Extraction of an Erythropoietin-Producing Factor from a Particulate Fraction of Rat Kidney

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

The extraction of erythropoietin with hypotonic phosphate buffer at pH 6.8 from the kidneys of hypoxic and anemic rats has recently been reported.\(^1\) Such extracts possessed more erythropoietic activity than could be accounted for by that determined to be present in residual trapped plasma and it was concluded that these extracts contained significant amounts of kidney erythropoietin. Erythropoietic activity has also been found in perfusates circulating through isolated kidneys.\(^2\) In general, these perfusion techniques probably expose the kidney to anoxia considerably more severe than would ordinarily be encountered physiologically. The recent studies of Erslev et al.\(^a\) with an isolated lung-kidney preparation and that of Mirand et al.\(^a\) with renal autotransplants support this view. Kuratowska, Lewartowski and Lipinski\(^b\) have obtained a substance from saline-perfused, isolated anoxic kidneys which becomes erythropoietically active in the presence of a plasma glycoprotein. This observation raises several important questions. Is this renal factor present in the kidneys of untreated normal rats, or is it produced only during the perfusion of an isolated anoxic kidney? Moreover, can this factor be extracted from the kidneys of untreated or mildly hypoxic rats?

The present report concerns the extraction of a renal erythropoietic factor from a particulate fraction of the kidneys of untreated and hypoxic rats. In addition, a number of studies were conducted on the chemical and biological properties of this factor.

Materials and Methods

Description of Experiments

In Experiment I, 38 female Long-Evans rats averaging 180 Gm. in weight were rendered hypoxic by continuous exposure to 0.5 atm. for 17 hours. The animals were then exsanguinated and the kidneys removed and kept on ice, but not frozen. Twelve kidneys were picked at random and frozen on dry ice. These frozen kidneys were then homogenized and extracted by a one-step hypotonic phosphate buffer procedure.\(^1\) The remaining 64 kidneys were homogenized and extracted by the two-step procedure described below. DEAE cellulose fractionation of the two-step renal extract was subsequently conducted. The plasma of these 38 rats was pooled and frozen at -20 C.

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In Experiment II, 23 female rats weighing approximately 180 Gm. were exposed to 0.4 atm. for 24 hours. Of the 46 kidneys collected after exsanguination of the rats, 34 were homogenized and fractionated as indicated in the subcellular fractionation scheme shown below. This fractionation method is essentially that of La Bella et al. The remaining 12 kidneys were frozen and extracted by the usual one-step hypotonic phosphate method. A portion of the pooled plasmas of these 23 rats was subjected to DEAE cellulose fractionation. Male rats of the Long-Evans strain weighing about 250 Gm. were used in the following experiments.

In Experiment III-A, 10 rats were exposed to 0.4 atm. for 24 hours and a second group of 10 rats was left untreated. Twelve rats exposed to 0.4 atm. for 18 hours were used in Experiment III-B. The kidneys of the rats employed in Experiments III-A and B were subjected to the homogenization and subcellular fractionation procedures used in Experiment II.

The dose response study constituting Experiment IV was carried out employing an extract of the light mitochondrial fraction obtained from the kidneys of 10 rats exposed to 0.4 atm. for 18 hours.

All renal and plasma extracts were tested for erythropoietic activity in hypoxia-induced polycythemic mice.

Procedural Details

A. Two-Step Extraction Procedure (Expt. I)

1. Into 100 ml. of cold phosphate-buffered isotonic saline (pH 7.0) were added 64 kidneys (42.8 Gm.). These kidneys were minced and homogenized in a Potter-Elvehjem homogenizer. The organs were not allowed to freeze.
2. The homogenate was then centrifuged at 37,000 g for 30 minutes in a refrigerated centrifuge. The supernate recovered represents the saline extract of the kidneys.
3. The sediment was rehomogenized in 38 ml. of isotonic saline and was centrifuged as in step 2. The saline supernates were combined.
4. The sediment was then rehomogenized in 75 ml. of 0.02 M phosphate buffer, pH 6.8, and centrifuged as in step 2.
5. Step 4 was repeated and the hypotonic phosphate buffer supernates were combined. This supernate is the two-step hypotonic extract of the kidneys.

B. DEAE Cellulose Fractionation of Two-Step Hypoxic Kidney Extract (Expt. I)

One-hundred and eleven ml. of two-step extract containing 809 mg. of protein were dialyzed overnight at 5 C. against 0.02 M phosphate buffer, pH 6.8. A 2 x 38 cm. DEAE column was prepared and a stepwise elution scheme for the extract was employed. The column was eluted with NaCl solutions of increasing molarity (0.02-1.00 M) buffered with 0.005 M PO₄ at pH 6.8. An effluent diagram is illustrated in Figure 1. Pooled fractions were dialyzed overnight against distilled water at 5 C. and then freeze-dried. The dried fractions were later redissolved in appropriate volumes of normal saline.

C. DEAE Fractionation of 24-Hour Hypoxic Rat Plasma (Expt. II)

Plasma from female rats exposed to 0.4 atm. for 24 hours was dialyzed against 0.02 M phosphate buffer, pH 6.8. The dialyzed plasma, containing 25.1 Units (U) of erythropoietin, was then passed through a column of the same dimensions as that used to fractionate the kidney extract. A simpler elution scheme was used in the fractionation of plasma than for the kidney extract (Fig. 2).

D. Subcellular Fractionation Scheme (Expts. II, III and IV)

The subcellular fractionation is described only for Experiment II. For Experiments III and IV, the procedures were identical except that, since the numbers of kidneys used were different, proportionate amounts of extraction medium were employed.

1. Thirty-four kidneys weighing 23.8 Gm. were homogenized in 214 ml. of 0.25 M sucrose.
2. The homogenate was centrifuged at 755 g for 10 minutes. The sediment containing nuclei and cell debris was resuspended in 16 ml. of 0.25 M sucrose and recentrifuged. Both supernates were combined.

3. These sucrose supernates were centrifuged at 6780 g for 15 minutes. The sediment obtained was washed once with 20 ml. of sucrose solution, and this supernate and the previously obtained 2 supernates were combined. The sediment remaining at this step is the heavy mitochondrial fraction.

4. The supernate was then centrifuged at 20,200 g for 15 minutes and the sediment
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Table 1.—Extractions of ESF\(^1\) from Kidney

<table>
<thead>
<tr>
<th>Procedure</th>
<th>ESF Conc. before NRS (Units/ml)</th>
<th>Total ESF after NRS Addition (Units)</th>
<th>Protein Conc. after NRS Addition (mg/ml)</th>
<th>Total ESF after NRS Addition (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Two-Step Procedure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline supernate</td>
<td>0.01</td>
<td>1.38</td>
<td>0.02</td>
<td>15.9</td>
</tr>
<tr>
<td>Hypotonic extract</td>
<td>0.03</td>
<td>4.50</td>
<td>0.11</td>
<td>7.3</td>
</tr>
<tr>
<td><strong>One-Step Procedure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypotonic extract</td>
<td>0.05</td>
<td>6.40</td>
<td>0.15</td>
<td>25.0</td>
</tr>
</tbody>
</table>

\(^1\)ESF—erythropoietin.
\(^2\)Normal rat serum.
\(^3\)Per 64 kidneys.

washed once with 20 ml. of sucrose solution. This sediment is the light mitochondrial fraction.
5. The remaining supernate was centrifuged at 37,000 g for 4-5 hours. The sediment recovered after centrifugation represents the microsomal fraction.
6. Each particulate fraction was resuspended in 50 ml. of 0.02 M phosphate buffer, pH 6.8, and frozen.
7. Each fraction was then thawed and rehomogenized. All were centrifuged at 37,000 g for 1 hour, with the exception of the microsomal fraction which was centrifuged for 5 hours. All centrifugation steps were performed at 5 C.
8. The supernates recovered after the final centrifugation step represent the extract of each particulate fraction. These extracts were then dialyzed overnight against cold normal saline.

E. Incubation Technic (Expts. I, II, III and IV)

In experiments involving the addition of normal rat serum to two-step extracts of kidney, one volume of serum was added to one volume of extract. Unless otherwise indicated, all incubations were conducted at 37 C. in buffered saline at pH 6.8. All reaction flasks were left open to the atmosphere. In some experiments, reactions were stopped by plunging the reaction vessels into an acetone-dry ice mixture. Two-step extracts were incubated for no more than 5 minutes. In Experiment IV, 1.0 ml. of the light mitochondrial extract of kidneys from rats exposed to hypoxia for 18 hours was added to 20 ml. of normal rat serum. This solution was incubated for 30 minutes and immediately injected.

F. Assay Methods (Expts. I, II, III and IV)

Erythropoietic activity of all preparations was determined in the plethoric mouse and potency was expressed in terms of Units (U) of Erythropoietin Standard A. The assay mice were made plethoric by exposure to 0.4 atm. 16 hours a day for over 200 hours. Five mice were used at each dose level for all materials tested. Assay mice received their entire 2 ml. dose i.p. on day 3 of the assay schedule. The calculations of erythropoietin concentration and 95 per cent confidence limits were done according to the methods outlined by Bliss. The assay procedure, recently further developed in our laboratory, now permits detection of quantities of erythropoietin as low as 0.02 U. (unpublished observations). Assay values provided in Tables 1–4 represent data extrapolated down to units per ml. of extract or incubation material.

RESULTS

Experiments I and II

A comparison of the two-step and one-step extraction procedures appears in Table 1. The isotonic saline supernate of 64 kidneys from rats exposed to
Table 2.—DEAE Fractionation of Kidney and Plasma

<table>
<thead>
<tr>
<th></th>
<th>ESF(^1) Conc. before NRS(^2) (Units/ml)</th>
<th>Protein Conc. (mg/ml)</th>
<th>Total ESF before NRS Addition(^3) (Units)</th>
<th>ESF Conc. after NRS Addition (Units)</th>
<th>Total ESF after NRS Addition (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Kidney Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KF 1</td>
<td>N.D.(^3)</td>
<td>4.8</td>
<td>---</td>
<td>N.D.</td>
<td>---</td>
</tr>
<tr>
<td>KF-2</td>
<td>0.03</td>
<td>1.3</td>
<td>0.69</td>
<td>0.02</td>
<td>0.40</td>
</tr>
<tr>
<td>KF-3</td>
<td>0.03</td>
<td>0.6</td>
<td>0.45</td>
<td>0.02</td>
<td>0.30</td>
</tr>
<tr>
<td>KF-4</td>
<td>0.01</td>
<td>1.1</td>
<td>0.40</td>
<td>0.04</td>
<td>1.60</td>
</tr>
<tr>
<td>KF-5</td>
<td>N.D.</td>
<td>0.3</td>
<td>---</td>
<td>N.D.</td>
<td>---</td>
</tr>
<tr>
<td>KF-6</td>
<td>N.D.</td>
<td>2.0</td>
<td>---</td>
<td>N.D.</td>
<td>---</td>
</tr>
<tr>
<td><strong>B. Plasma Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF-1</td>
<td>N.D.</td>
<td>20.0</td>
<td>---</td>
<td>---</td>
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</tr>
<tr>
<td>PF-2</td>
<td>0.37</td>
<td>10.7</td>
<td>14.77</td>
<td>---</td>
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</tr>
<tr>
<td>PF-3</td>
<td>N.D.</td>
<td>2.6</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PF-4</td>
<td>N.D.</td>
<td>0.7</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

\(^1\)ESF—erythropoietin.  
\(^2\)Normal rat serum.  
\(^3\)Protein concentration of freeze dried material redissolved in appropriate volumes of normal saline.  
\(^4\)Activity of entire fraction before incubation with NRS.  
\(^5\)Nondetectable erythropoietic activity.

Table 3.—Subcellular Extracts of Hypoxic Kidney

<table>
<thead>
<tr>
<th></th>
<th>ESF(^1) Conc. before NRS(^2) Addition (Units/ml)</th>
<th>Protein Conc. (mg/ml)</th>
<th>Total ESF Recovered before NRS Addition (Units)</th>
<th>ESF Conc. after NRS Addition (Units/ml)</th>
<th>Total ESF Recovered after NRS Addition (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose supernate</td>
<td>0.03</td>
<td>5.8</td>
<td>7.50</td>
<td>0.02</td>
<td>5.00</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.07</td>
<td>9.0</td>
<td>13.50</td>
<td>0.05</td>
<td>2.50</td>
</tr>
<tr>
<td>Light mitochondria</td>
<td>N.D.(^3)</td>
<td>8.0</td>
<td>N.D.</td>
<td>0.08</td>
<td>4.00</td>
</tr>
<tr>
<td>Heavy mitochondria</td>
<td>0.04</td>
<td>13.5</td>
<td>2.00</td>
<td>0.03</td>
<td>1.50</td>
</tr>
<tr>
<td>One-step whole kidney hypotonic extract</td>
<td>0.53</td>
<td>28.5</td>
<td>38.16</td>
<td>---</td>
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</tr>
</tbody>
</table>

\(^1\)ESF—erythropoietin.  
\(^2\)Normal rat serum.  
\(^3\)Nondetectable erythropoietic activity.

Hypoxia for 17 hours contained a total of 1.38 U. of erythropoietin. The homogenization of kidneys in an isotonic medium causes a minimal disruption of intracellular particles, but releases plasma erythropoietin present in renal blood vessels. Such an isotonic supernate should contain this residual plasma erythropoietin and some of intracellular origin. The pooled plasmas of these rats had an erythropoietin titer of 0.08 U./ml. Employing the method previously described,\(^1\) it was calculated that approximately 0.49 U. of erythropoietin present in the isotonic renal supernate represented residual plasma activity. The remaining 0.89 U. probably was due to intracellular erythropoietin released during homogenization. The hypotonic extraction of the sediment yielded a total of 4.50 U. of erythropoietin. Thus, approximately 75 per cent of the 5.88 U. extracted from these kidneys by the two-step method was recovered in the
Table 4.—Incubation Studies of Light Mitochondria Extract (LME) of Kidney and Normal Rat Serum

<table>
<thead>
<tr>
<th></th>
<th>NRS(^1) (ml.)</th>
<th>Normal LME (ml.)</th>
<th>Hypoxic LME (ml.)</th>
<th>Time (min.)</th>
<th>Units Erythropoietin/ ml. LME</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td>5</td>
<td>0.10</td>
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<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>10</td>
<td>0.09</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>15</td>
<td>0.10</td>
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<tr>
<td>1.0</td>
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<td>30</td>
<td>0.11</td>
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<td>5</td>
<td>0.05</td>
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<td>10</td>
<td>0.04</td>
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</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>30</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>B.</td>
<td>0.0</td>
<td>0.1</td>
<td>30</td>
<td>N.D.(^2)</td>
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</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>120</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>240</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>30</td>
<td>0.20</td>
<td></td>
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<tr>
<td>2.0</td>
<td>0.1</td>
<td>30</td>
<td>1.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>120</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>240</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>2.0</td>
<td>30</td>
<td>0.03</td>
<td></td>
<td></td>
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<tr>
<td>0.1</td>
<td>2.0</td>
<td>120</td>
<td>0.03</td>
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<tr>
<td>0.1</td>
<td>2.0</td>
<td>240</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>2.0</td>
<td>30</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Normal rat serum.

\(^2\)Nondetectable erythropoietic activity.

hypotonic extract. The number of units of erythropoietin recovered by the two-step and one-step extraction procedures was calculated on the basis of 64 kidneys. The one-step extraction procedure recovered a total of 6.40 U., or about 9 per cent more than the 5.88 U. yielded by the two-step procedure.

A quantity of the two-step extract of hypoxic kidneys containing 3.33 U. of erythropoietin and 809 mg. of protein was chromatographed on DEAE cellulose. The extract was resolved into six major fractions labeled KF-1 to 6 (Table 1A and Fig. 1). Erythropoietic activity was found in fractions KF-2, 3 and 4. Since the separation of KF-2 and KF-3 is probably incomplete, it is likely that the erythropoietic activity of KF-2 represents a contamination from KF-3. This seems probable because KF-3 contains twice the specific activity (approximately 50 U./Gm. protein) of KF-2 (approximately 23 U./Gm. protein). KF-4 had the lowest total recovery and specific activity (approximately 9 U./Gm. protein). The chromatographic purification of hypoxic kidney extract by DEAE cellulose resulted in a 27 per cent recovery of erythropoietin with greater than a 10-fold purification.

When normal rat serum containing no detectable erythropoietic activity was incubated with an equal volume of two-step extract of hypoxic kidney, a significant increase (approximately 165 per cent) in the total erythropoietin content was observed (Table 1). When kidney DEAE fractions KF-1 to 6 were added to normal rat serum, an increase in erythropoietic activity (4-fold) was
noted only with KF-4. Thus, incubation of KF-4 with normal serum now yielded the largest quantity of erythropoietin. KF-4 showed a typical protein spectrum with an optical density maximum at 280 μ.

Assuming a blood volume of 4.9 per cent body weight, it was calculated that the hypoxic rats used in Experiment I had an average total plasma erythropoietin level of 0.29 U./ rat. From the erythropoietin recovered in the hypotonic phase of the two-step extraction method, it was possible to compute an average kidney level of 0.14 U./ rat in the absence of added normal serum. In the presence of normal serum the kidney erythropoietin level was calculated to be 0.38 U./ rat. These figures represent minimal values due to losses occurring during extraction and to the presence of proteolytic enzymes. The latter point will be discussed in more detail below.

The fractionation of the plasma of 24-hour hypoxic rats (Expt. II) by DEAE cellulose showed that all of the erythropoietin was recovered in the 0.10 M NaCl fraction labeled PF-2 (Fig. 2 and Table 2B). This contrasts with the fractionation of kidney extract where 2 distinct elution areas containing erythropoietic activity were observed. In experiments involving the fractionation of plasma and renal extracts, the column, type and quantity of DEAE cellulose, and the eluting pH were kept constant. The erythropoietic material present in PF-2 and that in KF-3 were similar in their elution properties on DEAE cellulose and were stable at pH 4.5, while that of KF-4 was unstable at this pH. These 3 fractions had been adjusted to concentrations having the same activity before testing their resistance to acid pH. Fraction PF-4 had no erythropoietic activity in the presence or absence of normal rat serum and therefore could not be the plasma counterpart of KF-4.

In Experiment II (Table 3), the extract of each subcellular fraction from 24-hour hypoxic rat kidneys was assayed with and without the addition of normal rat serum. Of all the fractions tested, only the light mitochondrial extract (LME), when added to normal serum, produced a significant increase in erythropoietic activity. The erythropoietin content of this material increased from nondetectable levels to 0.08 U./ml. of LME. It was calculated that the LME extracted from the 34 kidneys used yielded a total of 4.00 U. of erythropoietin upon the addition of an equal volume of normal rat serum. Of the 7.50 U. recovered from the sucrose supernate, 5.80 U. were computed to be due to residual plasma erythropoietin. It is likely that the substance isolated in KF-4 was derived from particles in the light mitochondrial fraction of the kidney. Activity present in the microsomal and heavy mitochondrial extracts might have been due to slight contamination of these particles by the supernate.

Experiment III

Kidney LME was prepared as in Experiment II. The LME of equal numbers of kidneys from hypoxic and from normal unexposed rats were used in incubation studies. Table 4 indicates that when 1.0 ml. of normal rat serum was incubated with 1.0 ml. of LME from kidneys of rats subjected to hypoxia for 24 hours, a yield of approximately 0.1 U. erythropoietin/ml. of LME was obtained. No further increase in erythropoietic activity occurred beyond the
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first 5 minutes of incubation. When 1.0 ml. of normal rat serum was incubated with 1.0 ml. of LME from kidneys of normal unexposed rats, an average of approximately 0.05 U. erythropoietin/ml. of LME was observed in each group. On the basis of the volume of the LME of kidneys from normal and hypoxic rats, and the erythropoietin produced by 1.0 ml. of each in the presence of normal serum, an estimate of the erythropoietin-producing capacity of each extract could be made. A minimum of 3.00 U. of erythropoietin can be produced by the LME of 20 kidneys from rats exposed to hypoxia for 24 hours. The LME of 20 kidneys of normal rats not subjected to hypoxia was capable of producing a minimum of 1.20 U. The following studies indicate that these figures represent only a partial estimate of the erythropoietin-producing capacity of the LME. The data of Experiment III-B (Table 4) show that the largest amount of erythropoietin appeared within 30 minutes of incubation. At longer incubation periods the erythropoietin level decreased considerably. Such behavior suggests that reactions which inactivate erythropoietin are occurring simultaneously with those that produce or activate the factor. The presence of protease activity in the LME was determined by the method of Gianetto and De Duve.14 Such activity was detected in the LME and was found to increase at pH 5 and 6. For this reason the true erythropoietin-generating capacity or content of the LME cannot be precisely calculated. Table 4 also indicates that the greatest degree of erythropoietin generation occurred when 2.0 ml. of normal rat serum (120 mg. protein) were incubated for short periods with a small amount (0.1 ml. 0.8 mg. protein) of kidney LME from hypoxic rats. In the same experiment, 0.1 ml. of normal serum incubated with 2.0 ml. of LME produced much less erythropoietin. In this case, the normal serum level might have been the rate-limiting component. However, the presence of LME is definitely necessary for the production of erythropoietin.

Experiment IV

Here the mean responses (per cent RBC Fe³⁺ incorporation) were plotted against the log dose of the normal rat serum-LME incubation mixture (Fig. 3). No deviations from parallelism (p < 0.001) were found when compared with sheep plasma erythropoietin. This indicates that the interaction of the factor present in the renal LME with normal serum does result in the appearance of an erythropoietin, the activity of which, on the basis of the bioassay, is indistinguishable from the sheep plasma material.

It should be emphasized that Experiments I, II and III A and B were repeated on 4 different occasions with no statistically significant variations. Thus the descriptions above may be considered representative of the total observations made.

DISCUSSION

The results indicate that the major portion of erythropoietin present in kidneys of hypoxic or normal rats cannot be extracted by an isotonic medium. Initial isotonic saline extraction of the kidneys of hypoxic rats removes essentially all of the plasma-borne residual erythropoietin and a great deal of extraneous protein. The subsequent extraction of the resulting sediment in a
hypotonic phosphate buffer at neutral pH allows the recovery of about three-fourths of the total erythropoietin extractable from the kidney by the two-step procedure. This supports our earlier contention<sup>1</sup> that the bulk of the erythropoietin extracted from the kidney is most likely of intracellular origin, perhaps contained in some subcellular granule. Ion exchange chromatography of extracts of kidney from hypoxic rats indicated the possible presence of two species of erythropoietically active material (KF-3 and KF-4). This fractionation was repeated 4 times under various elution conditions, and in every case 2 distinct fractions containing erythropoietic activity were found. Similarly fractionated plasma from hypoxic rats contained only one erythropoietically active fraction (PF-2), corresponding in elution behavior and acid stability to kidney fraction KF-3. Since neither fraction (PF-2 nor KF-3) showed any increased erythropoietic activity in the presence of normal serum, it seemed likely that both contained the same type of erythropoietic material. Fraction KF-3 probably represents some unextracted residual plasma erythropoietin or newly produced activated factor. The properties of the erythropoietic material present in KF-4 differ greatly from those of plasma erythropoietin, and it is postulated that this fraction contains a renal erythropoietic factor (REF). The most significant distinguishing property of this REF is its ability to cause the production of erythropoietic activity in the presence of normal serum. The low level of erythropoietic activity detected when some kidney extracts of fractions containing the REF are assayed is probably due to interaction with the plasma of the assay mice.

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Fig. 3.—Log dose/response regressions for ESF standard (ESA) and light mitochondrial extract (LME) incubated with normal rat serum (NRS). Each point represents the mean (±S.E.m) per cent RBC Fe<sup>59</sup> incorporation of 5 mce.
EXTRACTION OF ERYTHROPOIETIN

The REF was found to be contained entirely in the LME. It was of interest that the LME of the kidneys of untreated, normal rats also caused the generation of erythropoietin when incubated with normal serum. The plasma of these rats contained no detectable activity and therefore the erythropoietic activity of the LME-normal serum incubates could not have been to residual plasma activity. Furthermore, this demonstrates that renal tissue possesses the capacity to produce erythropoietin without prior exposure to hypoxia.

The exact rate of the interaction of normal serum with the factor present in kidney LME cannot be determined because of difficulties inherent in the bioassay. The possibility exists that interactions may proceed for a time after a reaction mixture is injected into an assay animal. Studies did indicate that extended incubations (beyond 30 minutes) of LME with normal serum resulted in a decrease of erythropoietic activity. Small amounts of LME (0.1 ml., 0.8 mg. protein), when incubated with large amounts of normal serum (2.0 ml., 120 mg. protein) for 30 minutes, resulted in the appearance of the greatest quantity of erythropoietin. A volume of 0.1 ml. of LME is equivalent to 6 percent of the total LME prepared from one kidney. Because of the hydrolytic enzymes in the LME it is not surprising that 0.1 ml. of LME yielded more erythropoietin than did 1.0 ml. in the presence of similar amounts of normal serum. An increase in the quantity of LME incubated apparently did not increase the rate of appearance of erythropoietin as much as it augmented its rate of inactivation resulting from the action of these hydrolases. When LME was incubated with normal serum for 5–60 minutes, at pH 5 and 6, no erythropoietic activity could be detected. Since protease activity was found to increase at these pH ranges, the inability of the LME-normal serum incubation mixture to become erythropoietically active at acidic pH ranges may be due to a combination of the lability of the REF at pH 5 and 6, and to the increased proteolytic activity.

There seems at present no correlation between general protease activity in the LME and erythropoietin produced in the incubates. Kuratowska and co-workers obtained a factor from perfused anoxic kidneys which also required the addition of normal serum to manifest erythropoietic activity. These perfusates were found to contain no proteolytic activity. Anoxic perfusion may cause a disruption of cells and allow some of the cellular contents to appear in the perfusing medium. The proteolytic activity present in the extracts described in this report may have been due to the presence of lysosomal material. De Duve and co-workers have demonstrated that the lysosomes contain hydrolytic enzymes, including proteases, which are released at low pH or in hypotonic solution. The present study indicates that the REF is inactivated at low pH levels or in the presence of hydrolytic enzymes.

*The light mitochondrial fraction, from which the extract was derived, contains particles intermediate in size between the heavy mitochondria and the microsomes. It is a heterogeneous mixture which is known to include lysosomes and other cellular granules, but few or no true mitochondria.
Straus\textsuperscript{17,18} has reported that droplets from the normal kidney had a large range in size, the smallest of which sedimented at 15,000 g at 30 minutes. Such particles contained hydrolytic enzymes similar to those found in the lysosomes. The method of preparation of the LME described in this report would include such lysosome-like droplets. LME preparations from the livers of hypoxic rats did not show the presence of a factor similar to the REF when assayed for erythropoietic activity in our laboratory. It seems reasonable to postulate that the particles containing the REF are of the same general size as kidney lysosomes, but are activated by much milder conditions than those necessary for lysosomal activation in vivo. In the absence of destructive proteolytic enzymes of possible lysosomal origin, the kidney LME preparations would probably evoke considerably more erythropoietin than is presently indicated.

The above considerations suggest at least two possible mechanisms of erythropoietin production. The first is that the REF forms a complex with some serum protein. It seems unlikely that the primary function of such a complex is to protect the renal factor from proteolytic enzymes present in the tissues and body fluids, since erythropoietin derived from plasma, and therefore in the “complex” form, has been known to be inactivated by a variety of proteolytic enzymes.\textsuperscript{19,20} Such a hypothesis also implies the presence of a renal factor carrier in the serum of normal rats. The interaction of the REF with such a carrier might be necessary to produce an erythropoietically active molecular configuration. The REF, which by itself seems to induce erythropoietic activity, may be stored in intracellular granules and released into the renal circulation during hypoxia. The mechanism of such release may be more sensitive to hypoxia than is lysosomal disruption.

The second hypothesis envisions the REF as an enzyme capable of producing erythropoietin from a serum protein.\textsuperscript{*} A similar mechanism has been proposed by Penington.\textsuperscript{21} Neither the present report nor that of Kuratowska and co-workers\textsuperscript{8} has ruled out this possibility. In fact, the enzymatic production of erythropoietin seems a far simpler explanation, since large quantities of erythropoietin could conceivably be produced from the plasma pool available to the kidney. This reaction might involve a simple cleavage of a portion of a precursor substance present in the serum with the resultant production of erythropoietin. If the former or “active complex” hypothesis were the case, it would be necessary for the kidney to synthesize the REF continuously during periods of prolonged hypoxia. Preliminary studies in this laboratory indicate that little or no renal incorporation of isotopically labeled amino acids into protein occurs during prolonged hypoxia.\textsuperscript{22} An enzymatic mechanism would not necessitate the occurrence of any complex synthetic processes, such as protein synthesis, during periods of low oxygen availability. Studies are now proceeding to resolve this problem.

\textsuperscript{*}The possibility exists that the renal extracts used in these studies contain some renin. However, neither renin nor angiotensin has been found to stimulate erythropoiesis in plethoric mice (unpublished observations).
Summary

1. A two-step method for the extraction of erythropoietin from hypoxic kidneys has been developed which allows residual plasma erythropoietin in renal vasculature to be separated from that of intracellular origin.

2. Renal extracts have been purified by DEAE cellulose chromatography and found to contain 2 major erythropoietically active fractions. One bears strong resemblance to plasma erythropoietin. The other component is unique in that it has practically no erythropoietic-stimulating activity unless previously incubated with normal rat serum. This activation phenomenon is used to identify this kidney component as the renal erythropoietic factor (REF). The REF has the capacity to produce erythropoietin or become erythropoietically active when incubated with normal rat serum.

3. Differential centrifugation techniques revealed that the REF is confined to particles present in the light mitochondrial fraction of kidney.

4. Extracts of the light mitochondrial fraction of kidneys from normal rats produced significant amounts of erythropoietin when incubated with normal serum. The quantity found, however, was less than that evoked by similar extracts of kidneys from hypoxic rats.

5. The product of the incubation extracts of the renal light mitochondrial fraction with normal rat serum showed the same log dose/response regression as sheep plasma erythropoietin standard.

6. It is hypothesized that either (a) the REF is a precursor of erythropoietin which must be complexed with a carrier present in normal serum in order to become physiologically active, or (b) the renal factor is an enzyme which produces erythropoietin by its action on a particular serum protein.

Summario in Interlingua

1. Esseva disveloppate un metodo biphasic pro le extraction de erythropoietina ab renes hypoxic. Le metodo permette le separation de residue erythropoietin plasmatic in le vasculatura renal ab illo de origine intracellular.

2. Extractos renal eseva purificate per chromatographia a cellulosa die-thylaminoethanolic, con le constatation que illos contine duo major fractiones erythropoieticamente active. Le un presenta un forte similitude con le erythropoietin de plasma. Le altere es de typo unic in tanto che illo possede practicamente nulle activitate erythropoieticamente stimulatori si illo non es previemente incubate con normal sero de ratto. Iste phenomeno de ac-tivation es usate pro identifier iste componente renal como le renal factor erythropoietic. Le renal factor erythropoietic ha le capacitate de producer erythropoietina o de devenir erythropoieticamente active quando illo es in-cubate con normal sero de ratto.

3. Technicas de centrifugation differential revelava que le renal factor erythropoietic es restringite a particulias presente in le leve fraction mito-chondrial del ren.

4. Extractos del leve fraction mitochondrial del renes de rattos normal
produceva quantitates significative de erythropoietina quando illos esseva incubate con sero normal. Tamen, le quantitate trovate esseva inferior a illo evocate per simile extractos renal ab rattos hypoxic.

5. Le producto del incubation de extractos del fraction mitochondrial leve del ren con normal sero de rattos monstrava le mesme regression del logaritmo de dose/responsa como le standard de erythropoetina de plasma ovin.

6. Es postulate que (a) le renal factor erythropoietic es un precursor de erythropoietina que debe esser complexate con un vector presente in sero normal pro devenir physiologicamente active o que (b) le factor renal es un enzyma que produce erythropoietina su action super un proteina seral particular.

REFERENCES

EXTRACTION OF ERYTHROPOIETIN

Extraction of an Erythropoietin-Producing Factor from a Particulate Fraction of Rat Kidney

JOSEPH F. CONTRERA, ALBERT S. GORDON and ARTHUR H. WEINTRAUB