5. van Cauwenberge, H., Jaques, L. B., *Can. Med. Assn. J.*, in press.
6. Dale, D. U., Jaques, L. B., *ibid.*, 1942, v46, 546.
7. Lepp, E., Chubaty, W., Jaques, L. B., *Can. J. Med. Sc.*, 1953, v31, 173.
8. Jaques, L. B., Mogenson, G. J., Fisher, L. M., *Trans. Roy. Soc. Can. Third series*, 1956, v50, Sec. V, 9.
9. Bounameaux, Y., van Cauwenberge, H., Roskam, J., *Schweiz. Med. Wchschr.*, 1954, v84, 789.

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Purification of Erythropoietin by Ion-Exchange Chromatography.* (24120)

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We reported(1,2,3) that erythropoietic activity of acidified, boiled plasma filtrates prepared from phenylhydrazine anemic rabbits was associated with a protein having paper electrophoretic mobility of an alpha globulin and chemical characteristics of an acid glycoprotein. Small molecular weight, acidic glycoproteins are found in Cohn Fraction VI(4). In preliminary work with plasma from phenylhydrazine anemic rabbits and from a patient with polycythemia vera we found erythropoietic activity in Fraction VI (unpublished). The present study was therefore designed to isolate, by ion-exchange chromatography, acidic glycoproteins from the crude source of boiled anemic rabbit plasma.

Materials and methods. Acidified, boiled plasma filtrate (APF) obtained from phenylhydrazine anemic, adult, white male rabbits was prepared by modification of method of Gordon(5) as previously described(3). The filtrate was dialyzed in the cold 48 hours against repeated changes of distilled water and then lyophilized. One liter of plasma yielded 500 to 600 mg of lyophilizate. The lyophilizate, which possesses erythropoietic activity by our previously described method (3), was fractionated on diethylaminoethyl (DEAE) cellulose ion-exchange columns(6). Two separation procedures (I,II) were utilized. Procedure II was designed for a more definitive fractionation on the basis of results obtained with procedure I. The separation experiments are summarized in the elution diagrams in Fig. 1 and 2. Fraction pools as designated in Fig. 1 were dialyzed, lyophilized and redissolved in 0.9% NaCl. Along with unmodified APF starting material, and control saline injections, these fractions were assayed for erythropoietic activity. Fraction pools as designated in Fig. 2 were assayed directly, using a solution of 0.9% NaCl in 0.01 M sodium acetate buffer as a control injection. Female, Sprague-Dawley rats (average weight, 220 g) were injected subcutaneously daily for 4 days with 2 mg by dry weight (in 2 ml 0.9% NaCl) of the respective fractions from procedure I. The fractions from procedure II were injected on a similar schedule, with a daily dose of 0.5 mg by protein content. Protein content was estimated on the effluent fraction pools by the ratio of optical density measurements at 280 and 260 mμ according to the method of Warburg and Christian(7). On day 4, the animals were given an intravenous injection of 0.5 μc iron-59 as iron citrate contained in 0.25 ml of solution. The per cent of injected dose of iron-59 appearing in the total red cell mass in 24 hours was estimated by standard scintillation counting technics on whole blood obtained by aortic exsanguination under ether anesthesia. The total red cell mass was calculated from the microhematocrit assuming an average blood volume of 5.18 ml/100 g body weight(3). Prior to sacrifice, blood from tail vein was obtained for reticulocyte enumeration by the dry cresyl-blue method.

Results. Erythropoietic activity of isolated

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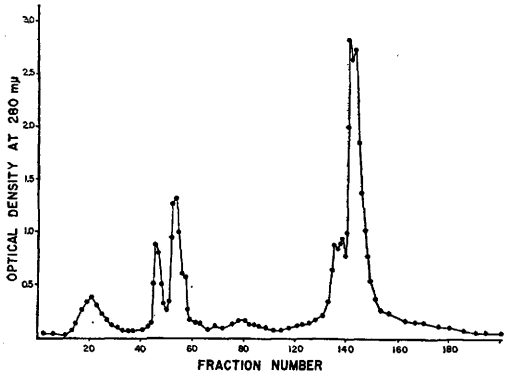

FIG. 1. Effluent diagram, procedure I. 500 mg APF dissolved in 30 ml 0.01 M sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (40 × 2.5 cm) which had been partially equilibrated to pH 6.1 with 0.01 M acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1-77. Gradient started at fraction 77 by continuous introduction of 0.01 M acetate buffer, pH 4.8 plus 0.25 M NaCl into a constant volume reservoir of 200 ml 0.01 M acetate buffer. Column operated at constant temperature of 25°C. Flow rate 0.65 ml/min. and fraction vol 6.5 ml. Fraction pool 44-56 (Fraction A), 132-138 (Fraction B), 142-150 (Fraction C).

fractions is presented in Tables I and II. Fraction A, washed from column in the solvent buffer, and Fraction B showed no erythropoietic activity. The small peak appearing in the elution diagram before Fraction A was also inactive, but was tested in only 2 animals. Fraction C contained the majority of erythropoietic activity and was more potent than the starting material. In 0.15 M NaCl the peak of Fraction C was homogeneous in the ultracentrifuge. The centrifuge data suggested a molecular weight of about 10,000. By free electrophoresis, barbiturate buffer pH 8.6, ionic strength 0.1, this peak yielded 3 components with mobilities (μ x 10^5) of –6.31, –5.23 and –4.49. These mobilities correspond to the mobilities of the alpha globulins.

In procedure II erythropoietic activity was almost entirely confined to Fraction G. The minimal response induced by Fraction F suggests that it is contaminated by a small amount of the active fraction. From the character of the effluent diagram, it appears that the material in Fraction G is homogeneous. On paper electrophoresis in veronal buffer pH 8.6, ionic strength 0.075, it migrates as a single component with a mobility between that of alpha-2 and alpha-1 globulin. It stains as a glycoprotein. From its behavior on the DEAE-cellulose column it has a low isoelectric point. Its small molecular size is indicated by the failure to sediment activity when APF is centrifuged at 103,000 g for 24 hours. Its protein content as estimated by the method of Warburg and Christian is 69.3%. Its sialic acid content as determined by Winzler's modification of the method of Ayala, *et al.*(9) is 15.6%. Its hexose content by the anthrone reaction is 7.7% as glucose equivalent, and its glucosamine content by modification of the method of Elson and Morgan(9) is 10%. Preliminary studies suggest that the glucose content is low, and that at least a part of the hexose is present as galactose. The sialic acid content of the material is relatively high when compared with that reported for other serum glycoproteins(8),

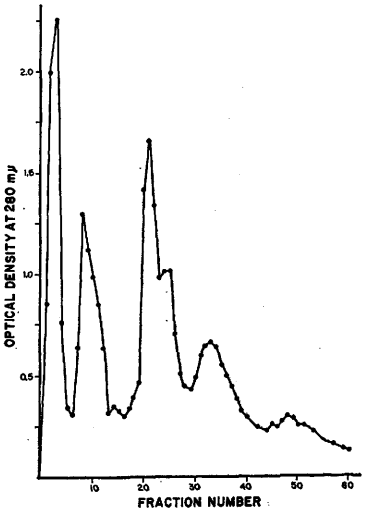

FIG. 2. Effluent diagram, procedure II. 300 mg APF dissolved in 20 ml 0.01 M sodium acetate buffer, pH 4.8. Solution clarified by centrifugation and supernate passed through a DEAE-cellulose column (30 × 1 cm) which had been partially equilibrated to pH 6.0 with 0.01 M acetate buffer, pH 4.8. Column washed with buffer and wash collected in fractions 1-13. Gradient started at fraction 13 by continuous introduction of 0.01 M acetate buffer, pH 4.8 plus 0.25 M NaCl into a constant volume reservoir of 125 ml 0.01 M acetate buffer. Column operated at constant temperature of 5°C. Flow rate 0.08 ml/min. Fraction vol: fractions 1-13, 6.5 ml; fractions 14-40, 3.5 ml. Fraction pool 1-4 (Fraction D), 7-12 (Fraction E), 19-26 (Fraction F), 30-40 (Fraction G).

and is probably responsible for its low isoelectric point. Fraction F contains 9% sialic acid, while Fractions D and E contain 7%.

Fraction G was obtained in a yield of 16 mg. Calculations based on amount of starting material indicate that the active fraction stimulates erythropoiesis when injected in 50 μg quantities. Preliminary dose response data using Fraction G suggest that the injection of 10 μg per day will induce a measurable erythropoietic response.

This separation and isolation of erythropoietin is reproducible, and similar results have been obtained from different lots of starting APF material. Studies are currently underway to further characterize this erythropoietic factor.

Summary. A method for purification of erythropoietin from the filtrate of acidified, boiled plasma prepared from phenylhydrazine anemic rabbits utilizing DEAE-cellulose ion-exchange columns has been described. The active erythropoietic factor thus prepared has been partially characterized and shown to be a low molecular weight, acidic glycoprotein.

TABLE I. Erythropoietic Activity of Effluent Fractions, Procedure I.

Fraction pool	Reticulocytes (%)*			Fe^{59} incorporation in RBC (%)*		
A	2.1	.4	(4)	26.7	4.3	(7)
B	1.6	.5	(4)	27.4	10.4	(4)
C	*6.1*	*1.5*	(4)	*50.5*	*2.2*	(9)
APF	*3.4*	*.6*	(4)	*39.9*	*4.4*	(4)
Saline control	1.8	.3	(4)	21.0	3.6	(7)

* Mean ± S.D.

Figures in parentheses indicate No. of animals in group.

Italicized values statistically significant, P = .01 or less.

TABLE II. Erythropoietic Activity of Effluent Fractions, Procedure II. (Four animals/group.)

Fraction pool	Reticulocytes (%)*		Fe^{59} incorporation in RBC (%)*	
D	2.7	.6	28.3	6.6
E	2.5	.4	26.9	4.5
F	2.6	.4	*29.6*	*1.0*
G	*6.5*	*1.3*	*47.6*	*2.5*
APF	*3.5*	*.5*	*40.7*	*6.9*
NaCl-acetate control	2.3	.4	21.0	2.6

* Mean ± S.D.

Italicized values statistically significant, P = .01 or less.

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1. Rambach, W. A., Cooper, J. A. D., Alt, H. L., *J. Lab. & Clin. Med.*, 1956, v48, 933.

2. ———, *Proc. VI Internat. Cong. Internat. Soc. Hem.*, Grune & Stratton, N. Y., p773, 1958.

3. Rambach, W. A., Alt, H. L., Cooper, J. A. D., *Blood*, 1957, v12, 1101.

4. Schmid, K., *J.A.C.S.*, 1955, v77, 742.

5. Gordon, A. S., Piliero, S. J., Kleinberg, W., Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255.

6. Peterson, E. A., Sober, H. A., *J.A.C.S.*, 1956, v78, 751.

7. Warburg, O., Christian, W., *Biochem. Z.*, 1941, v310, 384.

8. Winzler, R. J., *Methods of Biochemical Analysis*, vII, Interscience Publishers, N. Y., p299, 1955.

9. Palmer, J. W., Smyth, E. M., Meyer, K., *J.B.C.*, 1937, v119, 491.

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Method for Studying Translocation of Some Radioisotopes into Organs from Site of Injection.* (24121)

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Therapeutic application of a radioisotope is outlined by knowledge of its fate in the body. This information is acquired by testing amount of radioactivity in organs of animals

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