5. Elton, R. L., and Zarrow, M. X., *Anat. Rec.*, 1955, v122, 473.

6. Slover, G. A., Master's Thesis, Oklahoma State University, 1955.

7. Stamler, J., Bolene, C., Katz, L. N., Harris, R., and Pick, R., *Fed. Proc.*, 1950, v9, 121.

9. Wiseman, B. K., PROC. SOC. EXP. BIOL. AND MED., 1931, v28, 1030.

10. Shapiro, A. B., and Schechtman, A. M., *ibid.*, 1949, v70, 440.

11. Weller, E. M., and Schechtman, A. M., *ibid.*, 1949, v72, 370.

12. Dougherty, T. F., and White, A., *Endo.*, 1944, v35, 1.

13. Dougherty, T. F., and Kumagai, L. F., *ibid.*, 1951, v48, 691.

Received October 25, 1957. P.S.E.B.M., 1957, v96.

Probable Polypeptidic Nature of Erythropoietin.* (23555)

W. ROY SLAUNWHITE, JR., E. A. MIRAND AND T. C. PRENTICE
Roswell Park Memorial Institute, Buffalo, N. Y.

Previous work from this laboratory has been concerned with the erythropoietic stimulating effects of plasma extracts from anemic animals and human subjects(1-4) and from hypoxic hypoxic animals(5). The present communication describes certain chemical and physical properties of erythropoietic stimulating factor (EPF) from anemic rabbits. It is hoped that further study of this factor may reveal information relevant to the isolation and purification of the factor.

Materials and methods. Preparation of plasma extracts. New Zealand rabbits were made anemic by daily subcutaneous injections of 1 ml of neutralized (with NaOH to pH 7.0-7.5) (2.5%) aqueous solution of phenylhydrazine hydrochloride until the rabbits' hemoglobin was less than 5 g %. Plasma drawn in heparin (20 mg 100 ml blood) was pooled and frozen at −5°C until approximately 1 l was collected. It was then deproteinized by a modification of the method of Borsook *et al.*(6). The frozen plasma was melted and adjusted to pH 5.5 with 1 N HCl. It was then deproteinized in batches of 250 ml by placing in boiling water for 15 min. The precipitate, which was removed by filtration, was washed with 400 ml of boiling distilled water which was again placed in boiling water for 5 min. before filtration. The filtrates and washings were combined and concentrated *in vacuo* in a flash evaporator to ⅓ the original plasma volume (T < 55°C). Control plasma was obtained from normal untreated rabbits and deproteinized in the same manner. Extracts which received only this treatment are called "pH 5.5 extracts". Some extracts were treated further with trichloroacetic acid (TCA extracts) or by heating at pH 9 (pH 9 extracts). The preparation of the TCA extract was done essentially according to the method of Borsook *et al.*(6). To 1 volume of pH 5.5 extract at 5°C was added 0.1 volume of 50% TCA. After filtration the filtrate was extracted 3 times with equal volumes of ether to remove the TCA. Dissolved ether was removed in the flash evaporator. It was then dialyzed in 36/32" Nojax casing against running tap water for 48 hrs. (T = 9°C). An easier procedure which, however, removed less protein was afforded by heating at pH 9. Alkali (1 N NaOH) was added to the pH 5.5 extract until pH 9 was reached. The solution was then placed in a boiling water bath for 5 min. After the small amount of precipitate had been removed by centrifugation, it was dialyzed against running tap water. *Dialysis.* Dialysis was performed at 5°C against an equal volume of 0.16 M NaCl for 48 hrs. Plasma extract was contained in 20/32" Nojax cellulose casing. Both the dialysate and the dialysand (inside the casing) were tested.

* The authors express their thanks to Ruth Deckert, André Bulba, Helen Fox, Edward Dywinski, Jane Shoemaker, Gregory Makohon and Dr. Donald Pochedley. This investigation was supported in part by research grants from Natl. Cancer Inst., USPHS and the Atomic Energy Com.

TABLE I. Effect of Dialysis on Deproteinized Rabbit Plasma Extract (pH 5.5 Extract) Obtained from Anemic Rabbits and Assayed for Erythropoietin Activity in Rats Hypophysectomized at 60 Days of Age.

Fraction administered	No. animals	Avg % 24-hr Fe^{59} uptake and stand. dev.*
Exp. No. 1		
Anemic dialysate	5	35.3 ± 6.56
" dialysand	4	75.1 ± 5.88
" undialyzed	5	82.7 ± 6.48
Saline control	5	30.8 ±11.9
Untreated control	5	35.3 ± 7.64
Exp. No. 2		
Anemic dialysate	5	30.1 ± 4.12
" dialysand	4	72.9 ± 4.66
" undialyzed	5	69.2 ± 3.9
Saline control	5	37.9 ± 8.76
Untreated control	4	33.4 ± 6.65

* Hematocrits and reticulocyte counts were normal for 60-day-old hypophysectomized rats in every case and showed no consistent alteration as a result of EPF administration.

Effect of pH. Separate portions of plasma extract were adjusted to pH's 1, 9 and 13 and placed in boiling water for 5 min. After removal of the precipitate by centrifugation, the pH of the supernatant was returned to 5.5. *Effect of oxidation-reduction.* Mild oxidation was attempted by bubbling oxygen through plasma extract for 1 hr. Similarly, the effect of a mild reducing agent was tested by making the solution 1% with respect to cysteine. *Extractability.* Plasma extract was extracted twice with equal volumes of chloroform, breaking the emulsion formed by centrifugation. Dissolved chloroform was removed from the aqueous fraction in the flash evaporator. Evaporation of the organic layer left a greasy residue showing that this step did remove some material. This was not tested. *Enzymatic digestion.* Separate portions of a dialyzed pH 9 extract were incubated with pepsin at pH 2.0 and with trypsin and chymotrypsin at pH 7.5 Sufficient crystallized enzyme was added to attain a concentration of 0.6-0.8 mg/ml. Incubation was continued for 4 hrs. at 37°C. Controls contained no enzyme. One-half of each enzymatic digest was dialyzed against running tap water for 48-72 hrs. *Bioassay of erythropoietin.* Adult Sprague-Dawley rats aged 2-3 months and ranging in weight from 200-225 g were hypophysectomized. They were maintained on a diet consisting of Purina Laboratory Chow and bread and milk. Either 15 or 60 days after hypophysectomy each rat received 2 cc of plasma extract daily for 3 successive days subcutaneously. Twenty-four hours after the last plasma injection 1 μc of Fe^{59} in 1.0 ml of saline was given intravenously via jugular vein and 24 hours thereafter the rats were bled from the dorsal aorta. Radioactivity in the sample was measured in a Nancy Wood well-type scintillation counter. Radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Calculation of Fe^{59} uptake into red cells was carried out as described by Elmlinger *et al.* (7). Precaution was taken after each experiment to run hematocrits. Any animal with low hematocrit was not included in our results since animals with low hematocrits tended to exhibit higher iron uptakes.

Results. The results of the dialysis experiments are shown in Table I. Although employment of 60 day post-hypophysectomized rats caused the control values to be quite high, it is apparent ($P < 0.01$) that EPF did not dialyze through the membrane in detectable quantities.

From Table II it is obvious that EPF is unstable at pH 1 and 13 at 100°C. After this treatment there was little activity left in either the supernatant or the precipitate (data not shown). EPF was much more stable at pH 9 at 100°C, for while there was a statistically significant decrease in activity when compared to that of the untreated pH 5.5 extract, the drop was not pronounced. EPF is not markedly affected by oxidation-reduction or by extraction with chloroform. As can be seen from Table II there was a barely significant decrease in activity after these treatments. A TCA extract was also tested in a similar manner with substantially the same results.

The effect of enzymatic digestion on bioactivity is shown in Fig. 1. Pepsin, trypsin and chymotrypsin caused a pronounced decrease in activity as compared to their controls at the same pH. Upon dialysis of the digestion mixture activity rose in all 3. In

TABLE II. Effect of Various pH's, O_2, Cysteine and Chloroform Extraction on Erythropoietin Activity in pH 5.5 Extract Assayed in Adult Rats 15 Days after Hypophysectomy.

Treatment	No. animals	Avg % 24-hr Fe^{59} uptake and stand. dev.*
Plasma from anemic rabbits		
Supernatant from pH 1, 100°C, 5 min.	6	11.2 ± 7.09
Supernatant from pH 9, 100°C, 5 min.	7	46.1 ± 2.92
Supernatant from pH 13, 100°C, 5 min.	7	10.0 ± 6.42
O_2 bubbled through for 1 hr, 25°C	7	48.6 ± 3.18
1% cysteine added	6	47.6 ± 8.68
Aqueous fraction from chloroform extraction	7	48.8 ± 8.53
pH 5.5 extract, untreated	6	55.7 ± 7.03
Plasma from normal rabbits		
pH 5.5 extract	4	7.4 ± 4.33
Untreated controls	10	2.7 ± 1.73

* Hematocrits and reticulocyte counts were normal for 15-day hypophysectomized rats in every case and showed no consistent alteration as a result of erythropoietin administration.

the case of trypsin the rise was not statistically significant, but in the case of pepsin and chymotrypsin it was ($P < 0.05$ and < 0.01 respectively). Parenthetically, the pH 9 extract again possessed less activity than the pH 5.5 extract.

Discussion. The evidence indicates that EPF is a low molecular weight protein, or polypeptide, perhaps in the range of insulin or ribonuclease. Like ribonuclease(8), corticotrophin(9) and pituitary EPF(10) it is stable to high temperatures in weakly acidic or alkaline solution and like corticotrophin (11) it is stable to and not precipitated by TCA in the cold. However, it is inactivated by heat in strongly acidic or alkaline solution.

Like oxcorticotrophin and ribonuclease, EPF does not dialyze appreciably in water or saline solution at neutral pH. Dialysis against dilute acetic acid which markedly increases the dialyzability of corticotrophin(9), has not been performed yet. Van Dyke *et al.* (10) also found pituitary EPF to be nondialyzable.

It is not certain how stable EPF is to oxidation and reduction. Although the data in-

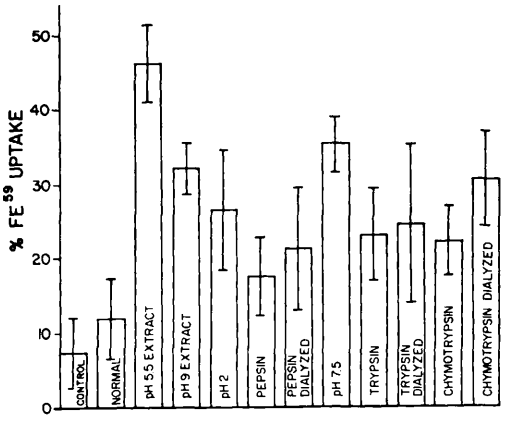

FIG. 1. Erythropoietic activity of various plasma extracts before and after treatment with proteolytic enzymes (see text for details). Averages and stand. dev. of 3 separate experiments are presented.

dicate a fair degree of stability, the presence of large amounts of protein may exert a modifying influence. Levels in plasma expressed as g% protein were: protein, 13; NPN, 30. Levels of total protein in various fractions were as follows: pH 9 extract, 77; TCA, extract, 32; pepsin treated and dialyzed, 25; trypsin treated and dialyzed, 8; chymotrypsin treated and dialyzed, 28. The lack of extractability with chloroform eliminates the possibility of EPF being a lipid.

All of the relationships discussed above are consonant with EPF being either a polysaccharide or a polypeptide. The digestion of EPF by proteolytic enzymes is in accord only with the latter possibility. However, the possibility of EPF being a glycoprotein or a lipoprotein is not excluded. The rise in bioactivity after dialysis of the digestion mixture is interesting. While such observations remain unexplained, conclusions regarding the polypeptidic nature of erythropoietin should be tentative.

Summary. Plasma from anemic rabbits containing erythropoietic stimulating factor was treated in numerous ways in order to determine some of the properties of the factor. Assay was performed by determining the Fe^{59} uptake in hypophysectomized rats. Since the factor remained after heating at pH 5.5 or pH 9 but was destroyed at pH 1 and pH 13 and since it was nondialyzable yet digested by pepsin, trypsin and chymotrypsin, it was

concluded that erythropoietin is probably a polypeptide. In the presence of the non-precipitable proteins (by heat) of plasma, it is fairly stable to mild oxidation and reduction.

1. Prentice, T. C., and Mirand, E. A., *Exp. Med. and Surg.*, 1956, v14, 226.
2. Mirand, E. A., and Prentice, T. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 473.
3. ———, *Blood*, 1957, v12, 993.
4. Mirand, E. A., and Prentice, T. C., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 164.
5. ———, *ibid.*, 1957, v96, 49.
6. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *Blood*, 1954, v9, 734.
7. Elmlinger, P. J., Huff, R. L., and Oda, J. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 16.
8. Jones, W., *Am. J. Physiol.*, 1920, v52, 203.
9. Dedman, M. L., Farmer, T. H., Morris, P., and Morris, C. J. O. R., *Rec. Prog. Hormone Res.*, 1952, v7, 59.
10. Van Dyke, D. C., Simpson, M. E., Contopoulos, A. N., and Evans, H. M., *Blood*, 1957, v12, 539.
11. White, W. F., *J. Am. Chem. Soc.*, 1953, v75, 503.

Received July 31, 1957. P.S.E.B.M., 1957, v96.

Factors Affecting Development of Hypertensive Vascular Disease After Renal Injury in Rats.* (23556)

ALVIN P. SHAPIRO† AND JULIAN MELHADO (Introduced by I. Arthur Mirsky)
Department of Internal Medicine, University of Texas, and Department of Clinical Science, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

Hypertensive vascular disease may result from various types of renal injury in the experimental animal and often is associated with primary renal disease in the human, but the responsible mechanisms remain unclear. Furthermore, following apparently identical renal injury, incidence and severity of hypertension show considerable variation; *i.e.*, in rats after figure-of-eight tie of one kidney and contralateral nephrectomy, hypertension ranging from mild and chronic form to severe and rapidly fatal disease may develop(1,2). Whether this variability is related directly to extent of renal injury is not readily apparent, since precise studies of its relationship to degree of impairment of excretory renal function, to hypertrophy of remaining renal tissue, or to other factors, have not been reported following this particular injury, and studied in only a limited fashion with other technics for renal injury in rats(3-5). The present study was undertaken to investigate this relationship of renal injury to severity in rats with a figure-of-eight tie of one kidney and contralateral nephrectomy. In addition, the effect of a behavioral disturbance devised to produce a chronic stress for the rat was evaluated.

Materials and methods. Fifty-four male Holtzman rats weighing 150 to 250 g were subjected to "figure-of-eight" tie of the right kidney and, a week later, to a left nephrectomy, according to the technic described by Grollman(1). The animals were lodged in groups of 8 to 10/cage and allowed free access to food and water. Under light ether anesthesia, systolic blood pressure was determined biweekly by tail plethysmographic method(6,7). Renal function was studied prior to sacrifice by testing phenosulfonphthalein (PSP) excretion and urine concentrating ability. PSP excretion was measured by injection of 10 ml of sterile 0.2% saline, intraperitoneally, followed one hour later by a second 10 ml with 1.5 mg of PSP dye; urine was collected over a 3-hour period and percentage of dye excreted determined spectrophotometrically. To determine urine concentration, rats were kept in metabolic cages without food and water for 48-hour period and osmolal concentration of samples col-

* Supported by PHS Grant and Hogg Foundation for Mental Hygiene.

† Present address: Department of Clinical Science, University of Pittsburgh School of Medicine, Pittsburgh, Pa.