BRIEF REPORT

Renal Erythropoietic Factor.
Lack of Effect on Hypertransfused Mice

By A. J. Erslev and L. A. Kazal

In 1957 Jacobson and co-workers firmly established the importance of the kidney for the production of erythropoietin. Since then numerous investigators have attempted to isolate erythropoietin from renal tissue but these attempts have in general been unsuccessful. In 1966 Contrera and Gordon provided a partial explanation by suggesting that the kidney rather than producing erythropoietin releases a factor, the Renal Erythropoietic Factor or R.E.F., and that this factor generates erythropoietin by activating a circulating erythropoietin precursor. This suggestion was based on in vitro studies of hypotonic renal extracts and it both confirmed and amplified previous studies by Kuratowska and co-workers. It also conformed with the activation kinetics of many other systems such as the coagulation and complement systems.

Since then, Gordon and his group have published much additional information about the R.E.F. pinpointing it to the light mitochondrial and microsomal fractions of renal extract and emphasizing that it is necessary to use EDTA treated serum for its identification because of the existence of an ion-dependent inactivator.

In September 1967 we became involved in the study of the R.E.F. using as assay animals hypertransfused mice rather than hypoxia-induced polycythemic mice as used by Gordon and co-workers. Employing this assay system we have in twelve separate studies been unsuccessful in demonstrating an erythropoietin-generating effect of renal extract.

MATERIAL, METHODS AND RESULTS

The bioassay employed in this study is a modification of the bioassay described by Rosse and Waldman and is the routine assay used in our laboratory since 1966. Basically, it depends on the sixty-six hour utilization of 59Fe injected eight days after the induction of a transfusion polycythemia in mice. On both days 0 and 1, 1 ml. of packed red cells are administered intraperitoneally, on both days 6 and 7, one half of the assay material suspended in 0.5 or 1.0 ml. of saline is given subcutaneously and on

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day 8, 0.5 uc. $^{59}$Fe as Fe Cl$_3$ is given intraperitoneally. Three days later the sixty-six hour utilization of iron is determined. For the calculation an estimated blood volume of 7 per cent of body weight is used. The hematocrit is also measured and results from all animals with hematocrits of less than 55 per cent are discarded. Each sample is assayed in at least five mice and a total of fifty to seventy assays are run each week. In study I and II (Table 1) carried out at the University of Copenhagen, female St/Eh mice were used, but in all the other studies, female I.C.R. mice were employed as assay animals. The dose response curve for the St/Eh mice was found to be very similar to that determined for the I.C.R. mice (Fig. 1). The apparent smoothness of the dose response curve is somewhat deceiving since the standard deviation of values at each point is quite considerable. This well-recognized crudeness of all bioassays for erythropoietin necessitates the use of at least five mice for each study and the need for several confirmatory studies. The baseline activity and the response to 0.05 units of erythropoietin are hardly distinguishable, but the response to 0.1 unit is significantly above baseline values.

The experimental procedures for the first ten studies were as outlined in Table 1. With the exception of the assay, technics and procedures were as far as possible identical with those published by Contrera and co-workers$^5$ and by Zanjani and co-workers.$^7$ A detailed communication from Dr. Zanjani also insured that the original procedures were duplicated. In Study XI, (Table 2) the renal material was prepared and provided by Dr. Zanjani and in Study XII, both renal material and normal rat serum dialyzed against EDTA were provided by Dr. Zanjani.

The results of our studies are given in Table 1 and 2 which show that with our assay system, crude and partly purified renal extracts fail to generate significant erythropoietic activity from normal, EDTA treated serum.

**DISCUSSION**

As shown in Tables 1 and 2, twelve separate studies made during a fifteen month period failed to indicate that renal extracts can generate “erythropoietin” from normal rat serum. In only one study (V) did the renal extract have significant erythropoietic activity, but this activity was present both after incubation with saline and with serum. These results have forced us to question the validity of the concept that the kidney releases an erythropoietin-activating enzyme rather than erythropoietin itself.

The major difference between studies reported by Gordon and his co-workers and the studies reported here lies in the assay system employed. The assay system used by the New York University group depends with one exception$^{13}$ on the utilization of $^{59}$Fe administered five days after the termination of two weeks of intermittent hypoxia.

The baseline utilization in this assay system is higher (about 1 per cent) than in the transfusion-polyarthyemia system (about 0.1 per cent) and the response to a given dose of erythropoietin is correspondingly higher. This difference may reflect the fact that there is a shorter period of acclimatization in the hypoxia system before $^{59}$Fe is injected (five days versus eight days in our assay), but it could also reflect the persistence of a hypoxia-induced expansion of the stem cell pool. Because of this latter possibility and because of the ease with which polycythemia can be induced by hypoxia we have on two occasions attempted to adopt this system for routine assays. Unfortunately, we could not detect a greater sensitivity and the higher utilizations observed were offset by the higher baseline and by a pronounced individual variation among assay mice. Consequently, we cannot accept
<table>
<thead>
<tr>
<th>Date</th>
<th>Kidney Donors</th>
<th>Anemia or Hypoxia</th>
<th>Material</th>
<th>Incubation mixture per mouse</th>
<th>Assay</th>
<th>R.E.F. + Saline 68 hour Utilization of $^{59}Fe$</th>
<th>R.E.F. + EDTA. Serum 68 hour Utilization of $^{59}Fe$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Sept. 67</td>
<td>Long Evans Female, white rats</td>
<td>0.4 Atmosp. 18 hours</td>
<td>Light Mitochondrial Fraction</td>
<td>1 ml. REF (0.5 Gm. Kidney tissue) + 1 ml. EDTA serum or saline 30 minutes at 37 C.</td>
<td>$^{59}Fe$ injected 8 days after the induction of transfusion polycythemia in female St/Eh mice</td>
<td>0.35% (5)</td>
<td>0.43% (5)</td>
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<tr>
<td>II Sept. 67</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>0.72% (6)</td>
<td>0.32% (7)</td>
<td></td>
</tr>
<tr>
<td>III Nov. 67</td>
<td>Wistar, Male, white rats</td>
<td>0.4 Atmosp. 23 hours</td>
<td>Hypotonic Kidney Extract</td>
<td>0.5 ml. REF (0.25 Gm. Kidney tissue) + 0.5 ml. EDTA serum or saline 30 minutes at 37 C.</td>
<td>$^{59}Fe$ injected 8 days after the induction of transfusion polycythemia in female I.C.R. mice</td>
<td>0.13% (5)</td>
<td>0.12% (5)</td>
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<tr>
<td>IV Jan. 68</td>
<td>&quot;&quot; Bled 25 ml./Kg. 24 hours</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>&quot;&quot;</td>
<td>0.14% (5)</td>
<td>0.31% (5)</td>
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<tr>
<td>V</td>
<td>Jan. 68</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>2.2%</td>
<td>(5)</td>
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<tr>
<td>VI</td>
<td>Nov. 68</td>
<td>Normal</td>
<td>Light Mitochondrial Fraction</td>
<td>0.5 ml. REF (0.50 Gm. Kidney tissue) + 0.5 ml. EDTA serum or saline 50 minutes at 37 C.</td>
<td>&quot;</td>
<td>0.13%</td>
<td>(5)</td>
</tr>
<tr>
<td>VII</td>
<td>Nov. 68</td>
<td>Bled 25 ml./Kg. 24 hours</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.08%</td>
<td>(5)</td>
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<tr>
<td>VIII</td>
<td>Nov. 68</td>
<td>Bled 25 ml./Kg. 24 hours</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.15%</td>
<td>(5)</td>
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<tr>
<td>IX</td>
<td>Nov. 68</td>
<td>Bled 25 ml./Kg. 48 hours</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.06%</td>
<td>(5)</td>
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<tr>
<td>X</td>
<td>Nov. 68</td>
<td>Bled 25 ml./Kg. 48 hours</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.08%</td>
<td>(5)</td>
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that the lack of confirmatory results is caused by a lower sensitivity of our assay system.

Since the R.E.F. in the hypoxia assay is injected three days after the discontinuation of hypoxia it undoubtedly will act on a bone marrow containing many more erythroid precursor cells than in our assay in which the R.E.F. is injected six and seven days after transfusion. Consequently, it is possible that part of the response observed by Gordon and his co-workers is caused by a factor acting primarily on maturing nucleated red cells. If this is true, the response to R.E.F. treated serum should be even more pronounced in normal mice or in mice rendered only moderately polycythemic, a possibility which should be studied further. However, the generation of an erythropoietic factor acting chiefly on stem cells could not be demonstrated in our studies, and we are forced to conclude that the existence of an inactive, erythropoietin-generating "renal erythropoietic factor," rather than a proven fact, is still a hypothesis.

The failure to demonstrate erythropoietin in kidney extracts does not demand the acceptance of this hypothesis since this failure may adequately be explained by assuming that the strong erythropoietin-inhibitor in renal
<table>
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<th>R.E.F. E.D.T.A. Serum</th>
<th>Saline</th>
<th>E.D.T.A. Serum</th>
<th>0.1 unit Erythropoietin</th>
<th>0.2 units Erythropoietin</th>
<th>1.0 units Erythropoietin</th>
</tr>
</thead>
<tbody>
<tr>
<td>XI</td>
<td>0.17% (9)</td>
<td>0.07%  (4)</td>
<td>0.08% (5)</td>
<td>-</td>
<td>-</td>
<td>15.1% (10)</td>
</tr>
<tr>
<td>Nov. 1968</td>
<td>0.15% (9)</td>
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<tr>
<td>XII</td>
<td>0.18% (8)</td>
<td>0.15%  (4)</td>
<td>0.14% (10)</td>
<td>0.48% (2)</td>
<td>5.7% (3)</td>
<td>12.0% (5)</td>
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<tr>
<td>Dec. 1968</td>
<td>0.07% (7)</td>
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extracts inactivates erythropoietin during the extraction procedure. Further studies by other laboratories are obviously needed in order to resolve this extremely important question.

**Summary**

In twelve separate studies employing hypertransfused mice, attempts were made to demonstrate the generation of erythropoietin from normal serum incubated with the "Renal Erythropoietic Factor." Despite the use in some studies of preparations found effective in another laboratory, these attempts were all unsuccessful. It is concluded that the hypothesis that erythropoietin is produced by the enzymatic activation of an inactive circulating protein needs much further study before it can be accepted.

**SUMMARIO IN INTERLINGUA**

In dece-duo separate studios con le uso de muses hypertransfusionate le effortio esseva facite de demonstrar le generation de erythropoietina ab sero normal incubate con le "factor erythropoietic renal." Ben que in plures del studios preparatos esseva usate le quales se habeva previemente provar efficace in un altere laboratorio, iste effortio esseva nonsuccessose. Es conclusionate que le hypothese que erythropoietina es producite per le activation enzymatic de un inactive proteina circulante require multe studios additional ante que illo pote esser acceptate.

**ACKNOWLEDGMENT**

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**REFERENCES**

11. Gordon, A. S., Cooper, G. W., and


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