Extraction of Erythropoietin from Kidneys of Hypoxic and Phenyldiazine-Treated Rats

By Joseph F. Contrera, James F. Camiscoli, Arthur H. Weintraub, and Albert S. Gordon

The contention that the kidney is a major site of erythropoietin (ESF) production or activation is well supported. Several attempts have been made to extract ESF from kidneys of anemic and normal animals. Renal homogenates obtained at neutral pH from both anemic and normal dogs, rabbits, or rats showed erythropoietic activity although the responses observed with the anemic preparations were generally greater than those with the homogenates derived from normal animals. Boiled, acidified (pH 5.5) homogenates of normal and anemic rat kidney were also effective, but activity in supernates of such homogenates appeared only in preparations from anemic donors. Rambach and co-workers, however, found that supernates obtained at 40,000 g. from both anemic and normal kidney homogenates at neutral pH exerted little erythropoietic effect. This implies that activity was present only in suspensions of these homogenates. All of the above studies employed isotonic extraction media as well as a variety of nonspecific and/or relatively insensitive assay methods; furthermore, the probable contamination of these extracts by the ESF present in trapped renal plasma of anemic animals has been ignored. Finally, the question of whether the erythropoietic activity of these preparations is attributable to the same factor as that found in the plasma is still unresolved.

The relatively low specific erythropoietic activities of kidney homogenates coupled with their tendency to induce irritative effects in assay animals would seem to necessitate better extraction procedures and more careful assay methods. This report presents data on the extraction of renal ESF in a hypotonic medium and its subsequent quantitation through the use of the radioiron-polycythemic mouse assay. This sensitive method permits detection of ESF in quantities as low as 0.05 units.

Materials and Methods

Female Long-Evans rats, ranging in weight from 180–300 Gm., maintained on Purina lab chow in a temperature-controlled room, were employed as donor animals. In Experiment I, 23 rats were injected i.p. with 10 mg. of neutralized phenylhydrazine hydrochloride, daily for 3 days. On the fourth day, each rat received 5 mg. On day 5, the animals were exsanguinated by aortic puncture under ether anesthesia. Hematocrits averaged 20 per cent at the time of bleeding. Thirty-nine Gm. of kidney and 180 ml. of plasma were obtained from these animals. In another experiment (II), 10 rats were...
rendered hypoxic by continuous exposure to a simulated altitude of 23,000 feet for 17.5 hours in a low pressure chamber. A total of 16 Gm. of kidney and 35 ml. of plasma was obtained from these animals within 1 hour after their return to ambient pressure. In experiment III, 52 ml. of plasma and 18 Gm. of kidney were collected from a group of 13 rats 3 hours after discontinuation of the same hypoxic stimulus employed in experiment II. Plasma (42 ml.) and kidney (17.5 Gm.) were also secured from 12 normal rats (experiment IV).

In all cases, blood was immediately centrifuged at 3000 g., the plasma removed, and both the kidneys and plasma were stored at -10 C. until used. Protein concentrations of plasma samples and kidney extracts were measured by the Biuret method.

**Extraction Procedure**

Three ml. of hypotonic 0.02 M phosphate buffer (pH 6.8) were added per Gm. (wet weight) of frozen kidney and the kidneys were homogenized in a Waring blender while still in a frozen state. The homogenate was centrifuged in the cold for 30 minutes at 37,000 g. and the supernate filtered through glass wool. The filtrate was then dialyzed overnight against normal saline at 5 C. and frozen.

**Assay Methods**

Rat standard ESF was prepared from the plasma of phenylhydrazine-treated rats (experiment I). The plasma was fractionated with ammonium sulfate and the 40–80 per cent saturated fraction was collected. This fraction contained most of the erythropoietic activity. After dialysis against normal saline, this material was standardized against an ESF laboratory standard (ELS*) and was found to have a potency of 0.96 units per ml. (95 per cent confidence limits: 64–154 per cent). The protein concentration of the rat standard was 12.3 mg. per ml.

Erythropoietic activity of all preparations was determined by use of the radioiron-polycythemic mouse assay and potency was expressed in terms of units of Erythropoietin Standard A (ESA†). Five to 6 mice were employed at each dose level for all materials tested. The calculation of potencies and other statistical parameters was done according to methods outlined by Bliss.11 Details of these procedures have been described elsewhere.12

**Estimation of Kidney Residual Plasma Volume**

The amount of plasma remaining in excised kidneys was estimated by i.v. administration of approximately 0.4 μc. of I131-labeled bovine serum albumin to 6 normal and 6 hypoxic rats. The latter were rendered hypoxic in the manner indicated for experiment II. Thirty minutes after isotope injection, each animal was bled by aortic puncture and plasma and kidneys collected. The kidneys were then weighed and dissolved in 2M KOH. The radioactivity present in the resultant solution and that present in plasma were determined in a well-type scintillation counter. The residual plasma volume was then computed from the following relation:

\[
\text{Residual Plasma Volume} = \frac{\text{cpm./ml. plasma}}{\text{cpm./Gm. kidney}}
\]

Little difference was found after exsanguination of the rats between the amount of residual plasma present in the kidneys of normal rats (mean of 0.17 ml. per Gm.) and

*ELS—Step 4 sheep plasma erythropoietin (Armour Lot K-103124, originally assaying at 25 units per mg. protein). Preparation supplied by Hematology Study Section of the National Institutes of Health.

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those exposed to hypoxia (mean of 0.21 ml. per Gm.). A value of 0.19 ml. per Gm. (wet weight) of kidney was used for the computation of residual plasma volume in all experiments. This figure is somewhat higher than that obtained by others using the Fe^59-labeled RBC technic^1^ and probably represents a maximum value.

RESULTS

A summary of the results obtained in all four experiments appears in table 1. In experiment IA, 33.6 units of ESF were recovered from 39.0 Gm. of kidney derived from phenylhydrazine-treated rats. Of the total yield, 17.0 units were due to residual plasma activity present in the kidney whereas the remaining 16.6 units were considered to represent true kidney ESF. Little change in total yield (36.0 units) was noted when the renal extracts were reassayed at a later date (experiment IB). Thus, approximately 50 per cent of the total amount of factor extracted represents ESF present in the renal tissue itself.

In experiment II, where the rats were sacrificed 1 hour posthypoxia, 8.10 units of ESF were extracted from 16.0 Gm. of kidney. Since 2.92 units were due to residual plasma activity, 5.18 units or about 64 per cent of the total extractable activity represented true kidney ESF. When rats were sacrificed 3 hours after removal from the low pressure chamber (experiment III), the fraction of true kidney ESF was quite similar to that noted above (about 62 per cent). Plasma levels of the factor in this group (0.46 units per ml.) were about 52 per cent lower than the levels noted in the group bled 1 hour posthypoxia (0.96 units per ml.). The decrement in plasma activity was accompanied by an approximately similar percentage fall in kidney ESF titers: 0.32 units per Gm. in the 1 hour group versus 0.14 units per Gm. in the group bled 3 hours posthypoxia. Both of these values were less than those noted in kidneys of the phenylhydrazine-treated rats (0.42, 0.49 units per Gm.).

Erythropoietic activity was not detected in extracts of kidney from normal rats (experiment IV).

No untoward effects resulted from the administration of renal extracts to assay animals.

When mean responses (per cent RBC Fe^{59} incorporation) were plotted against log dose, no deviations from parallelism (p < 0.001) were found to exist among sheep plasma ESF (ELS), rat plasma standard, and anemic or hypoxic kidney extracts (fig. 1).

DISCUSSION

The present report indicates that significant amounts of ESF can be extracted from anemic or hypoxic rat kidneys homogenized in a hypotonic phosphate buffer. These extracts contained low concentrations of protein as compared to whole homogenate suspensions used by others^5-^9 and were well tolerated by the assay mice. Accurate estimation of the potencies of these preparations is attested to by: (1) the parallelism noted in 2 + 2 dose assays and (2) by the relatively low lambda values obtained in all assays. Although others have previously assayed erythropoietic activity in renal extracts, this report represents the first occasion that such activity has been compared to ESF standards by valid assay methods. The parallelism of the
Table 1.—Erythropoietin Activity in Extracts of Plasma and Kidney from Anemic and Hypoxic Rats

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Assay Design</th>
<th>R.P.* (units/ml.)</th>
<th>95% Limits (%)</th>
<th>Residual Plasma Vol. (ml.)</th>
<th>Residual ESF† (units)</th>
<th>Total ESF Ext.‡ (units)</th>
<th>Kidney ESF Ext.§ (units)</th>
<th>Kidney Wt. (Gm.)</th>
<th>Units ESF per Gm. Kidney</th>
<th>Prot. Conc. (mg./ml.)</th>
</tr>
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<tbody>
<tr>
<td>IA. Anemic</td>
<td>Plasma</td>
<td>2 + 1</td>
<td>2.30</td>
<td>62–168</td>
<td>0.25</td>
<td>—</td>
<td>—</td>
<td>414.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2 + 2</td>
<td>0.28</td>
<td>59–170</td>
<td>0.24</td>
<td>7.41</td>
<td>17.04</td>
<td>33.60</td>
<td>16.56</td>
<td>39.0</td>
</tr>
<tr>
<td>IB. Anemic</td>
<td>Kidney</td>
<td>2 + 1</td>
<td>0.30</td>
<td>76–132</td>
<td>0.28</td>
<td>7.41</td>
<td>17.04</td>
<td>36.00</td>
<td>18.96</td>
<td>39.0</td>
</tr>
<tr>
<td>II. Hypoxic</td>
<td>Plasma</td>
<td>2 + 1</td>
<td>0.96</td>
<td>62–157</td>
<td>0.13</td>
<td>—</td>
<td>—</td>
<td>33.60</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2 + 2</td>
<td>0.18</td>
<td>66–166</td>
<td>0.11</td>
<td>3.04</td>
<td>2.92</td>
<td>8.10</td>
<td>5.18</td>
<td>16.0</td>
</tr>
<tr>
<td>III. Hypoxic</td>
<td>Plasma</td>
<td>2 + 2</td>
<td>0.46</td>
<td>74–138</td>
<td>0.14</td>
<td>—</td>
<td>—</td>
<td>32.20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>2 + 2</td>
<td>0.08</td>
<td>68–148</td>
<td>0.11</td>
<td>3.42</td>
<td>1.57</td>
<td>4.16</td>
<td>2.59</td>
<td>18.0</td>
</tr>
<tr>
<td>IV. Normal</td>
<td>Kidney</td>
<td>2 + 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>17.5</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* R.P. = Relative potency as compared to ELS (step 4 sheep ESF).
† Residual ESF = (residual plasma volume) × (relative potency of corresponding plasma).
‡ Total ESF Extracted = (R.P.) × (volume of plasma or extract).
§ Kidney ESF Extracted = (total ESF extracted) – (residual plasma ESF).
log-dose responses observed with active kidney extracts and the sheep and rat ESF standards suggests that these materials exert similar physiologic effects.

The extract of normal rat kidneys showed no activity, although the protein concentration of this extract was approximately the same as in those derived from hypoxic or anemic rats. There are a number of nonspecific factors which may increase radioiron incorporation in assay animals, such as (1) trapped hemolyzed red cells in tissue extracts, (2) calorigenic effects of injected material, or (3) the lowering of plasma iron levels by the injected material resulting in an increased specific activity of radioiron. Such nonspecific effects, which may have been produced when normal kidney homogenates were given to assay animals, were not observed in the present study. This may be a reflection of differences in assay method and/or the use of the more selective extraction procedure.

It was calculated that 50 to 64 per cent of the activity extracted from the kidneys of anemic or hypoxic rats represents true kidney ESF whereas the balance of the extracted activity is due to contaminating residual plasma. Since a maximum value for residual kidney plasma volume was employed, the actual contribution of true kidney ESF may be somewhat larger and the specific renal activity proportionately higher. A second extraction of the kidney residue, remaining after the first phosphate buffer extraction, resulted in no further recovery of erythropoietic activity, although about 20 per cent more protein was extracted. This suggests that the initial extraction is quantitative and that no significant amounts of ESF can be recovered by further extraction with 0.02 M phosphate. Preliminary studies, employing supernates
obtained by the centrifugation of isotonic homogenates of anemic kidney at 37,000 g. proved unsatisfactory. This finding is in agreement with that of Rambach and co-workers. Some evidence suggests a relation between the granularity of the juxtaglomerular cells of the kidney and ESF. It is conceivable that hypotonic solutions induce a more complete destruction of these subcellular granules, resulting in greater solubilization and recovery of ESF.

Stohlman and Brecher have reported that the ESF present in the plasma of rats, returned to ambient pressure after 16 hours of continuous hypoxia at 23,000 feet, displayed a half-life of 3 to 5 hours. Our data indicate that the ESF level in the plasma of rats at 3 hours posthypoxia is about one-half that of the 1-hour posthypoxia group. This would correspond to a half-life of somewhat less than 3 hours.

The amount of ESF extracted from kidneys of phenylhydrazine-treated rats was calculated to be 2.3 per cent of the total ESF present in the plasma (assuming a blood volume for the rat of 4.9 per cent body weight). In hypoxic rats sacrificed 1 hour posthypoxia (experiment II), kidney ESF was 12 per cent of total plasma ESF. Stohlmans has shown that the concentration of ESF in the plasma of hypoxic rats attains a maximum between 16 and 24 hours of continuous hypoxic stimulation. The relatively high levels of ESF present in the kidney after 17.5 hours of continuous exposure to 23,000 feet observed in this study is consistent with Stohlmans findings. Evidently the production and release of ESF from the kidney represents a dynamic process in which only a fraction of the circulating level is present in renal tissue at any one time.

Our data suggest that a causal relation exists between kidney and plasma ESF levels. Preliminary attempts to purify further the kidney extracts indicate that this material possesses chemical properties that may be different from plasma ESF. Fractionation has been conducted of the active kidney extract by ion-exchange chromatography on DEAE cellulose after dialysis against 0.005 M phosphate buffer at pH 6.8. The active material was found to be adsorbed by the column and could be eluted at the same pH with 0.1 M NaCl. However, adjustment of the pH of the kidney extract to 4.5, as is usually done in fractionation of the plasma factor, resulted in complete disappearance of erythropoietic activity. Such loss of biologic activity may imply that the kidney ESF is more acid labile. A possible difference in the isoelectric points of the plasma and renal factors is under current investigation.

**SUMMARY**

1. Significant amounts of ESF have been extracted from kidneys of anemic and hypoxic rats by the use of hypotonic phosphate buffer at pH 6.8.
2. A minimum of 50 per cent of the ESF extracted from the kidneys was not due to activity contained in the trapped residual plasma.
3. Bioassay of sheep and rat plasma ESF and the kidney extracts by the radioiron-polycythemic mouse method indicated that the above preparations were parallel in their log-dose regressions, suggesting that these substances are similar in physiologic behavior.
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SUMMARIO IN INTERLINGUA

1. Quantitates significative de factor erythropoieto-stimulatori (FES) esseva extrahite ab le renes de rattos anemic e hypoxic per le uso de un tampon de phosphato hypotonic a pH 6.8.
2. Un minimo de 50 pro cento del FES extrahite ab le renes non esseva le producto de activitate inherente in le trappate plasma residue.
3. Bio-essayage de FES in plasma de ove e de ratto e del extractos renal per le metodo a muses polycythemic con radio-ferro indicava que le mentionate preparatos esseva parallel in lor regressiones logarithmo-dose, lo que suggere que iste substantias es simile in lor comportamento physiologic.

REFERENCES

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