WBC types is the same after isolation as in whole blood argues strongly that the cells are a representative rather than a resistant population. In this study yields have been sacrificed for the sake of purity. Leukocyte purities greater than 90% have been uniformly achieved by washing and resuspending isolated WBC in a phosphate buffer which lyses residual red cells while leaving white cells intact. This result is considerably better than the optimal 50% purity recorded by Skoog and Beck(2) for the fibrinogen technic or the 52.5% and 83.8% for normal and leukemic bloods, respectively, calculated from data of Li and Osgood(3). The phytohemagglutination technic with phosphate buffer is recommended to those workers who require white cell suspensions of high purity.

*Summary.* Methods for preparing human leukocytes from venous blood have been investigated. Sedimentation of erythrocytes by fibrinogen or phytohemagglutinin of *Phaseolus vulgaris* have given best results. With white cell purity as a goal, the phytohemagglutination method has been clearly superior, giving preparations of 72.7% and 96.5% from normal and leukemic bloods, respectively. These values have been increased to 93.8% and 98.8% by utilization of a phosphate buffer which preferentially lyses red cells while leaving white cells intact.

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## Effect of Divalent Cations on Proteolysis of Fibrinogen.* (23860)

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Calcium, in conjunction with an unidentified serum factor, exerts an effect on the fibrinogen-fibrin conversion such that the final clot differs significantly from that formed in the absence of one or both of these components. The clots differ in solubility in dilute acid(1), in concentrated urea(2), and in concentrated lithium bromide(3). They differ also in tensile strength(4) and in ease of conversion from $\alpha$- to $\beta$-keratin structures, as demonstrated by x-ray diffraction patterns(5). It has also been demonstrated(6) that the intermediate polymers formed in the conversion are longer and that the rate and extent of their dissociation is diminished in the presence of Ca. Recently, Thomas† observed the inhibition by Ca of streptokinase-induced clot lysis. The conditions of the experiment were such that the effect of Ca could have been on the nature of the clot formed; Ca does not inhibit activity of plasmin(7).

Since these effects might have a common origin in the initial proteolytic attack of thrombin on fibrinogen, it appeared of interest to investigate the effect of Ca on hydrolysis of fibrinogen by trypsin. Both thrombin(8) and plasmin, the streptokinase-induced fibrinolysin(9), possess proteolytic specificity similar to trypsin. Chymotrypsin, a proteolytic enzyme of differing specificity, was studied for comparison.

*Methods and materials.* Fibrinogen, 90-94% clottable by the method of Laki(10), was prepared from bovine plasma fraction I‡ by the methods of Morrison(11) and Laki (12). Other reagents were obtained commercially. The assay was similar to that of Kunitz(13). A 0.9 ml aliquot of a solution

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† W. A. Thomas, personal communication.

‡ Generously supplied by Armour Laboratories, Chicago, Ill.

TABLE I. Effect of Various Divalent Cations on Proteolysis of Fibrinogen by Trypsin.

| Substance added | Increase, $OD_{280}$ |
|---|---|
| .005 M EDTA | .355 |
| Pyrophosphate | .365 |
| .001 M $CaCl_2$ | .225 |
| $MgCl_2$ | .240 |
| $CoCl_2$ | .237 |
| $ZnCl_2$ | .255 |
| $MnCl_2$ | .233 |
| $CdCl_2$ | .225 |

of fibrinogen in 0.05 M Tris§ buffer, pH 7.4, containing sufficient NaCl to bring the final ionic strength to 0.40, was warmed in a constant temperature bath at 38°C. One-tenth ml trypsin (1.0 mg/ml) or chymotrypsin (0.2 mg/ml) was added with shaking. After 20 minutes 1.0 ml 5% trichloroacetic acid was added, the mixture was allowed to stand for one hour, centrifuged for 20 minutes, and the optical density of the supernatant was measured at 280 mμ on the Beckman Model DU spectrophotometer. Appropriate blank determinations were run with addition of trichloroacetic acid before addition of the enzyme. Trypsin autolysis did not measurably contribute to the optical density of the supernatant. A preliminary experiment indicated that the increase in optical density was approximately linear in the time interval 0-30 minutes, and the 20-minute incubation period was chosen for convenience as a measure of the initial rate of proteolysis.

*Results.* Table I demonstrates that pyrophosphate, which would also be expected to reduce the activity of Ca ions, is effective as EDTA in enhancing the tryptic digestion of fibrinogen. This table also indicates that the other divalent cations tested were approximately equal to Ca in effectiveness. It was found that the rate of proteolysis without addition of chelating agent or metal ion was variable from one preparation to another, indicating contamination by traces of metals. The rates of some preparations approached that obtained in the presence of chelating agents.

In Table II, the averages of determinations at various Ca concentrations are expressed as percentage maximum initial rate. It is apparent that the rate of proteolysis is diminished at very low Ca concentrations, and that 50% reduction occurs at about $10^{-3}$ M.

§ Sigma Chemical Co.'s trishydroxymethylaminomethane.

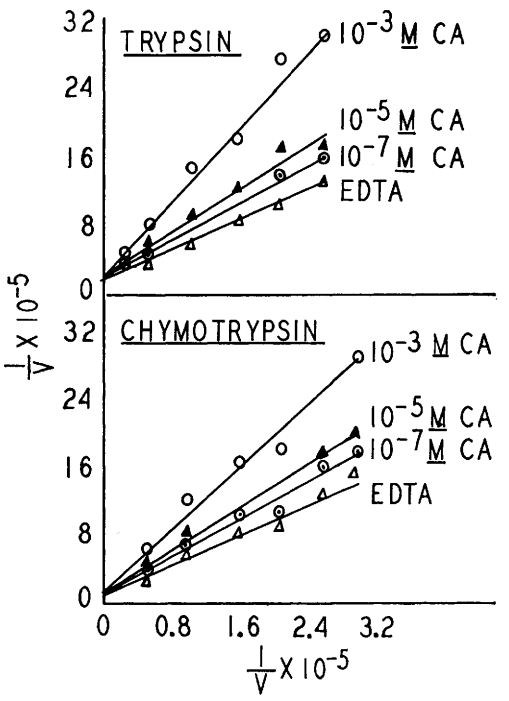


FIG. 1. Lineweaver-Burk reciprocal plot of effect of various concentrations of calcium on kinetics of proteolysis of fibrinogen by trypsin (0.1 mg/ml final conc.) and by chymotrypsin (0.02 mg/ml final conc.).

The results of varying fibrinogen concentration are expressed in Fig. 1 in a Lineweaver-Burk reciprocal plot. The kinetic curves are typical of a competitive inhibition.

*Discussion.* Since maximal rates of proteolysis were obtained in the presence of pyrophosphate as well as with EDTA, it would appear that the ability of these anions to enhance proteolysis of fibrinogen lies in their Ca-binding capacity rather than in a specific

TABLE II. Comparison of Effects of Varying Concentrations of Calcium on Rates of Tryptic and Chymotryptic Digestion of Fibrinogen, Expressed as Percentage of Maximum Initial Rate.*

| Substance added | % max initial rate — Trypsin | % max initial rate — Chymotrypsin |
|---|---|---|
| .005 M EDTA | 100 | 100 |
| $10^{-7}$ M $CaCl_2$ | 79.3 ± 5.1 | 87.7 ± 5.4 |
| $10^{-5}$ M " | 74.6 ± 3.8 | 77.6 ± 2.6 |
| $10^{-3}$ M " | 49.8 ± 6.8 | 54.0 ± 1.3 |

* The exp. errors are expressed as stand. dev.

interaction with the protein. The only other anion present in the test system was chloride.

The apparent competitive inhibition of the reaction could most simply be explained by a competition between Ca and substrate for reactive sites on the enzyme. However, it has been demonstrated that Ca does not inhibit the activity of trypsin on synthetic substrates, on casein, or on denatured hemoglobin; rather, it has been shown to stabilize the enzyme(14). It has been further shown that Ca enhances the activity of chymotrypsin for these substrates(15). Since Ca inhibits the proteolysis of fibrinogen by both of these enzymes, it may be inferred that the action of Ca is not upon the enzyme but is to stabilize the substrate.

Gorini and co-workers(16) have demonstrated a similar effect of Ca and Mn on the tryptic digestion of serum albumin and lysozyme and have correlated this effect with the ability of those cations to decrease the rate of thermal denaturation of those proteins. They suggest that the metal exerts its effect upon an equilibrium between 2 forms of the protein, only one of which is susceptible, at least rapidly, to proteolytic action or to irreversible thermal denaturation. This concept follows from the suggestion by Linderstrøm-Lang(17) that the primary step in proteolysis is a reversible denaturation.

In view of the concepts of Linderstrøm-Lang and of Gorini, it is suggested that the role of Ca is to shift an equilibrium between two forms of fibrinogen, only one of which is rapidly attacked by the enzyme. The other form is attacked relatively slowly or not at all by the enzyme, but by reversibly combining with the active site(s) serves, in effect, as a competitive inhibitor of the reaction.

There is ample precedent for the concept of reversible conformation changes of proteins in solution(18), and it is not unlikely that the role of the divalent cation is to stabilize the tertiary structure of fibrinogen.

*Summary.* The effects of various divalent cations on proteolysis of fibrinogen by trypsin and chymotrypsin were studied. Calcium, Mg, Mn, Co, Zn, and Cd were approximately equally effective in reducing the rate of proteolysis by trypsin. At levels as low as $10^{-7}$ M, Ca began to decrease the rate of proteolysis by either trypsin or chymotrypsin, and a 50% reduction of the rate occurred at about $10^{-3}$ M. The effects of Ca on both tryptic and chymotryptic digestion appeared to yield the kinetics of a competitive inhibition; and the suggestion is made that Ca acts to affect an equilibrium between two forms of fibrinogen, the one susceptible, the other inhibitory, to proteolysis.

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