The Renal Erythropoietic Factor. V. Studies on Its Purification* (32944)

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A renal erythropoietic factor (REF) extracted from the kidneys of hypoxic rats was reported by Contrera and Gordon (1). The REF is located primarily in the light mitochondrial fraction of kidney homogenates (2–4) and is distributed throughout the renal cortex and medulla (2). Studies suggest that the REF is an enzyme, which upon incubation with normal serum *in vitro,* generates erythropoietin (ESF) (4,5).

This report deals with the purification of the REF by ion-exchange chromatography and gel filtration. Reinvestigation was also made of the REF activity among the subcellular fractions of kidney homogenates in order to determine the possible existence of additional sites of REF origin.

Methods. Preparation of subcellular fractions from hypoxic rat kidneys. Long-Evans rats (250–300 gm) were rendered hypoxic by exposure to 0.42 atm in a low pressure chamber for 18 hours. The rats were sacrificed by decapitation immediately after termination of the hypoxic period. The kidneys were minced and then homogenized with an Elvehjem–Potter homogenizer in 10 volumes (w/v) of 0.25 *M* sucrose. Separation of the subcellular fractions was carried out by differential centrifugation at 5°C as follows (6). The homogenate was sedimented at 6300*g* for 15 min (step 1). The supernatant was decanted and centrifuged at 21,000*g* for 30 min (step 2). After separation from the sediment, this supernatant was centrifuged at 37,000*g* for 30 min (step 3). Finally, the supernatant obtained from step 3 was sedimented at 105,000 *g* for 30 min (step 4).

The sediments from steps 1 and 2 were each resuspended in 2 volumes of distilled water per gm of kidney while those from steps 3 and 4 were each resuspended in 1 volume. One drop of a detergent, Cutscum[1] was added to each of these 4 suspensions which were immediately frozen at —20°C. The preparations were subsequently thawed and resedimented at 37,000*g* for 20 min. The freezing and thawing served to disrupt the membranes of the subcellular particles. The supernatants obtained at this stage represented extracts of the nuclear-heavy mitochondrial, light mitochondrial, submicrosomal and microsomal fractions, respectively. In addition, the supernatant obtained from step 4 was designated as the soluble fraction. Each fraction was assayed for REF activity. The amount of protein contained in each fraction was determined by the Biuret method (7).

Purification of the REF in the light mitochondrial extract. I. *Ammonium sulfate precipitation.* Ammonium sulfate was added gradually to the light mitochondrial extract at 5°C until a 30% saturation was attained. After standing for an hour in the cold, the protein suspension was centrifuged at 6000*g* for 20 min. The precipitate was separated and the supernatant was brought to 70% saturation with ammonium sulfate. The suspension was allowed to stand in the cold for an hour and further sedimented at 6000*g* for 20 min. Both precipitates were redissolved in a small volume of distilled water and dialyzed against cold distilled water overnight.

II. *Fractionation by diethylaminoethyl (DEAE) cellulose ion-exchange chromatography.* Microgranular DEAE (DE-52) cellulose[2] was neutralized with 1 N HCl, and packed in a 2 × 30-cm column. The column was equilibrated overnight with 0.005 *M* phosphate buffer, pH 6.8. The dissolved material recovered from the 30–70% saturation fraction of the ammonium sulfate precipitations was applied to the column and eluted by discontinuous gradients of NaCl solutions at concentrations of 0.02, 0.1, 0.2, 0.3, and 1.0 *M* all prepared in 0.005 *M* phosphate buffer,

* Supported by USPHS Grant 2-R01-HE03357-11.
[1] Fisher Scientific Company, Union, New Jersey.
[2] Reeve Angel Co., Clifton, New Jersey.

pH 6.8. The protein content of 9.0-ml volumes of the eluates was indicated by the UV absorbance at 280 mμ. The column flow rate was maintained at 45 ml/hour by applying pressure with nitrogen from a cylinder. Each protein fraction was dialyzed overnight against distilled water, and its volume was reduced by evaporation in the cold.

III. *Fractionation by Sephadex G100 gel filtration.*[3] A 4 × 25-cm Sephadex G 100 column was equilibrated with 0.05 M phosphate buffer, pH 6.8. The REF-active fraction from the DEAE cellulose chromatography was then applied to this column for further purification. The REF was eluted with the same 0.05 *M* phosphate buffer at a flow rate of 40 ml/hour. The protein content of 9.0-ml eluate fractions was traced by UV absorbance at 280 mμ. The protein fractions were dialyzed against distilled water and their volumes reduced by evaporation at 5°C. The amount of protein in each fraction of the various purification steps was determined by the method of Lowry (8).

Assay of REF activity. The REF activity was determined by incubating 6 ml of each of the fractions with an equal volume of EDTA-dialyzed normal rat serum at 37°C for 1 hour (3). Two ml of each of the incubation mixtures were injected intraperitoneally into ex-hypoxic polycythemic mice to determine erythropoietic activity (4,9). Five mice were used for assay of each sample. One unit of REF activity was defined as that amount which, after incubation for 1 hour with an equal volume of a standard sample of EDTA-dialyzed normal rat serum, would generate one International Reference Standard unit of ESF activity in the assay mice.

Electrophoresis. The REF-active fractions obtained from DEAE-cellulose chromatography and Sephadex G 100 gel filtration were also subjected to electrophoresis. This was carried out with Sepraphore III cellulose polyacetate strips using a barbiturate buffer at pH 8.6 in the Gelman electrophoretic apparatus.[4]

Results. The REF activity was found in extracts of the light mitochondrial and microsomal fractions of hypoxic rat kidneys. No activity was detected in the nuclear-heavy mitochondrial, submicrosomal and soluble fractions. Calculations indicated that 78% of the total activity in the kidneys was located in the light mitochondrial fraction with the remaining activity in the microsomal fraction (Table I).

TABLE I. Distribution of REF Activity Among Subcellular Fractions of Kidney.

Six ml of each fraction were incubated with an equal volume of EDTA-dialyzed normal rat serum followed by assay for ESF content. Values indicated are means ± SEM of 4 separate experiments.

Subcellular fraction (*g*)	% of REF activity
Nuclear-heavy mitochondrial (6300)	0
Light mitochondrial (21,000)	78.0 ± 7.7
Submicrosomal (37,000)	0
Microsomal (105,000)	23.0 ± 4.3
Soluble (supernatant after 105,000)	0

The REF was successfully purified by sequential ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography and Sephadex G 100 gel filtration. The data show that the REF activity was associated with the fraction precipitated in 30–70% saturation of ammonium sulfate. Figure 1

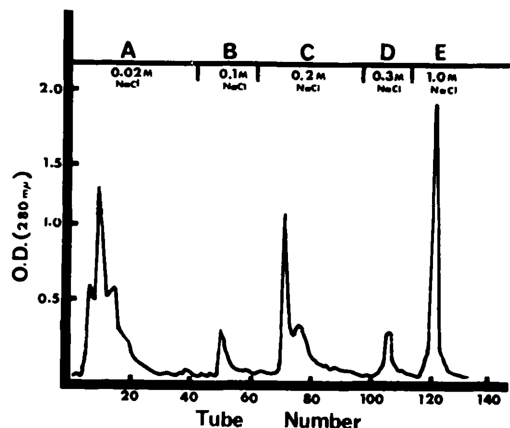

FIG. 1. Elution pattern of REF through DEAE-DE52 column. Material was fractionated with $(NH_4)_2SO_4$ (precipitation between 30–70% saturation prior to the ion-exchange chromatography). The REF activity was found to be associated with the protein fraction eluted with 1.0 *M* NaCl. For specific activity, see Table II.

[3] Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

TABLE II. Purification of REF by Ammonium Sulfate Precipitation, DEAE–Cellulose Column Chromatography and Sephadex Gel Filtration.

The REF activity was calculated by comparison with the sheep plasma ESF standard used in the assay.

	Total protein (mg)	Total REF activity (milli-units/hour)	Specific activity (milliunits/mg per hour)	Purification factor
Light mitochondrial extract	543	6210	11.4	1 X
$(NH_4)_2SO_4$ precipitation, 30–70% saturation	85.8	5400	63.0	5.5X
DEAE–cellulose column chromatography, fraction eluted with 1.0 *M* NaCl	8.98	2940	326.0	28.6X
Sephadex gel filtration, fraction eluted with phosphate buffer, pH 6.8	0.62	1620	2620.0	230 X

indicates the elution pattern from the DEAE cellulose column. A protein fraction was obtained in every elution gradient applied. However, REF activity was found only in the fraction eluted with 1.0 *M* NaCl. This suggests that the protein of the REF is acidic and tightly bound to the DEAE–cellulose. Three protein peaks were observed in the Sephadex G 100 gel filtration (Fig. 2). The REF activity was found to be associated with the second fraction. The total amount of REF

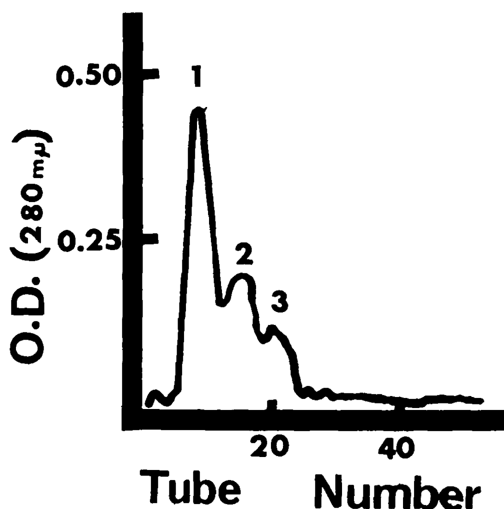

FIG. 2. Elution pattern of REF through gel filtration (Sephadex G 100). Starting material was obtained from DEAE ion-exchange chromatography. The REF activity was found to be associated with the second peak. For specific activity see Table II.

and the specific activity obtained in each purification step in a typical experiment are summarized in Table II. A single band of protein was observed in the electrophoresis pattern for the final active fraction.

Discussion. The present experiments confirm and extend our previous studies (2–5) indicating that the major REF activity is present in the light mitochondrial fraction of kidneys from rats rendered hypoxic for 18 hours. Two possibilities exist for presence of additional REF activity in the microsomal fraction: (a) this activity may have originated in the light mitochondrial fraction but partial breakdown or swelling of the light mitochondrial particles during preparation may have reduced their size or density to those present in the microsomal fraction. This would imply that the microsomal fraction contains modified light mitochondrial particles; (b) the REF activity may have actually originated in the microsomal fraction. Since REF activity was absent from the submicrosomal fraction, which is intermediate between the light mitochondrial and the microsomal fractions, it would seem more likely that the activity observed is actually microsomal in origin.

Summary. The REF is located primarily in the light mitochondrial fraction and, to a lesser extent, in the microsomal fraction of

[4] Gelman Instruments Co., Ann Arbor, Michigan.

kidneys from rats rendered hypoxic for 18 hours. This factor has been purified approximately 230-fold by ammonium sulfate precipitation, DEAE–cellulose column chromatography and Sephadex gel filtration.

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Received Jan. 2, 1968. P.S.E.B.M., 1968, Vol. 128.

Plasma Growth Hormone in the Infant Undergoing Deep Hypothermic Cardiovascular Surgery*† (32945)

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Growth homone in man is involved in fuel metabolism as well as in the promotion of growth (4). Both its stimulatory effect on lipolysis (11) and its anti-insulin effect (4) relate it to lipid and carbohydrate pathways. The effect of growth hormone on free fatty acid mobilization and on insulin suggests that it may have a metabolic role in alterations of heat production. Therefore, the release of growth hormone has potential physiologic importance in hypothermic man.

Utilization of plasma glucose is diminished at low body temperatures in several mammals (23,18), including man (1,6,18). In many situations associated with decreased glucose utilization, the release of growth hormone is increased, as with the administration of 2-deoxy-D-glucose (14) and in diabetic patients with severe ketoacidosis (16). Furthermore, cold stress itself has been found to stimulate the release of growth hormone in nonprimate species (8,9). These observations suggesting increased release of growth hormone under conditions of low body temperature prompted an investigation of plasma growth hormone in patients undergoing deep hypothermic cardiovascular surgery.

This investigation represents a departure from earlier studies (8,9,14,16) in that the subjects were young infants and each was cooled to a body temperature below 20°C. Since hypothermia was surface induced, all had intact cardiovascular systems during cooling, eliminating any effect that might have been contributed by the use of cardiopulmonary bypass.

Methods. Six infants, age 1.5 to 16 months, who underwent cardiovascular surgery were studied (Table I). Corrective heart surgery was performed in all except L.G., who had a palliative operation. Body temperature was lowered to 15–20°C by surface cooling. Hyperventilation with high concentrations of oxygen was used to induce respiratory alkalosis during cooling and rewarming in order to offset the development of metabolic acidosis (10). Deep ether anesthesia was used from

* Supported by a Grant from the Montana Heart Association.

† Presented in part at the International Symposium on Growth Hormone, Milan, Italy, September 13–15, 1967.