anemic plasma to the hypophysectomized assay animal elicits a 2- to 3-fold increase in Fe^{59} incorporation. The mechanism of rapid reduction of erythropoiesis that follows hypophysectomy in rats and its relationship to increased sensitivity of the hypophysectomized animal to anemic plasma are discussed briefly.

1. Grant, W. C., and Root, W. S., *Physiol. Rev.*, 1952, v32, 449.

2. Erslev, A. J., *Blood,* 1955, v10, 954.

3. Bonsdorff, E., and Jalavisto, E., *Acta Physiol. Scandinavica,* 1948, v16, 150.

4. Reisman, K. R., *Blood,* 1950, v5, 372.

5. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F., *J. Lab. Clin. Med.,* 1955, v46, 671.

6. Stohlman, F., Jr., and Brecher, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 1.

7. Gray, S., and Sterling, K., *J. Clin. Invest.,* 1950, v29, 1604.

8. Plzak, L., Fried, W., Jacobson, L. O., and Goldwasser, E., to be submitted for publication.

9. Meyer, O., Stewart, G. E., Thewlis, E. W., and Rusch, H. P., *Folia Haematol.,* 1937, v57, 99.

10. Van Dyke, D. C., Contopoulos, A. N., Williams, B. S., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Acta Haematologia,* 1954, v11, 203.

11. Contopoulos, A. N., Ellis, S., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Endocrinology,* 1954, v55, 808.

Received April 19, 1956. P.S.E.B.M., 1956, v92.

Adrenocortical Properties of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Meticorten) and $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione (Meticortelone).* (22429)

S. TOLKSDORF, M. L. BATTIN, J. W. CASSIDY, R. M. MACLEOD, F. H. WARREN, AND P. L. PERLMAN.†

Biochemistry Department, Schering Corp., Bloomfield, N. J.

The therapeutic usefulness of cortisone and hydrocortisone prompted a search for agents possessing anti-inflammatory actions similar to these steroids but producing a lower incidence of undesirable side effects. Efforts in these laboratories led to the development of two new steroids, Meticorten ($\Delta^{1,4}$-pregnadiene-17α, 21-diol-3,11,20-trione) and Meticortelone ($\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione), which differ from cortisone and hydrocortisone in possessing an additional double bond between C_1 and C_2 in the A ring(1-3). Their effectiveness in the treatment of rheumatoid arthritis was first demonstrated by Bunim *et al.*(4).

The glucocorticoid potency of Meticorten and Meticortelone was found to be 3 to 4 times greater than that of cortisone and hydrocortisone by 3 different bioassay methods. Further comparison of the adrenocortical activities of the new steroids and their parent substances included 1) the life maintaining action in adrenalectomized rats and 2) the degree of hypercorticism induced in intact rats by prolonged steroid administration as well as the regression of these changes after cessation of treatment. The results of electrolyte excretion studies will be reported separately. Preliminary biological data have been reported previously(5,6). It is the purpose of this paper to present in detail the biological work carried out with Meticorten and Meticortelone.

Methods. 1. *Eosinophil response.* The procedure employed was based on the methods of Speirs and Meyer(7) and Rosemberg and co-workers(8). Steroids were screened in adrenalectomized C_{57} brown, male mice‡ at a dose of 24 μg without pretreatment with epinephrine. Preliminary potency estimates were performed with 3-fold dilutions of Meticorten and Meticortelone (6, 2, and 0.67 μg) and cortisone and hydrocortisone (12, 4, and 1.33 μg). The potency was calculated on

* Meticorten and Meticortelone, Schering Corp. brand of prednisone and prednisolone were used.

† The authors wish to thank Mrs. Shirley W. Bolles, Miss Janet Maley and Mrs. Alide Tali for valuable technical assistance. Mr. Gordon B. Thomas carried out statistical analysis of data, and members of the Chemical Research Division provided the steroids.

‡ Obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me. We wish to thank the members of our Pharmacology Department for performing the adrenalectomies.