TABLE I. Simultaneous Inulin and B_{12} Clearances in 10 Subjects by Individual Periods at Plasma Levels of 12-26 mμg B_{12}/ml.

Subject	Age	Clearance, ml/min.	Period I	II	III	IV	Mean clearance
Bn	17	Inulin	110.3	114.9	107.9	113.2	111.6
		B_{12}	122.5	121.4	133.8	131.6	127.3
Df	35	Inulin	131.1	134.4	122.3	114.7	125.6
		B_{12}	99.6	124.1	115.8	143.5	120.8
Cn	51	Inulin	160.5	140.7	128.7	141.5	142.8
		B_{12}	147.8	127.4	130.7	154.5	140.1
Ti	52	Inulin	51.9	49.7	56.1	47.2	51.2
		B_{12}	63.7	55.3	58.3	76.6	63.5
Le	53	Inulin	103.3	108.0	107.3	104.7	105.8
		B_{12}	86.4	115.0	83.0	117.3	100.4
Po	59	Inulin	73.4	76.1	68.5	76.1	73.5
		B_{12}	85.0	80.4	78.3	82.9	81.6
Ro	64	Inulin	99.5	98.5	94.7	89.5	95.6
		B_{12}	134.2	107.2	98.7	116.6	116.7
Sm	75	Inulin	50.8	50.8	51.5	53.1	51.6
		B_{12}	46.1	56.9	61.8	44.7	52.4
Fv	77	Inulin	66.6	84.1	78.3	80.1	77.3
		B_{12}	86.9	88.6	94.4	100.9	92.7
Mu	88	Inulin	25.8	26.8	25.5	25.9	26.0
		B_{12}	34.4	37.5	38.6	27.4	34.5

tration rate. 3. No relation of renal clearance of B_{12} with advancing age was noted except for that resulting from the gradual reduction in glomerular filtration rate with age.

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Received March 16, 1961. P.S.E.B.M., 1961, v107.

Partial Purification of Erythropoietin by Alcoholic Fraction. (26584)

W. C. GRANT, W. H. LINKENHEIMER, E. L. PATTERSON AND H. BERGER
Veterans Administration Hospital, Coral Gables, Fla. and American Cyanamid Co., Pearl River, N. Y.

A number of methods for concentration of the erythrocyte stimulating factor or erythropoietin in the plasma of anemic rabbits have been devised. Chemical methods of varying complexities have been employed and assays have been performed on both normal and abnormal animals(1). The method to be described utilized simple fractionation procedures and all assays were performed on normal adult albino rats. A preliminary report was made in 1958(2).

Methods. Production of anemia. Adult

male New Zealand rabbits 2-3 Kg were injected s.c. daily with 0.5 to 1.0 ml of a 2.5% solution of phenylhydrazine. Since the most active plasmas were obtained from animals with hematocrit values of 10-12% from 5-7 days after start of injections, it was necessary to measure the hematocrit values of the rabbits daily to obtain maximal erythropoietic titers. Blood was drawn by cardiac puncture, heparinized, centrifuged and the plasma frozen until used. Apparently heparin did not interfere, since similar activity was found in serum.

Normal male albino rats (Carworth Farms) weighing 180 to 200 g were used in all assays. Hematocrit values of tail blood were measured using Van Allen tubes and reticulocyte percentages were estimated by counting 1000 cells after staining with New Methylene Blue.

Approximately 0.3 mc of Fe^{59} (as $FeCl_3$ in 0.3 ml) was injected into the saphenous vein of the rat 24 hours after the plasma fraction or saline as control had been given by i.p. injection. Eighteen hours after Fe^{59} injection, 0.1 ml of blood was obtained from the tail, cells were separated, washed in saline and counted in a scintillation counter. Iron uptake values were expressed as per cent of total iron administered. In all Fe^{59} uptake studies, at least 4 rats were used in each group.

Alcohol fractionation. Plasma obtained from anemic rabbits was adjusted to pH 5.5, boiled for 15 minutes and filtered as described by Borsook(3). The opalescent filtrate was adjusted to pH 7.3 and ethanol added to 60% by volume. The alcohol was added slowly with stirring and temperature was maintained at 25°C. After standing 60 minutes, the white flocculent precipitate was easily separated by centrifugation and discarded. Ethanol was added to the supernatant to 80% concentration. The resultant 60-80% ethanol precipitate was separated by centrifugation after 30 minutes and was quite active. It was further purified by dissolving in water, adjusting to pH 5.5 and adding ethanol to 50% concentration. The precipitate was removed by centrifugation and discarded. Ethanol was added to the supernatant to achieve a concentration of 80%. This active 50-80% precipitate was separated by centrifugation after 30 minutes standing. By narrowing the alcohol range to 70 to 75%, for example, it was possible to achieve a greater degree of purity although a considerable loss of substance was experienced. For injection, all fractions were dissolved or suspended in saline up to a volume equivalent to that of the original plasma filtrate and injection volumes were maintained constant.

Various organs obtained from anemic rabbits were prepared for assay by grinding with an equal volume of saline, adjusting to pH 5.5, boiling for 15 minutes and filtering. The filtrate was neutralized and injected into rats as were the plasma fractions.

Results. Rats were injected s.c. 6 days a week for 4 weeks with 1 ml of the plasma filtrate obtained from anemic rabbits, 0-60% ethanol precipitate, 60-80% ethanol precipitate or saline (Fig. 1). In the groups receiving the plasma filtrate and the 60-80% precipitate significant increases in both reticulocytes and hematocrit values occurred. No significant changes appeared in the groups receiving either saline or 0-60% ethanol precipitate. When rats were injected s.c. 6 days a week with the untreated plasma from anemic rabbits, no increase in hematocrit was noted and death occurred in a few weeks. The reaction is probably immunologic and has been noted by Borsook(3) and Gordon (4).

A single i.p. injection of 4 ml of various active plasma fractions 24 hours before Fe^{59}

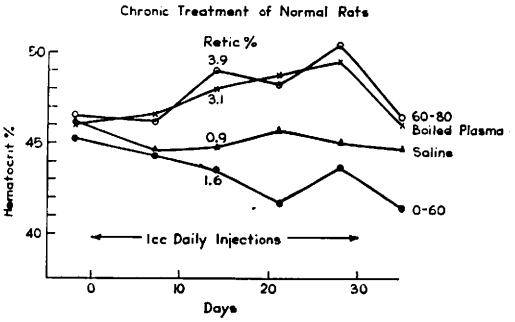

FIG. 1. Effects of boiled plasma filtrate, 60-80% ethanol precipitate, 0-60% ethanol precipitate and saline on normal rats.

TABLE I.

Fractions of anemic rabbit plasma	% Fe^{59} uptake	Amt of solids in fraction inj. (mg)
Control (saline)	33	—
Raw plasma	42	280.0
Boiled plasma filtrate	46	58.0
60-80% ethanol, pH 7.3	46	1.76
50-80% " , pH 5.5	47	.16
70-75% " , pH 7.3	44	.08

injection produced a 30-40% increase in Fe^{59} uptake by circulating red blood cells (Table I). Degree of concentration achieved by these relatively simple fractionation procedures is indicated by the reduction in solids of the various fractions (Table I). Greater purification could be achieved by narrowing the alcohol fractions, but results were not easily reproducible.

Table II lists 30 tissues, organs or body fluids from anemic rabbits which were tested for erythropoietic stimulating activity. Consistent, positive results were not obtained from any of these sources even though the plasmas obtained from the same animals were active. This finding confirms the work of Gordon *et al.*(5).

Despite the crude nature of all fractions, certain properties of the stimulating material could be established. It is water soluble, resists boiling for 15 minutes and is insoluble in 100% alcohol or acetone. It will not dialyze and can not be ultrafiltered across an alundum candle coated with 10% parlodion.

TABLE II. Sources Tested for Erythropoietin Activity.

Bone marrow	Skin	Thyroid
Spleen	Bone	Adrenal
Brain	Erythrocytes	Testis
Kidney	Leucocytes	Ovary
Liver	Embryo	Urine
Skeletal muscle	Thymus	Bile
Stomach	Lymph gland	Lymph
Intestine	Salivary "	Aqueous humor
Lung	Mammary "	Milk
Pancreas	Pituitary	Feces

It is active when administered by the usual parenteral route but inactive by mouth.

Discussion. Ethanol fractionation of the boiled filtrate provided a simple and reproducible means of partial purification of the erythropoietic stimulating principle. Concentration of activity on a weight basis amounted to 3000 fold. An indication of the lack of purity is obtained by comparison of Tiselius electrophoretic migration patterns of the 50-80% ethanol precipitates obtained from normal rabbits and those obtained from anemic animals. Although the material from normal rabbits was inactive, the electrophoretic patterns were indistinguishable.

The use of chromatography columns composed of hydroxyapetite, celite or DEAE cellulose as a means of fractionation was inefficient in our hands. The procedures were elaborate and although some purification was achieved, a large amount of the active material was lost.

Summary. The erythropoietic stimulating principle present in the filtrate of plasma obtained from anemic rabbits was partially purified by ethanol fractionation. This material was active in increasing Fe^{59} intake of *normal* rats and in producing a reticulocytosis and hematocrit elevation in repeatedly injected *normal* rats. Fe^{59} uptake was stimulated by as little as 0.08 mg of the fraction representing a concentration of 3,000 fold over that occurring in the plasma.

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