Effects of Cobalt on the Renal Erythropoietic Factor and Kidney Hydrolase Activity in the Rat

By Robert J. Smith and James W. Fisher

In an experiment to determine the effects of cobalt on the renal erythropoietic factor and kidney hydrolase activity in the rat we obtained the following results: Cobalt produced significant increases in renal erythropoietic factor activity and plasma levels of erythropoietin which reached peak activity 12 hr after treatment. It also produced an increase in the activity of renal hydrolases, cathepsins A and B, which paralleled the increase in renal erythropoietic factor activity. Enzyme inhibitors which are specific for proteases, esterases, and metalloenzymes inhibited the activity of the renal erythropoietic factor in vitro. Polycythemic mice exposed to 7- and 8-day posthypoxic intervals still retained their ability to respond to in vitro generated erythropoietin when compared to mice treated on the fourth posthypoxic day. The erythropoietic activity generated by the light mitochondrial extract–normal rat serum (LME-NRS) reaction mixture was blocked by the antibody to erythropoietin. The relative concentrations of smooth and rough endoplasmic reticulum (microsomes) and vesicles (lysosomes) were approximately the same in the light mitochondrial fractions of kidneys from normal and cobalt-treated rats. Marker enzyme studies revealed primarily alkaline phosphatase activity in the light mitochondrial fraction. These studies correlate with electron micrographs of the LME which indicate a fraction composed mainly of microsomes. In addition, these data suggest a possible relationship between renal lysosomal hydrolase activity and the renal erythropoietic factor (Erythropoietin).

The kidney has been postulated to play a primary role in the control of erythropoiesis. Cobalt is well known to stimulate erythropoiesis by increasing the production of erythropoietin (ESF, erythropoietic-stimulating factor) by the kidney. Gordon proposed that kidney production of erythropoietin involved the action of a renal enzyme on a plasma protein substrate probably through a hypoxic mechanism. Renal extracts from animals exposed to erythropoietic stimuli, such as cobalt or hypoxia, have been demonstrated to generate increased erythropoietic activity in vitro when incubated with normal serum as compared with renal extracts from untreated animals. Contrera et al. have reported that the “light mitochondrial fraction” (LMF) of the kidney contains the highest concentration of this erythropoietic-generating activity, which has been designated the renal erythropoietic factor (REF, erythropoietin). A hypotonic extract (light mitochondrial extract) of the LMF has been reported to contain a high concentration of the REF. The action of REF was inhibited by EDTA and enhanced by calcium ion. In addition, the

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Supported by USPHS Grant AM 13211.
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REF-serum interaction was demonstrated to exhibit reaction kinetics which are characteristic of an enzymatic mechanism.17,18 The present studies attempt to correlate increases in REF activity with an elevation in a class of renal hydrolases (Cathepsins) in the light mitochondrial extract of the rat kidney following cobalt stimulation. In addition, the effects of several enzyme inhibitors on the in vitro generation of ESF by the REF are reported.

MATERIALS AND METHODS

Preparation of Normal Dialyzed Serum

Male Sprague-Dawley rats (250–300 g) were anesthetized with ether and blood withdrawn from the abdominal aorta with a plastic syringe. The blood from four to five rats was pooled and allowed to clot for 24 hr at 4°C. The serum was collected and dialyzed against 100 volumes of 0.005 M ethylenediaminetetraacetic acid (pH 7.0, 4°C) for 24 hr. The serum was again dialyzed against 100 volumes of distilled water (pH 7.0, 4°C) for 24 hr. The serum was frozen at -70°C until used in the incubation studies.

Preparation of Renal Erythropoietic Factor (REF, Erythropoietin)

Male Sprague-Dawley rats (250–350 g) received an injection of cobaltous chloride-hexahydrate (250 μmoles/kg, subcutaneous) and were sacrificed at 1, 4, 8, 12, 18, 24, 48, and 72 hr after injection. At each time interval the rats in groups of six were anesthetized and subsequently bled and nephrectomized. Plasma was prepared and frozen until used. The renal erythropoietic factor was prepared from the kidney tissue by a modification of the method of Gordon et al.19 The kidneys were minced, suspended in 0.25 M sucrose (10 ml/g kidney, pH 7.0), and homogenized in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 6000 g for 10 min (4°C) in a Sorvall RC2-B refrigerated centrifuge. The sediment was discarded and the supernatant centrifuged at 21,000 g for 30 min (4°C). The supernatant was then discarded, and the sediment (LMF) resuspended in cold distilled water (2 ml/g kidney, pH 6.8). Three drops of a detergent (CUTSCUM) were added to the LMF which was then frozen. The LMF was frozen and thawed two times, and then centrifuged at 35,000 g for 15 min (4°C). The sediment was discarded and the supernatant (LME, light mitochondrial hypotonic extract) containing the renal erythropoietic factor was frozen until used.

Incubation Procedure for REF and Normal Dialyzed Rat Serum (NRS)

The reaction mixture consisted of equal volumes of the light mitochondrial extract (REF) and normal dialyzed rat serum. The pH was adjusted to 6.8 and the incubation carried out at 37°C for 60 min in a shaking incubator. The reactions were stopped by immersing the reaction vessels into ice water. The REF-NRS mixtures were then administered to the exsanguinated polycythemic mice at the end of the incubation period. The same procedure was followed for all of the inhibitor studies.

Unless otherwise stated, the LME was preincubated with the respective inhibitors for 30 min prior to incubation with NRS.

The final concentration of inhibitors in the incubation mixture were as follows: Trasylol, 500 KIU; diisopropyl fluorophosphate (DFP), 1 × 10^{-5} M; epsilon- amino-n-caproic acid (EACA), 1 × 10^{-5} M; diethylthiocarbamic acid (DEDTCA), 1 × 10^{-4} M; 2-mercaptoethanol (2-ME), 1 × 10^{-3} M; 3-mercaptopropionic acid (3-MPA), 5 × 10^{-3} M.

The experiments involving the ESF antibody were carried out as follows: Equal volumes of LME and'NRS were incubated for 60 min at 37°C (pH 6.8) in a shaking incubator. Anti-ESF was then reacted with the LME-NRS mixture for 60 min. Goat antirabbit gamma globulin (GARGG) was then added to the reaction vessels, and the entire mixture was allowed to react for 18 hr at 4°C. Following centrifugation, the supernatant was assayed for ESF activity in 8-day posthypoxic polycythemic mice.
The Exhypoxic Polycythemic Mouse Assay

HAM/ICR (CD-I) female Charles River mice (22-26 g) were made polycythemic according to a modification of the method of Cotes et al.20 by exposure to 0.42 atmosphere for 14 days in a hypobaric chamber. The mice were removed from the chamber for 2 hr each day at which time they were given water and food. At the end of the 2-wk period the mice were removed from the chamber and allowed to equilibrate at normal atmospheric pressure for 4 days. On the fourth posthypoxic day the mice were injected intraperitoneally with the test material to be assayed. Except for the REF-NRS mixtures, all samples (1.0 ml) were injected in a divided dose (subcutaneously) on the fourth and fifth posthypoxic days. On the sixth posthypoxic day each mouse received 0.5 μCi of 59Fe citrate via the tail vein. On the eighth posthypoxic day the mice were bled via cardiac puncture, blood counted on a Packard Tri-Carb Scintillation counter with an Auto-Gamma Spectrometer, and the per cent 59Fe incorporation in RBC was converted to units of erythropoietin from a 4-point log dose-response curve for the International Reference Preparation of (IRP) of erythropoietin.

In order to evaluate the effects of extending the posthypoxic period on the response of the assay mice to the REF-NRS incubation mixture, the mice were removed from the chamber at the end of the 2-wk period and allowed to equilibrate at normal atmospheric pressure for 7 and 8 days. They received the test sample on the seventh or eight posthypoxic day, 59Fe on the ninth or tenth posthypoxic day, and were bled on the eleventh or twelfth day following removal from the chamber.

Determination of Enzyme Activities

Cathepsin A activity was measured according to a modification of the method of Misaka et al.21 Cathepsin B and Cathepsin C (dipeptidyl transferase) activity was measured according to the method of Mycek.22 Cathepsin D activity was measured according to a modification of the method of Misaka et al.21 The reaction mixture (5.0 ml) contained 20 mg of acid denatured hemoglobin, 100 μmoles of sodium acetate buffer, pH 4.0, and diluted REF. Incubations were carried out at 37°C for 30 min, and reactions were stopped by adding 1 ml of 15% trichloroacetic acid to the reaction mixture. The precipitated protein was removed by centrifugation, and the released amino acids were determined by the ninhydrin method of Moore.23 Cathepsin E activity was assayed in a manner similar to that of Cathepsin D. However, acid denatured bovine serum albumin served as substrate, and the reaction was run at pH 2.5. Alkaline phosphatase activity was measured according to the method of Garen and Levinthal.24 Acid phosphatase activity was measured in a reaction mixture which contained 3.0 ml of 0.001 M p-nitrophenyl phosphate in 0.15 M sodium acetate buffer, pH 4.0. At zero time, 0.1 ml of enzyme (REF) was added to a test cuvette which contained the substrate, and absorbancy changes were recorded over a 5-min period at 410 μm. The reaction was read against a suitable blank.

Cytochrome oxidase activity was measured according to the method of Cooperstein and Lazarow.25 The protein concentration of the REF, serum, and plasma preparations employed in these experiments was determined by the biuret method of Gornall et al.26 The statistical evaluation of the data was made by the use of Dunnett’s method for comparing several treatments with a single control,27 and by the Student’s t test as indicated in the appropriate table and figures.

RESULTS

Figure 1 illustrates the effects of cobalt on ESF production and renal REF activity. The REF reached peak activity (0.160 ± 0.04 U) 12 hr after the cobalt injection, as compared to the control value of 0.05 ± 0.006 U. When REF was incubated with an equal volume of saline at 37°C for 60 min (pH 6.8), no significant increase in erythropoietic activity was seen at any time interval.

The plasma ESF levels (Fig. 1) reached a maximum 12 hr after the cobalt injection (2.10 ± 0.6 U) as compared to control values (zero time) of 0.069 ±
Fig. 1. Effect of cobalt on renal erythropoietic factor activity and erythropoietin production. Each value represents the mean ± SEM of four experiments. The activities for the REF (○—○) and ESF (×—×) are expressed as IRP units of erythropoietin. The asterisks indicate values which are significantly (p < 0.05) different from the control (zero time) value.

Fig. 2. Cathepsin A activity in the light mitochondrial extract of kidneys from cobalt-treated rats. Each value represents the mean ± SEM of four experiments. The asterisks indicate values which are significantly (p < 0.05) different from the control (zero time) value.
0.01 U. When 2 ml of a mixture of normal dialyzed serum and saline were injected into the assay mice intraperitoneally, no detectable activity was seen.

The effect of cobalt on cathepsin activity in the light mitochondrial extract of kidneys from normal and cobalt-treated rats were examined. Cathepsin A activity (Fig. 2) was significantly ($p < 0.05$) increased over the control value at 4, 8, and 12 hr after cobalt treatment. The activity curve for cathepsin B (Fig. 3) was significantly increased over the control value at 8, 12, 18, and 24 hr after cobalt treatment. Cobalt had no effect on the activity of cathepsin C (Fig. 3), or Cathepsins D and E (Fig. 4).

Figure 5 represents the effects of several enzyme inhibitors on the activity of the renal erythropoietic factor. Diisopropyl fluorophosphatase, a general esterase inhibitor, produced a significant ($p < 0.05$) decrease (54%) in the in vitro generation of erythropoietin by the light mitochondrial extract. Trasylol, an inhibitor of trypsin, chymotrypsin, plasmin, and several kinin-forming enzymes also produced a significant ($p < 0.05$) inhibition (42%) of the erythrogenin capacity of this extract. It has been reported that EDTA will block the activity of erythropoietin in vitro. Therefore, it was of interest to investigate the effects of several chelating agents on the activity of REF in vitro. 3-Mercaptopropionic acid (3-MPA) is a sulfhydryl chelating agent which inhibits metalloenzymes. 3-MPA caused a 45% inhibition of REF activity in vitro (Fig. 5). In that 3-MPA is only one member of a certain class of enzyme inhibitors, other examples of this class
**Fig. 4.** Cathepsin D and Cathepsin E activity in the light mitochondrial extract of kidneys from cobalt-treated rats. Each value represents the mean ± SEM of four experiments. The asterisks indicate value which are significantly (p < 0.05) different from the control (zero time) value.

**Fig. 5.** Effects of enzyme inhibitors on the in vitro generation of erythropoietin. Each value represents the mean ± SEM of five to six experiments as indicated by the number at the bottom of the bars. During these studies the LME was preincubated with the respective inhibitors for 30 min prior to incubation with EDTA dialyzed-normal rat serum. The asterisks indicate significantly (p < 0.05) different from control (LME + NRS) values. Statistical analyses were made by use of the Student's t test. DFP, diisopropyl fluorophosphate. LME, light mitochondrial extract. NRS, normal (EDTA dialyzed) rat serum.
were examined for their ability to inhibit REF activity (Fig. 6). 2-Mercaptoethanol (2-ME) caused a significant ($p < 0.05$) inhibition (53\%), while diethyl-dithiocarbamic acid produced a 60\% inhibition of the erythropoietin-generating activity of the light mitochondrial extract. Epsilon-amino-n-caproic acid (EACA), an inhibitor of several exopeptidases, failed to inhibit the in vitro production of erythropoietin when incubated with the REF extract.

Throughout these studies, the activity of the LME and saline were significantly ($p < 0.01$) less than that of the LME and serum incubation mixtures. In addition, when the inhibitors were incubated with serum or saline, and injected into the assay mice, they failed to produce an erythropoietic effect.

The possibility still existed that the inhibitors tested in our system were not blocking REF activity, but rather inhibiting the activity of the erythropoietin generated. However, when the inhibitors in the concentrations shown in the Materials and Methods section were incubated with the erythropoietin (0.1 U) standard and the reaction mixtures assayed in polycythemic mice, no inhibition of erythropoietic activity was observed.

The ability of the REF to generate ESF in vitro was evaluated in mice which were allowed to equilibrate in an exhypoxic environment for 4, 7, and 8 days, respectively. As indicated in Table 1, the levels of erythropoietin generated from the REF-NRS mixtures were significantly ($p < 0.05$) greater than that of the controls (REF + Saline and NRS + Saline). However, the activity seen in the mice which were subjected to 7- and 8-day posthypoxic periods was consider-
Table 1. Effects of Renal Erythropoietic Factor Activity on $^{59}$Fe Incorporation in Red Cells of 4-, 7-, and 8-Day Posthypoxic Polycythemic Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Mice</th>
<th>Mean Hematocrit (%)</th>
<th>% $^{59}$Fe Incorporation in RBC of Polycythemic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fourth posthypoxic day†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>59 ± 1.16*</td>
<td>3.81 ± 1.27</td>
</tr>
<tr>
<td>NRS + saline</td>
<td>7</td>
<td>66 ± 2.10</td>
<td>2.59 ± 0.69</td>
</tr>
<tr>
<td>REF + saline</td>
<td>6</td>
<td>60 ± 2.90</td>
<td>9.29 ± 1.64</td>
</tr>
<tr>
<td>REF + NRS</td>
<td>7</td>
<td>63 ± 1.62</td>
<td>16.29 ± 3.79†</td>
</tr>
<tr>
<td>Seventh posthypoxic day†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>7</td>
<td>64 ± 2.10</td>
<td>1.84 ± 0.818</td>
</tr>
<tr>
<td>NRS + saline</td>
<td>5</td>
<td>64 ± 3.28</td>
<td>0.67 ± 0.206</td>
</tr>
<tr>
<td>REF + saline</td>
<td>6</td>
<td>64 ± 2.94</td>
<td>2.03 ± 0.799</td>
</tr>
<tr>
<td>REF + NRS</td>
<td>8</td>
<td>62 ± 2.35</td>
<td>5.30 ± 0.417†</td>
</tr>
<tr>
<td>Eighth posthypoxic day†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>9</td>
<td>57 ± 1.46</td>
<td>0.75 ± 0.095</td>
</tr>
<tr>
<td>NRS + saline</td>
<td>7</td>
<td>56 ± 2.34</td>
<td>1.00 ± 0.148</td>
</tr>
<tr>
<td>REF + saline</td>
<td>6</td>
<td>56 ± 1.36</td>
<td>2.07 ± 0.494</td>
</tr>
<tr>
<td>REF + NRS</td>
<td>11</td>
<td>59 ± 1.60</td>
<td>6.66 ± 0.589</td>
</tr>
</tbody>
</table>

†Each mouse received an intraperitoneal injection of the test sample on the fourth, seventh, or eighth posthypoxic day following removal from the hypobaric chamber.

1NRS, Normal (EDTA diolyzed) rat serum.

§REF, Renal erythropoietic factor contained in the light mitochondrial extract (LME)

*Each value represents the mean ± SEM

†Significantly different (p < 0.05) from control (REF + saline and NRS + saline).

‡Significantly different (p < 0.01) from control (REF + saline and NRS + saline).

Statistical analyses were made by use of the Student’s t-test.

ably less than that of the mice maintained at normal atmospheric pressure for a 4-day period.

The effects of the antibody to erythropoietin on the REF-NRS mixture are seen in Table 2. The erythropoietic activity generated by the REF-NRS reaction mixture is significantly (p < 0.01) decreased by the anti-ESF.

Marker enzyme studies were also performed on the light mitochondrial extract from kidneys of normal and cobalt-treated rats. In all extracts studied, alkaline phosphatase, a microsomal enzyme, showed significantly more activity (Fig. 7) than that of acid phosphatase or cytochrome oxidase, markers for lysosomes and mitochondria, respectively.

With reference to the histological composition of the light mitochondrial fraction separated in our studies where cobalt was used as an histotoxic hypoxic stimulus, electron micrographs were made from sections of the LMF (21,000 g fraction) from normal kidneys and kidneys taken from rats 12 hr after cobalt treatment. Cobalt did not produce a detectable alteration in the histological appearance of the LMF. The LMF from kidneys of both normal and cobalt-treated rats consisted of smooth and rough endoplasmic reticulum, comprising the microsomal fraction. In addition, the electron micrographs indicate the presence of a heterogeneous population of vesicles, probably indicative of the presence of lysosomes.
Table 2. Effect of Anti-ESF on the Erythropoietic Activity of the REF-NRS Reaction Mixture in 8-Day Posthypoxic Polycythemic Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Mice</th>
<th>Mean Hematocrit (%)</th>
<th>%^{59}Fe Incorporation in RBC of Polycythemic Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>9</td>
<td>57 ± 1.46†</td>
<td>0.75 ± 0.095</td>
</tr>
<tr>
<td>ESF (0.2 U) + saline</td>
<td>7</td>
<td>57 ± 1.39</td>
<td>15.04 ± 2.330</td>
</tr>
<tr>
<td>NRS + saline</td>
<td>7</td>
<td>56 ± 2.34</td>
<td>1.00 ± 0.148</td>
</tr>
<tr>
<td>REF + saline</td>
<td>6</td>
<td>56 ± 1.36</td>
<td>2.07 ± 0.494</td>
</tr>
<tr>
<td>REF + NRS</td>
<td>11</td>
<td>59 ± 1.60</td>
<td>6.66 ± 0.589</td>
</tr>
<tr>
<td>(REF + Saline + Anti-ESF) + GARGG I</td>
<td>7</td>
<td>56 ± 1.66</td>
<td>3.01 ± 0.924</td>
</tr>
<tr>
<td>[(REF + NRS) + Anti-ESF] + GARGG I</td>
<td>10</td>
<td>59 ± 2.31</td>
<td>1.47 ± 0.276**</td>
</tr>
<tr>
<td>[(ESF (0.2 U) + saline) + Anti-ESF] + GARGG I</td>
<td>9</td>
<td>56 ± 1.68</td>
<td>1.46 ± 0.163**</td>
</tr>
</tbody>
</table>

*Each mouse received an intraperitoneal injection of the test sample on the eighth posthypoxic day following removal from the hypobaric chamber.
†Each value represents the mean ± SEM.
§NRS, normal (EDTA dialyzed) rat serum.
$REF, renal erythropoietic factor contained in the light mitochondrial extract (LME).
\#GARGG, Goat antirabbit gamma globulin.
$Anti-ESF was added to the REF-NRS reaction mixture at the end of the 60-min incubation period. The remaining free anti-ESF was then removed from the incubation mixture with GARGG before injection into the polycythemic mice.
**Significantly different (p < 0.01) from control (REF + NRS or ESF + saline).
Statistical analyses were made by use of the Student's t test.

Fig. 7. Marker enzyme studies on the light mitochondrial extract of kidneys from normal and cobalt-treated rats. Each value represents the mean ± SEM of four experiments. The asterisks indicate significantly (p < 0.05) different from the control (zero time) value.
DISCUSSION

The present experiments indicate that cobalt causes an increase in kidney levels of the renal erythropoietic factor and an elevation in plasma erythropoietin. REF (erythrogenin) activity, which was localized in the "light mitochondrial fraction" of the kidney, reached a peak 12 hr after an injection of cobalt. Plasma ESF titers also reached a peak 12 hr after cobalt.

In that the generation of ESF might possibly involve the interaction of a renal proteolytic enzyme and a plasma protein, experiments were undertaken to investigate the effects of cobalt as the erythropoietic stimulus on a class of kidney hydrolases (Cathepsins), in the light mitochondrial extract. The light mitochondrial fraction, from which the extract is derived, is known to contain particles intermediate in size between the heavy mitochondria and microsomes. It is a heterogeneous mixture which is known to include lysosomes and other cellular organelles, but very few true mitochondria. We have prepared electron micrographs of the LMF (21,000 g fraction) in the present studies from kidneys of normal and cobalt-treated rats. Cobalt had no observable effect on the integrity of the organelles composing this fraction of renal tissue.

The cathepsins are hydrolytic enzymes which have been localized in the lysosomal\(^2\) and light mitochondrial fractions\(^2\) of various tissues, including the rat kidney. It has been shown by DeDuve and Beaufay\(^2\) that if an organ is made ischemic, in time the membranes of the organelles which contain the cathepsins become labile and eventually rupture. In this way, the cathepsins as well as other hydrolases are released into the ischemic tissues and initiate the process of tissue autolysis.\(^2\) The mechanism of membrane labilization might involve to some extent a decrease in intracellular pH, which might serve to activate several of the hydrolases in the lysosomes and other organelles. Cobalt, by inducing histotoxic hypoxia, may simulate a condition in tissues which is similar to that found following ischemia. Levy et al.\(^4\) and Yastrebov\(^4\) observed that cobalt inhibited certain oxidative enzymes of the rat kidney in vitro. This effect of cobalt was accompanied by a decrease in kidney respiration and a fall in oxidative phosphorylation. It is possible that cobalt might also act to cause the release and/or activation of the REF. The observation that the activity curve for REF parallels to some extent the curves for cathepsins A and B following cobalt stimulation may indicate a possible relationship between cathepsins A and B and renal erythropoietin production.

The work of Contrera\(^4\) and Zanjani et al.\(^7\) indicates that the REF-serum interaction possesses several enzymatic characteristics. Therefore, known inhibitors of various classes of enzymes were tested for their ability to block the in vitro production of ESF by the renal erythropoietic factor. The polypeptide, Trasylol, a potent inhibitor of many proteolytic enzymes, significantly \((p < 0.05)\) inhibits the erythropoietin-generating activity of REF. The inhibition of REF activity by diisopropyl fluorophosphate, a general esterase inhibitor, indicates that REF possesses "serine enzyme" activity. This observation substantiates the work of Contrera,\(^4\) who demonstrated that the esterase activity of the LME was inhibited by diisopropyl fluorophosphate. Several investigators\(^1\) have shown that dialyzing REF against EDTA caused a decrease in the ability of REF to generate erythropoietin when incubated with normal dialyzed rat serum. We have demonstrated in the present studies that sulphydryl chelating
agents were effective in reducing the erythrogenic capacity of the REF in vitro. Diethyldithiocarbamic acid, 3 mercaptopropionic acid, and 2-mercaptoethanol were found to be potent inhibitors of REF. These findings suggest that REF might possess metalloenzyme activity, in that the agents tested are inhibitors of metal-dependent enzymes.

In an attempt to resolve the question as to whether or not the time of injection of the REF-NRS incubation mixture following exposure to hypoxia had an effect on the response of the polycythemic mice to this reaction mixture, we studied the effects of erythropoietin generated by REF in polycythemic mice which were allowed to equilibrate at normal atmospheric pressure for 4-, 7-, and 8-day periods. It is very difficult to determine when erythropoiesis is maximally suppressed because of the variability of the exhypoxic polycythemic mouse bioassay systems used in different laboratories. However, OKunewick et al.43 reported that radioactive iron incorporation in polycythemic mice reached the lowest point between 7 -10 days after removal from hypoxia. It is clear from our data that the baseline iron incorporation in red cells of mice maintained exhypoxic for 7 and 8 days was reduced when compared to that of the 4-day exhypoxic mice. However, significant erythropoietic activity was seen in polycythemic mice injected with the REF-NRS incubation mixture while using the 7- and 8-day posthypoxic injection schedule. Furthermore, we have demonstrated that the erythropoietic activity of the product(s) of the REF-NRS reaction can be effectively blocked by the antibody to erythropoietin in the 8-day posthypoxic polycythemic mice, indicating that the activity generated by REF in vitro is erythropoietin. It does not, however, completely preclude the possibility that substances other than erythropoietin might have been generated which are erythropoietically active. Nevertheless, polycythemic mice maintained for a longer (8-day) posthypoxic period still retained their ability to respond to the REF-NRS reaction mixture.

In 1954, DeDuve and Berthet44 found that hydrolases (e.g., Cathespins) are segregated within lysosomal particles which are impermeable to their respective substrates. This is believed to be a mechanism for the storage of these hydrolases in normal intact cells. The cathepsin-like activity observed in the present studies most probably originated in the lysosomal particles of the light mitochondrial fraction. It is quite possible that cobalt may cause labilization of the membrane of renal lysosomes, thus permitting an interaction between the lysosomal hydrolases and their respective substrates. If cobalt produced membrane labilization in lysosomes in cells near the wall of renal blood vessels, the REF released could then act upon its protein substrate in the cells and/or plasma to generate ESF. An alternative possibility is that cobalt, acting as an erythropoietic stimulus, might enter the lysosomal particles, catalyze the conversion of an inactive REF to its active form, and labilize the lysosomal membrane to cause the release of active REF. This active REF could react intracellularly or in plasma with a protein substrate to produce ESF.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Robert D. Yates, Dr. Paul M. Heidger, Mr. J. A. Mascarro, and Mr. Jesse Brookins for their expert assistance, and to Mrs. Gilda Chabert for her help in the preparation of this manuscript.
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