over which y_{10} is activated is not sufficient for the realization of the steady states, then the gain of the enzyme amplifier will be less than that indicated by Eq. 5. The effect of progressively shorter time intervals, a, on $F(t)$ is shown (Fig. 1) in the instance of a three-stage process. Though the choice of constants is arbitrary the curves make clear the nature of this dependency.

A second point with reference to Eq. 5 is that the gain involves the products of several single-stage terms. This means that small shifts in the rate constants or the intial conditions can collectively produce significant overall effects. Thus a decrease of only 2 percent in k_i and y_{10} together with a corresponding increase in K_i results in an eight-stage process, such as blood coagulation, in an overall decrease in gain of nearly 40 percent. This sort of effect has bearing on negative and positive feedback which effects several stages simultaneously.

SUMNER N. LEVINE
State University of New York
Stony Brook, Long Island

References and Notes

1. R. G. MacFarlane, *Nature* **202**, 498 (1964).
2. G. Wald, *Science* **150**, 1028 (1965).
3. I thank J. L. Barr for programming the equations on the computer. Sponsored by the Air Force Office of Scientific Research contract AF-AFOSR-667-64.

6 January 1966

Erythropoietin: Production by a Particulate Fraction of Rat Kidney

Abstract. *An erythropoietic factor was extracted with hypotonic phosphate buffer from the kidneys of hypoxic rats. Normal rat serum enhanced the activity of this factor, which is associated with the light mitochondrial fraction. The data suggest that the renal factor is not physiologically active unless it interacts with a serum carrier or activator, or that the factor may be an enzyme which produces erythropoietin from some serum substrate.*

Erythropoietin has been extracted from the kidneys of hypoxic and anemic rats. The extract obtained by homogenization in 0.02*M* phosphate buffer, *p*H 6.8 (*1*), contains a mixture of erythropoietin, some derived from residual plasma in the renal vasculature, and some of intracellular origin (renal erythropoietic factor). We now report details of the extraction and properties of the renal erythropoietic factor.

Thirty-two adult female Long-Evans rats were rendered hypoxic by continuous exposure to 0.5 atm of air for 17 hours. The animals were then immediately exsanguinated, and their kidneys (43.9 g) were removed, minced, and homogenized in 100 ml of cold isotonic saline (*p*H 7.0). The homogenate was centrifuged at 37,000*g* for 30 minutes, and the sediment was washed in 38 ml of isotonic saline. The isotonic supernatants were combined. The sediment was homogenized again in 150 ml of 0.02*M* phosphate buffer at *p*H 6.8, and the supernatant was collected by centrifugation. This two-step procedure resulted in separation of plasma-borne residual erythropoietin (isotonic supernatant) from the renal erythropoietic factor (hypotonic supernatant). Erythropoietic activity was assayed in mice rendered plethoric by discontinuous exposure to hypoxia (*2*, *3*). Values obtained for incorporation of radioactive iron into circulating red cells were converted to unit equivalents of standard A (*3*). All materials were administered intraperitoneally in a volume of 1 to 2 ml. The assay was sufficiently sensitive to detect as little as 0.02 unit of erythropoietin.

The hypotonic extract contained approximately 75 percent of the total erythropoietin recovered from the kidney (Table 1*A*). This indicates that the bulk of the erythropoietin in the kidneys of hypoxic rats may be contained within particles. Based on the method for estimating residual plasma volume (*1*), the activity in the isotonic saline extract was due mainly to residual plasma erythropoietin. Both extracts were incubated at 37°C for 30 minutes with an equal volume of normal rat serum. The erythropoietic activity of the hypotonic extract increased more than twofold after such incubation, whereas the isotonic saline extract did not (Table 1*A*). The normal rat serum contained no activity when assayed alone. This activation phenomenon is believed to be a characteristic property of the renal erythropoietic factor. Perfusates of kidneys of anoxic rats also enhance erythropoietic activity when incubated with normal serum (*4*). Association of the erythropoietic factor with the nuclei of kidney cells has been recently reported (*5*).

To clarify further the nature of the renal erythropoietic factor, a subcellular fractionation of the kidney was undertaken (*6*). Thirty-four kidneys (23.5 g) from adult female Long-Evans rats made hypoxic by exposure to 0.4 atm of air for 17 hours were used. The nuclear, heavy-mitochondrial, light-mitochondrial, and microsomal fractions were collected, and each fraction was resuspended in 50 ml of 0.02*M* phosphate buffer (*p*H 6.8) and centrifuged at 37,000*g* for 30 minutes. The supernatant of each fraction was then assayed for erythropoietic activity before and after incubation with an equal volume of normal rat serum. The extract of the light-mitochondrial fraction exhibited no detectable activity when assayed alone but showed the largest amount of erythropoietic activity after incubation with normal rat serum (Table 1*B*). This extract had a protein concentration of 8 mg/ml. Enhancement was noted only with the light-mitochondrial extract. It seems, therefore, that the renal erythropoietic factor is localized in particles intermediate in density between the heavy mitochondria and microsomes.

An increase in the time of incubation or the amount of light-mitochondrial extract results in a decrease in activity (Table 2). Such effects may be due to the hydrolytic enzymes—some of probable lysosomal origin—present in the fraction (*7*) since erythropoietin is known to be inactivated by proteolytic enzymes (*8*). Regression data show no significant deviation from parallelism between standard A-erythropoietin and the product of the interaction of the light-mitochondrial extract with normal rat serum. This implies that the product is physiologically indistinguishable from erythropoietin. Extracts of the light-mitochondrial fraction of 24.0-g kidneys from nonhypoxic rats were obtained, and these also engendered erythropoietic activity when incubated with normal rat serum (Table 1*C*).

Active renal extract, obtained from adult male Long-Evans rats made hypoxic by 17-hour exposure to 0.4 atm of air, was acidified to *p*H 5.0 and then quickly neutralized. This extract no longer evoked erythropoietic activity

Table 1. Erythropoietic activity of extracts from kidneys of hypoxic and nonhypoxic rats. ESF, erythropoietin; NRS, normal rat serum; N.D., not detectable.

Extract	ESF before NRS addition		ESF after NRS addition	
	In incubation mixture (units/ml)	Total (units)	In incubation mixture (units/ml)	Total (units)
A. Two-step extract of hypoxic-rat kidneys				
Isotonic saline	0.02	2.60	0.02	2.60
Hypotonic phosphate	.06	8.40	.15	21.00
B. Subcellular extracts of hypoxic-rat kidneys				
Sucrose supernatant	0.03	6.90	0.02	4.60
Microsomes	.05	2.50	.04	2.00
Light mitochondria	N.D.		.08	4.00
Heavy mitochondria	0.04	2.00	.03	1.50
C. Subcellular extracts of nonhypoxic-rat kidneys				
Sucrose supernatant	N.D.		N.D.	
Microsomes	N.D.		N.D.	
Light mitochondria	N.D.		0.03	1.50
Heavy mitochondria	N.D.		N.D.	

Table 2. Factors influencing the erythropoietic-enhancing activity of light-mitochondrial extracts (LME) of kidneys from hypoxic rats. ESF, erythropoietin; N.D., not detectable.

Light mitochondrial extract		Normal rat serum (ml)	Time (min)	ESF/ml LME (units)
Amount (ml)	Treatment			
1.0	none	2.0	30	0.12
0.1	none	2.0	30	1.80
.1	none	2.0	120	0.50
.1	none	2.0	240	.50
1.0	none	1.0	30	.08
1.0	heat	1.0	30	N.D.
1.0	*p*H 5.0	1.0	30	N.D.

when incubated with normal rat serum at *p*H 7.0. Similarly treated erythropoietin obtained from whole plasma of hypoxic rats showed no significant loss in activity. In another experiment, a portion of the extract was heated to 80°C for 5 minutes prior to incubation with an equal volume of normal rat serum. When the mixture was incubated for 30 minutes no erythropoietic activity could be detected. Erythropoietically active plasma from hypoxic rats, diluted to an erythropoietin concentration comparable to that produced during the incubation of light-mitochondrial extracts, was similarly heat-treated and exhibited only a slight decrease in activity (from 0.1 to 0.07 unit/ml). The renal erythropoietic factor therefore does not have the acid and heat stability of erythropoietin derived from plasma (Table 2). The lability of the renal factor may be due in part to proteolytic enzymes in this extract. Many proteases are known to be most active at acid *p*H.

Since cobalt is a stimulant of erythropoiesis, the effect of this metal on the interaction of the renal factor with normal rat serum was investigated. The addition of 0.8 μmole of $CoCl_2$ to an incubation mixture made up of equal volumes of light-mitochondrial extract in normal rat serum did not result in further increase in erythropoietic activity.

The question as to whether the renal factor is an erythropoietin precursor or an erythropoietin-producing enzyme has not yet been resolved. Evidence does indicate the possible binding with serum protein of a highly purified preparation, derived from sheep plasma, known as step-4 erythropoietin (*9*). In one experiment, 2 units of step-4 erythropoietin dissolved in 2 ml of isotonic saline were added to 4 ml of 10 percent trichloroacetic acid (TCA). The solution was allowed to stand for 15 minutes and then was centrifuged (no precipitate appeared). The resulting solution (solution B) was then adjusted to *p*H 7.0 with 1*M* NaOH. Solution C differed in that 2 units of step-4 erythropoietin were added to 2 ml of normal rat serum prior to the addition of TCA. In solution D, 4 ml of TCA were added to 2 ml of rat serum prior to the addition of 2 units of step-4 erythropoietin. After the erythropoietin was added, the solution was centrifuged, and the supernatant was adjusted to *p*H 7.0. All solutions were dialyzed overnight against normal saline, after which their volumes were all adjusted to 10 ml with saline. One milliliter of each solution, including the untreated solution A, was administered intraperitoneally to each mouse being tested. Purified erythropoietin was unaffected by TCA when no serum proteins were present. In the presence of such proteins, all erythropoietic activity was lost after the addition of TCA. Solution D lost only 30 percent of its activity, which is not considered significant in view of the probable mechanical loss of erythropoietin on the flocculated serum proteins. It would appear that the step-4 erythropoietin is not being precipitated by indiscriminate association with denatured serum proteins, but is bound strongly to some protein component of the serum only when this component is in the native state. Possibly such a component is functioning as an erythropoietin carrier. Erythropoietin preparations of higher purity than step-4 have been shown to be highly unstable, and it has been suggested that purification strips away a protective protein shell surrounding the basic erythropoietin molecule (*9*). The renal erythropoietic factor present in the light-mitochondrial extract may represent the basic erythropoietin molecule before it has become associated with a protective and possibly activating serum protein. The possibility cannot be precluded at this time that the renal factor is an enzyme which produces erythropoietin from some serum substrate.

JOSEPH F. CONTRERA
ALBERT S. GORDON
Department of Biology,
Graduate School of Arts and Science,
New York University, New York

References and Notes

1. J. F. Contrera, J. F. Camiscoli, A. H. Weintraub, A. S. Gordon, *Blood* **25**, 809 (1965).
2. A. S. Gordon and A. H. Weintraub, in *Erythropoiesis*, L. O. Jacobson and M. Doyle, Eds. (Grune and Stratton, New York, 1962), pp. 1–16.
3. A. H. Weintraub, A. S. Gordon, J. F. Camiscoli, *J. Lab. Clin. Med.* **62**, 743 (1963).
4. Z. Kuratowska, B. Lewartowski, B. Lipinski, *ibid.* **64**, 226 (1964).
5. Z. Kuratowska, *Bull. Acad. Polon. Sci.* **8**, 385 (1965).
6. F. S. La Bella, R. J. Reiffenstein, G. Beaulieu, *Arch. Biochem. Biophys.* **100**, 399 (1963).
7. W. Straus, *J. Biol. Chem.* **207**, 745 (1954); *J. Biophys. Biochem. Cytol.* **2**, 513 (1956).
8. P. H. Lowy and H. Borsook, in *Erythropoiesis*, L. O. Jacobson and M. Doyle, Eds. (Grune and Stratton, New York, 1962), pp. 33–38; J. W. Winkert and A. S. Gordon, *Biochem. Biophys. Acta* **42**, 170 (1960).
9. E. Goldwasser, W. F. White, K. B. Taylor, in *Erythropoiesis*, L. O. Jacobson and M. Doyle, Ed. (Grune and Stratton, New York, 1962), pp. 43–49.
10. Supported by PHS grant 5-ROI-HE3357-09. We thank the National Heart Institute for supplies of step-4 sheep erythropoietin.

15 February 1966