Agents Providing Nonenzymic Prothrombin Activation. (26864)

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An unusual nonenzymic prothrombin activation was firmly established by demonstrating thrombin formation in mixtures of synthetic poly-L-lysine (polyLYS) and homogeneous horse prothrombin(1). Reaction characteristics were a high, optimum *weight* ratio of synthetic protein to prothrombin, a low temperature optimum, high sensitivity to ionic strength changes, and insensitivity to diisopropylphosphofluoridate (DFP). Several basic materials now substitute for the polyLYS, including synthetic poly-L-ornithine (polyORN) and polymyxin B. The prothrombin activation in protamine sulfate solution, for which an autocatalytic mechanism was suggested(2), has several characteristics of this form of nonenzymic reaction.

Nonenzymic prothrombin activation is inhibited by heparin, poly-L-glutamic acid (polyGLU), and poly-L-aspartic acid (polyASP).

Materials and methods. Horse prothrombin isolated from citrated horse plasma in 60% yield was thrombin-free and immunochemically and physicochemically homogeneous after equilibrium ion-exchange chromatography(3,4). Synthetic tri-L-lysine (triLYS) was kindly donated by Dr. Bernard Erlanger of Columbia University. Poly-L-histidine (polyHIS), approximately 6,000 molecular weight, was contributed by Dr. Gerald Fasman, Children's Cancer Research Foundation, Boston, Mass., and Dr. F. R. N. Gurd, School of Medicine, Indiana University. Synthetic polyLYS, polyORN, polyGLU, and polyASP were commercial products. Antibiotics, protamine, and heparin were commercial preparations free of solubilizers and extraneous salts.

Prothrombin and thrombin were titered according to the methods of Ware and Seegers(5), using polyethylene tubes to dilute thrombin samples.

Substitutes for polyLYS in the nonenzymic prothrombin activation were screened by testing 4.5 to 5.0 mg of each with 3.0 to 3.5 mg samples of horse prothrombin in 2.0 ml of 0.08 *M* Tris buffer, pH 8.0, at 25°C. Reactions were followed by thrombin and prothrombin titrations; the degree of activation is recorded in Table I. Controls included equivalent mixtures of preformed thrombin with each test material. Inhibition of the nonenzymic reaction by heparin, polyGLU, and polyASP was tested by adding 3 mg of each to systems containing polyLYS and prothrombin in the above proportions (Table I). The optimum activation temperatures of prothrombin-polymyxin B and prothrombin-protamine mixtures were determined for comparison with the synthetic polyLYS-prothrombin reaction(1) (Fig. 1).

To rule out enzymic contamination, samples of an aqueous protamine sulfate solution were heated 15 minutes at 100°C or incubated 30 minutes in 5×10^{-3} *M* DFP. Following dialysis, these samples and the original solution activated horse prothrombin at pH 7.5. Subsequently, 3.2 mg of horse pro-

TABLE I. Nonenzymic Prothrombin Activation.

Substance added	% activation 2 hr	8 hr
PolyLYS	74	83
PolyORN	65	75
Polymyxin	65	70
Protamine	60	70
Neomycin	6	16
Streptomycin	3	10
PolyLYS + heparin	0	0
PolyLYS + polyGLU	0	0
PolyLYS + polyASP	0	0
TriLYS	0	0
PolyHIS	0	0
PolyGLU	0	0
PolyASP	0	0
Bacitracin, Novobiocin, Vancomycin, Ristocetin, Erythromycin, Amphotericin B, Nystatin	0	0

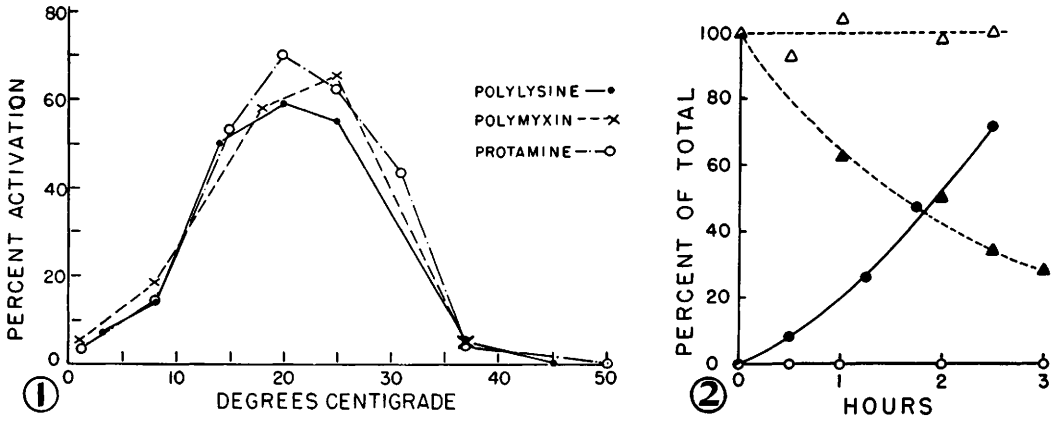

FIG. 1. Effect of temperature on nonenzymic prothrombin activation. With polymyxin and protamine for 2 hr and with polyLYS for 1.5 hr.

FIG. 2. Per cent thrombin (spheres) and prothrombin (triangles) in the following systems: Prothr. + protamine (●——●); prothr. + protamine + 3.8 × 10^{-3} M DFP (○——○) and (▲---▲); and prothr. + saline + DFP (△---△).

thrombin and 6.0-mg samples of heat-treated or DFP-treated and dialyzed protamine were combined in 2.0 ml of 0.05 *M* imidazole buffer, pH 7.5, which contained 3.8 × 10^{-3} *M* DFP. DFP was omitted from an otherwise identical reaction mixture, and saline was substituted for protamine in another control. Prothrombin consumption in the DFP-containing systems and thrombin formation in the DFP-free-systems are plotted in Fig. 2.

Results. At the optimum conditions characterizing nonenzymic polyLYS-prothrombin activation, polyORN, polymyxin B, and protamine also activate the zymogen. Neomycin and streptomycin may also function to a slight degree in the same process. Synthetic triLYS, polyHIS, polyGLU, and polyASP, as well as a variety of peptide and nonpeptide antibiotics, failed to activate horse prothrombin. Heparin, polyGLU, and polyASP completely inhibited the activation by polyLYS (Table I). Optimum temperatures for the polymyxin B and protamine reactions were identical to the characteristic low (15° to 28°C) optimum of the polyLYS-prothrombin activation (Fig. 1).

The prothrombin activating property of protamine was stable on pretreatment at 100°C or with DFP. Prothrombin consumption in the prothrombin-protamine-DFP mixtures occurred concomitantly with thrombin formation in the DFP-free systems suggesting that the reaction itself is resistant to DFP. No measurable thrombin appeared in the reactions containing DFP (Fig. 2).

In proper proportions for nonenzymic activation the mixtures of prothrombin with polyLYS, polyORN, and protamine form heavy coprecipitates which do not affect the activation reactions. No such precipitates form at optimum polymyxin B-prothrombin mixtures.

Discussion. Thrombin formation in polyORN (free δ-NH_2 groups) and in polymyxin B (5 free γ-NH_2 groups per mole (6-8)) excludes the absolute specificity for the ε-NH_2 groups of polyLYS in nonenzymic prothrombin activation. However, inhibition of the reaction with the polyanions (heparin, polyGLU, and polyASP) supports requirement for primary amino groups and/or strong positive charges. Prothrombin activation in polyLYS but not with an equal weight of triLYS suggests that the reaction requires proper spatial conformation of the free amino groups and/or positive charges.

Prothrombin activation in protamine solution resembles the polyLYS-prothrombin system in having a low temperature optimum and similar kinetics of prothrombin consumption and thrombin formation in presence and absence of DFP(1). Inhibition of thrombin as it forms in systems containing DFP tends to rule out an autocatalytic process. Unless

extraordinarily resistant, a prothrombin activating enzyme is an improbable contaminant of the heterogeneous protamine because activity resists 100°C heat and DFP treatment.

The ability of polymyxin to activate prothrombin by the nonenzymic process may be a toxic mechanism of this drug as well as of some other antibiotics. Interestingly, by simply adding heparin to the antibiotics, Higginbotham(9) diminished the lethal effect in mice of polymyxin, neomycin, viomycin, and dihydrostreptomycin while not affecting neomycin antibiotic properties.

If a model for physiologic processes, the mechanism of nonenzymic prothrombin activation may *initiate* blood coagulation at bleeding sites(10). Similarities between this reaction and the action of the combined calcium ion, tissue thromboplastin, and accessory factors include insensitivity to and progression in DFP, whereas autocatalytic activation is inhibited by small amounts of DFP (1,11). If not a physiologic process, nonenzymic prothrombin activation still remains a potential *nonspecific* contributor to *in vitro* coagulation experiments, particularly those involving purified protein reactants in high concentration. Extreme care should be exercised in controlling, ruling out, or including this reaction when interpreting coagulation experiments.

Summary. Synthetic polyORN, polymyxin B, and protamine activate horse prothrombin by processes resembling the nonenzymic activation with polyLYS. Neomycin and streptomycin may function to a slight degree in the same mechanism. Nonenzymic prothrombin activation with polyLYS is inhibited by heparin, polyGLU, and polyASP. The capacity of protamine preparations to activate prothrombin is stable to heat, DFP, and dialysis. Prothrombin consumption in prothrombin-protamine-DFP mixtures is concomitant with thrombin formation in DFP-free systems. Nonenzymic prothrombin activation may be associated with some drug toxicities as well as with physiologic hemostasis.

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Cholesterol in Vitamin B_{12}-Deficient Chick Embryo. (26865)

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Vitamin B_{12} has been implicated in cholesterol metabolism in various species. The blood cholesterol level was found to be abnormally low in pernicious anemia patients in relapse(1,2,3). After effective liver therapy, owing presumably to the action of the Vit. B_{12} present, cholesterol level increased. Forbes and Patterson(4) showed that the livers of Vit. B_{12}-deficient rats had higher levels of cholesterol than the control livers. Hsu

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