the increase of these elements in the circulation. However, the reticulocyte releasing effect of other stimuli on the spleen occurs whithin a few h[19] i.e., it is early and transient. In contrast the increase in ^{59}Fe uptake and number of circulating reticulocytes appeared with a delay of 12 h and lasted up to 24 h after PLE administration. Replenishing of the reticulocyte pool in the spleen takes only a few h, the observed protracted splenic erythropoietic depression can therefore not be interpreted solely on the basis of a short-lasting reticulocyte depletion.

The association of an increased RBC ^{59}Fe uptake with a shorter half life of plasma radioiron probably reflects a genuine increase in the rate of hemoglobin synthesis and not a mere mobilization of reticulocytes.

This time-course for the changes also makes it improbable that PLE had an Epo-like effect, since this would have demanded a longer time for the effects to be apparent in peripheral blood.

The most plausible interpretation of the effects of phospholipids on the erythropoietic parameters here measured may be based on an action on erythroid precursors younger than the reticulocyte. This interpretation is strongly supported by the effects observed after administration of PLE to polycythemic recipients after CO-Epo stimulation as is shown in figure 2. In this experiments PLE were given at a time when nucleated erythroid precursors in the organs were not beyond the stages of basophilic or earlier polychromatic erythroblasts.

Although the data reported here do not provide direct evidence that lipids act as regulatory factors on erythropoiesis, they suggest an erythropoietic activity of some sphingolipids. A mere nutritional effect of lipids on the growth of red cells appears unlikely since mouse plasma contained presumably non-limiting amounts of sphingolipids prior to injection. These observations also indicate that the erythroblastic compartment is capable of significant changes in the rate of erythrocytic output.

1 Acknowledgments. This work was supported by a grant from the Consejo Nacional de Investigaciones cientificas y Técnicas de la República Argentina. We thank Isabel Zingariello and Maria Amalia Nicastro for excellent technical assistance.

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Enhanced enzyme activity in concentrated salt solutions

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Summary. The enzyme activity of thrombin, β-factor Xa-E and trypsin was enhanced in 1.0 M sodium citrate solution. Thrombin activated factor X. Synthetic substrate S-2222, designed for factor Xa, was hydrolyzed more rapidly with factor Xa or with thrombin in 1.0 M sodium citrate solution than in dilute salt solutions.

In 1949 it was found that prothrombin complex preparations spontaneously generated thrombin in 1.0 M sodium citrate solutions[2]. Other salt solutions such as ammonium sulfate, magnesium sulfate, sodium sulfate, potassium citrate, potassium oxalate and dipotassium hydrogen phosphate had a similar effect[3]. Factor X converted to factor Xa by autocatalysis. It may be that many enzyme reactions progress more rapidly in certain strong salt solutions, and in support of that idea we are reporting on a study of thrombin, β-factor Xa-E, and trypsin. All three had their activity enhanced in 1.0 M sodium citrate solution. For the experiments all of our proteins were single components as measured by polyacrylamide gel electrophoresis, and were prepared and assayed by methods developed and described in this laboratory.

In physiological saline solution slow conversion of factor X to factor Xa occurred with thrombin. Only 8% of the potential activity generated in 2 h (fig. 1). This low yield is in accord with the general view that factor X is not activated by thrombin. Complete activation was, however, achieved in 1.0 M sodium citrate solution. Using the same quantities of factor X and thrombin in 3 M ammonium sulfate solutions also fulfilled conditions for accelerated activation but not as well as was the case with sodium citrate. The bond broken by thrombin is in the sequence Arg: Ile-Val-Gly-Gly-Arg-Asp. Commonly the bond bro-

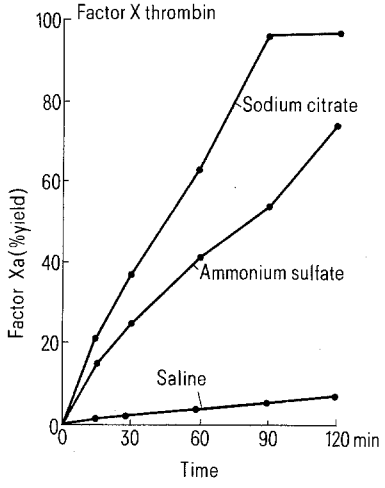

Figure 1. Purified bovine thrombin (25 µg/ml) was mixed with purified α-factor X (100 µg/ml) in physiological saline at pH 7.2, 3.0 M ammonium sulfate, or 1.0 M sodium citrate solution at 37 °C. The yield of factor Xa is recorded in percent.

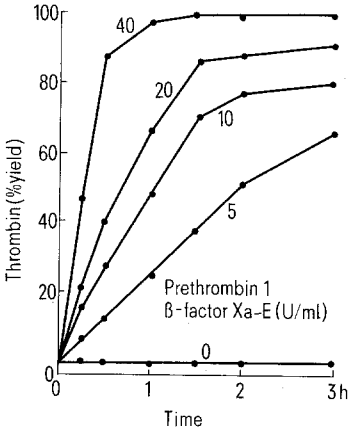

Figure 2. Purified bovine prethrombin 1 (250 µg/ml) was mixed with purified bovine β-factor Xa-E at 5, 10, 20 and 40 units/ml (40 = 25 µg) in 1.0 M sodium citrate solution at 37 °C. Thrombin yield is given in percent.

ken by thrombin is arginyl with glutamic acid or aspartic acid as well as the respective amides positioned at 6, 7 or 8 amino acid residues away from the cleavage bond. Such a bond in factor X was made susceptible for cleavage by using 1.0 M sodium citrate solution.

β-Factor Xa-E is the smallest (mol. wt 34,500) known degraded form of factor Xa and has virtually no prothrombinase activity. The latter term refers to the complex formed by factor Xa, phospholipid, factor V, prothrombin fragment 1 · 2 and calcium ions[5]. Prothrombin fragment 1 · 2 or a combination of prothrombin fragment 1 plus prothrombin fragment 2 is essential for prothrombinase activity[5]. β-Factor Xa-E had practically no prothrombinase activity because the γ-carboxyglutamic acids were removed from the molecule. Maximum generation of thrombin from prethrombin 1 with β-factor Xa-E was found at enzyme concentrations that are inadequate in physiological saline solutions (fig. 2). The proteolytic activity of β-factor Xa-E functioned in concentrated salt solutions even though it is a drastically modified form of α-factor Xa, and cannot participate in prothrombinase activity.

In earlier work trypsin[6] was used in physiological saline solution to generate thrombin from prethrombin 1. On a

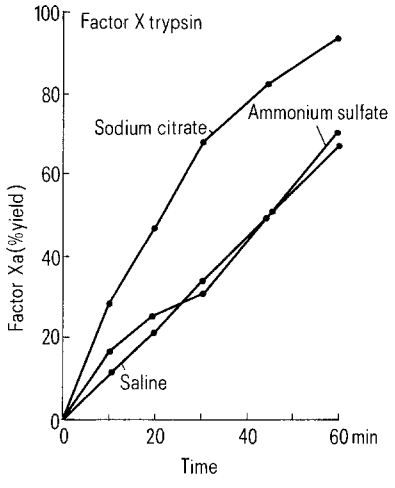

Figure 3. Purified bovine α-factor X (200 µg/ml) mixed with trypsin (1 µg/ml) at 37 °C in physiological saline pH 7.2, 3.0 M ammonium sulfate, or 1.0 M sodium citrate solution. Yield of factor Xa given in percent. Acceleration occurred only in the sodium citrate solution.

w/w basis prethrombin 1 converted to thrombin with trypsin at about the same rate as with α-factor Xa. There was however, a lag phase with the latter[6]. In 1.0 M sodium citrate solution there was some enhancement of trypsin activity (fig. 3), but in 3.0 M ammonium sulfate solution we obtained the same results as in physiological saline solution.

In the 3 illustrations thrombin activity was measured using fibrinogen and factor Xa by our prothrombinase assay[7]. At the same time we carried out assays for thrombin with synthetic substrate S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide dihydrochloride) and for factor Xa with synthetic substrate S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride). The fibrinogen and prothrombinase assays correlated with synthetic substrate assays.

With synthetic substrate S-2222, designed to meet the specificity requirements of factor Xa, the enzyme activity of β-factor Xa-E was potentiated in 1.0 M sodium citrate solution. The same response was observed with thrombin, but not so much with thrombin on S-2238. Stated another way, enhancement in the case of thrombin was not with the substrate designed for it but the one designed for factor Xa. This correlates with effectiveness of thrombin in activating factor X and the autocatalysis of factor X. We did not determine kinetic constants, but estimate about a 5-fold increase in amidolytic activity on S-2222 with either enzyme. The information is consistent with the conclusion that bonds in synthetic substrates as well as protein substrates may be cleaved more effectively in 1.0 M sodium citrate than in dilute salt solution. These are the same bonds cleaved slowly in dilute salt solutions by the enzymes we studied.

1 Recipient of a Fogarty International Research Fellowship (TW-02743-02).
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