The Renal Erythropoietic Factor (REF). III. Enzymatic Role in Erythropoietin Production.* (32132)

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The existence of a kidney factor that interacts with a serum protein to produce erythropoietin (ESF) was reported by Kuratowska *et al*(1). The recent extraction of a renal erythropoietic factor (REF) from the kidneys of hypoxic rats has strengthened this contention(2,3). The REF, when administered alone, exhibits no erythropoiesis stimulating activity, but when incubated *in vitro* with normal serum, engenders the production of ESF. Recent evidence has indicated that the REF has chemical and physiological properties different from renin, and that renin, angiotensin II and bradykinin are ineffective in stimulating erythropoiesis (5).

Kinetic studies of ESF production by the REF in this *in vitro* system have been hampered by an inactivation reaction which occurs simultaneously with the ESF-generating process, but at a slower rate. This inactivation has been prevented by dialyzing the normal serum used in the incubation system against ethylenediamine tetra-acetate (EDTA)(4,5). The elimination of the inactivation process makes it possible to investigate the kinetics of the REF-serum interaction and to determine whether ESF production is an enzyme-catalyzed reaction, or simply the result of a coupling or conjugation of the REF with a serum protein.

Materials and methods. Serum from 280 male Long-Evans rats (240-280 g) was dialyzed for 24 hours in cellulose tubing against 50-100 volumes of 0.005 M Na_2-EDTA and subsequently dialyzed against 50-100 volumes of deionized water at 4°C for an additional 24 hours to remove the excess EDTA. A mean increase of 25% in the volume of the serum within the casing was observed after dialysis. Adjustment for this increase was not made in the serum volumes used in the incubations. All dialyzed serum was frozen until used. The REF was prepared from the "light mitochondrial" fraction of kidneys from 100 hypoxic rats as previously described(2,3). Incubations of the REF and dialyzed whole serum were conducted in a water bath with shaking at 37°C in vessels open to the atmosphere. The ESF content of the reaction mixtures was determined by a standard assay involving the incorporation of radioiron into the circulating red cells of hypoxia-induced polycythemic mice(6). Five or six mice were injected i.p. with one 2 ml dose of the reaction mixture immediately following incubation. Each mouse received 48 hours later an i.v. injection of 0.5 μc ^{59}Fe and 48 hours after the radioiron injection the per cent RBC-^{59}Fe incorporation was estimated (6). Per cent RBC-radioiron incorporation values were converted to ESF International units by reference to the dose-response curve for assay mice injected with the International Reference Preparation for ESF(7). All assays were conducted at least 2 times.

Results. Fig. 1 shows the total quantities of ESF produced from a mixture of 1 ml of REF (equivalent to 0.5 g hypoxic rat kidney) and 48 ml of dialyzed normal serum after varying times of incubation. A progressive increase in ESF formation was noted with the prolongation in incubation time. When the REF concentration was doubled, with the amount of dialyzed serum, total volume of the mixture and incubation time held constant, approximately twice as much ESF was produced (Fig. 2). When the volume of dialyzed serum was varied (0.5 ml to 6.0 ml), the REF content (6 ml), total volume (12 ml) and incubation time (15 minutes) held constant, the reaction rate was found to in-

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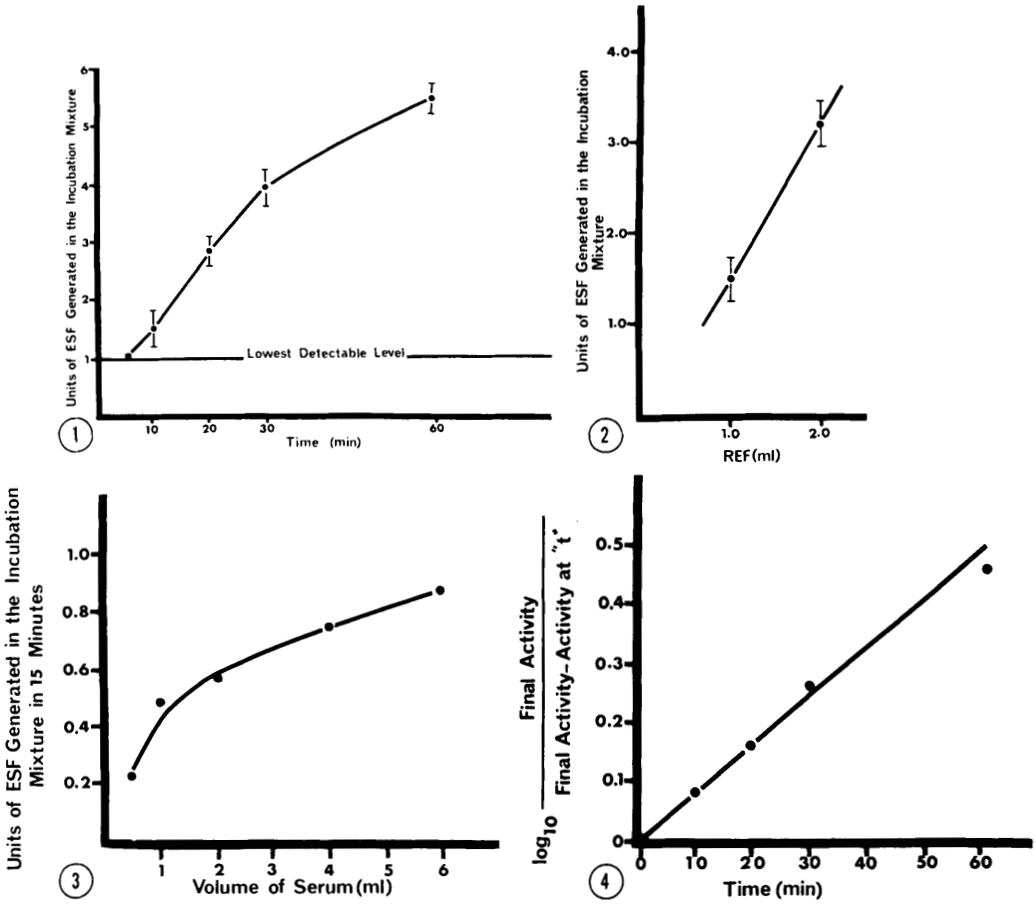

FIG. 1. Formation of ESF in the REF-serum reaction mixture as a function of time. Volume of the dialyzed serum was 48 ml; volume of the REF was 1.0 ml. Each point represents the mean ± SEM of 3 separate experiments.

FIG. 2. Formation of ESF in the REF-serum reaction mixture as a function of REF concentration. Volume of the dialyzed serum was 48 ml and incubation time 15 min. The volumes of the reaction mixtures were brought to 54 ml by addition of deionized water. Each point represents the mean ± SEM of 3 separate experiments.

FIG. 3. Relation of volume of dialyzed serum to quantity of ESF produced in 15 min. Volume of the REF was 6 ml. Volumes of all reaction mixtures were brought to 12 ml by addition of deionized water. Each point represents the mean of 2 separate experiments.

FIG. 4. Logarithmic function of the remaining concentration of substrate plotted against time. Each point represents the mean of 2 separate experiments.

crease with an increase in the serum content (Fig. 3).

Discussion. Fig. 1 showing the effect of incubating REF with serum substrate for varying periods of time, suggested the occurrence of a first-order reaction. First-order reaction kinetics were demonstrated by plotting a logarithmic function (y) of each point in Fig. 1 against the incubation time,

$$\text{with } y = \log_{10} \frac{\text{Final activity}}{\text{Final activity—activity at time ``t''}}$$

Final activity was the estimated maximum ESF produced under the incubation conditions described (8.6 U/49 ml of incubation mixture) and was determined by the amount of substrate present in the serum at zero time(8). A straight line was obtained (Fig. 4), showing that the reaction followed first-order kinetics under the indicated conditions of incubation, and within the accuracy of the experimental method. The yield of ESF obtained after 60 minutes of incubation (0.12

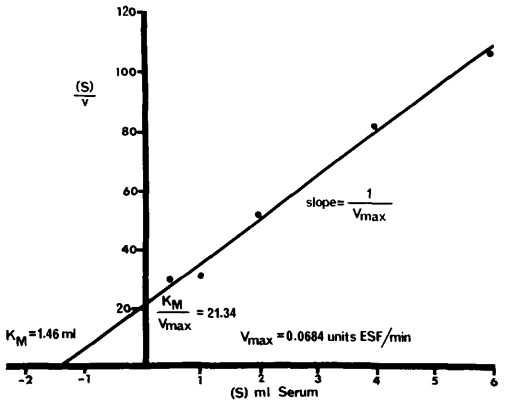

FIG. 5. Modified Lineweaver-Burke plot of the relation of rate of ESF production to the level of serum substrate. The reaction velocity (v) is expressed in Units of ESF formed per minute. Volume of the incubation mixture was 12 ml and incubation time was 15 min. Each point represents the mean of 2 separate experiments.

U of ESF/ml of serum) was approaching, but had not yet reached maximum. Prolonged incubation for periods exceeding one hour would have produced maximal amounts of ESF, estimated at about 0.18 U/ml of serum. However, some inactivation of the enzyme and/or product probably still occurred during long-term incubation of the REF. The most effective procedure for generating maximal quantities of ESF was to incubate serum with a large excess of the enzyme for short periods of time (10-15 minutes). Under these conditions, one ml of the standardized dialyzed serum yielded 0.18-0.28 U of ESF. It was also necessary, for accurate and reproducible results, to use the same batch of REF and dialyzed serum. Serum collected at different times was found to vary in substrate content.

The production of ESF by the action of the REF on a serum substrate was observed to be proportional to the concentration of the REF (Fig. 2). This behavior is typical of both first-order and zero-order enzymatic reactions (9). The reaction rate increased when substrate concentration was increased (Fig. 3). The modified Lineweaver-Burke plot of these data (Fig. 5) showed a linear relation between substrate level (S) and (S)/v (substrate divided by the rate of the reaction) (10). The reciprocal of the slope of the line gave the maximum velocity of the reaction (V_{max}) as 0.068 U ESF/min which would be observed when the enzyme was saturated with the serum substrate. This value was somewhat greater than the rate of ESF formation (0.056 U ESF/min) noted when 6 ml of dialyzed serum was present in the reaction mixture. In these experiments, the rate of ESF production was still rising, the enzyme was not yet saturated with the substrate, and therefore a higher rate of reaction was still possible. The Michaelis constant (K_M) is indicated on the graph by the intercept on the abscissa, 1.46 ml. This behavior is characteristic of a first-order enzyme-substrate reaction.

The second possibility regarding the *in vitro* formation of ESF by the REF involves the coupling or conjugation of the REF to a serum protein. Since two substrates are required in this type of reaction, second-order kinetics would be expected to follow. As first-order kinetics were demonstrated for the ESF-generating reaction, the hypothesis that the ESF is formed as a result of activation of the REF by a serum protein moiety does not seem to be tenable. Moreover, if a non-enzymatic coupling of the REF to a serum protein constituted the mechanism of ESF formation, it would be necessary for the kidney to synthesize the REF continuously during hypoxia. Although there was significantly greater ^{14}C-isoleucine incorporation into kidney proteins during exposure of rats to one hour of hypoxia, these values did not differ significantly from the controls after longer exposures (2-18 hours) (11). An enzyme mechanism would not require continuous synthesis of the REF.

Summary. The properties of the renal erythropoietic factor (REF)-serum reaction, in which ESF is generated *in vitro,* are described. The amount of serum substrate converted to ESF in a given time is proportional to the REF concentration, when the serum level is kept relatively high and constant. Reaction rate is also directly dependent on serum concentration. The production of ESF as a function of time of incubation of the REF with serum, conforms to a first-order reaction. The data support the contention that the REF is an enzyme which acts on a substrate present in normal serum to produce the ESF.

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Effect of Estrogen and Progesterone on Uterine Acid and Alkaline Phosphatase and β-Glucuronidase Activity in Mated Ovariectomized Rats. (32133)

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The role of uterine enzymes in the process of implantation has been the subject of numerous investigations but still remains poorly understood(1). Confusion exists because many of the uterine enzyme activities may be altered differently by hormone administration, by treatment with deciduomatogenic agents or by the process of nidation itself(2-8).

The search for enzymological evidence of the hypothetical "estrogen surge" required for implantation, which is thought to occur on day 3 following mating in the rat, has also contributed to contradictory findings [an increase in β-glucuronidase on the day prior to implantation reported(9) as evidence of an "estrogen surge" could not be confirmed by us(2)]. Manning *et al*(2) described a technique which permitted simultaneous evaluation and segregation of endocrine and nidatory effects on uterine enzymes in the same animal. Thus rats were surgically prepared with unilateral section and ligation of the fallopian tubes ("USLT" rats). When mated, such animals became unilaterally pregnant, permitting measurement of enzyme activities in the uterine horn subjected to the influence of fertilized ova in comparison with the sterile contralateral horn. Using USLT rats, it was found(2) that during the implantation process a large increase in alkaline phosphatase activity occurred at the implantation sites of the pregnant horn. Enzyme concentration in the inter-implantation areas as well as in the sterile horn remained similar to that observed *in uteri* from non-pregnant animals. It was therefore postulated that the increased alkaline phosphatase activity resulted from an interaction of the blastocyst with the hormonally conditioned endometrium rather than from direct hormonal influences on the uterus(2).

In the present experiments, USLT rats were bilaterally ovariectomized one or four days following proven mating. Exogenous 17β-estradiol and/or progesterone in doses which succeeded or failed to induce nidation were studied for effects on uterine alkaline and acid phosphatase and on β-glucuronidase in the fertile as compared with the sterile horns.

Material and methods. Mating, tissue separation and homogenation, as well as unilateral tubal ligation and section were carried out as previously reported(2). Occurrence of copulation was determined within 3 hours by vaginal smear and this time was designated as day zero of pregnancy.