tive transport system is involved since fluoride would not travel against a concentration gradient across the intestinal wall nor was the diffusion rate influenced appreciably by enzyme poisons or temperature changes. In addition, the rate of diffusion varied with the diffusion area as was expected and diffusion was inhibited by physiological concentrations of calcium and magnesium.

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Preparation of Water Insoluble Thrombin.* (28897)

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The preparation of water insoluble proteolytic enzymes such as trypsin(1) and papain (2) has been successfully accomplished. It is possible to remove such enzymes quantitatively and rapidly from reaction mixtures by physical means such as filtration and centrifugation. This permits the investigator to use these insoluble enzymes in the study of intermediate steps in progressive proteolytic reactions. The study of blood clotting has been hampered by the difficulty of isolating intermediate reactions. A central component of the clotting process, thrombin, not only clots fibrinogen, but also appears to accelerate the early stages of its own formation in an autocatalytic manner. We therefore felt that an insoluble thrombin would be particularly advantageous in the study of clotting processes involving activation or degradation of other clotting factors. We have prepared such a water insoluble thrombin by coupling soluble thrombin with a copolymer of p-amino-DL-phenylalanine and L-leucine, and report here some of its properties.

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Materials and methods. Preparation of insoluble thrombin. Bovine thrombin (thrombin-topical, Parke-Davis & Co.) was chromatographed on Amberlite, C-G-50 type II by modification of the method of Rasmussen (3). The inactive protein was washed off the column with 0.1 M sodium phosphate buffer pH 7.5. The thrombin was then eluted by 0.3 M sodium phosphate buffer pH 8.0 containing 1 M sodium chloride. The active effluent was concentrated by dialyzing against 10% polyvinylpyrrolidone for 24 hours followed by dialysis against 0.154 M NaCl for 4-6 hours at 4°C. This concentrated thrombin had a specific activity of 665 NIH units/mg protein as compared to the starting material, 57 NIH units/mg protein.

A copolymer of p-amino-DL-phenylalanine and L-leucine was prepared at the Weizman Institute of Science, Rehoboth, Israel by the method of Bar-Eli and Katchalski(1).† This copolymer has a molar ratio of L-leucine and p-amino-DL-phenylalanine of 2.1:1. Coupling of the thrombin to the copolymer was

† This was given to us through the generosity of Professors Ephraim Katchalski and Michael Sela.

accomplished in a manner identical to that for preparation of insoluble papain(2).† The diazotization of the copolymer resulted in an insoluble brownish-orange particle which was subsequently washed in phosphate buffer, pH 7.5. Thrombin (5000 NIH units in 5 ml of buffer) was mixed with the copolymer (100 mg weight before diazotization), and stirred at pH 7.5, 5°C for 24 hours. The preparation was washed free of reaction products with sodium phosphate buffer, 0.1 M, pH 7.5. Siliconized glassware was used. Addition of 50% glycerol to all solutions appeared to improve the yield(4).

An alternative method used the principle of coupling prothrombin to the copolymer followed by thromboplastic activation. Since Factors VII, IX, and X would be expected to increase the yield of thrombin, no attempt was made to eliminate them from the preparation. Normal oxalated beef plasma was adsorbed with $BaSO_4$ (100 mg/ml). The $BaSO_4$ was eluted with trisodium citrate (0.14 M, pH 7.5), dialyzed against phosphate buffer (0.05 M, pH 7.0) and chromatographed on DEAE cellulose column. The fraction containing prothrombin was eluted with 0.3 M sodium phosphate buffer and 1 M NaCl, pH 8.0, and concentrated and dialyzed as the thrombin was. The activity expressed per mg of protein showed a purification of 132-fold. This preparation (containing 41.25 mg protein in 12.5 ml) was coupled to the copolymer as above. It was then washed in buffer and activated by a thromboplastic mixture containing Russell Viper venom (Stypven®, Burroughs Wellcome Co.), Factor V, and calcium. The resulting insoluble thrombin had properties apparently identical with those of insoluble thrombin prepared by direct coupling.

Esterase activity was tested using p-toluenesulphonyl-L-arginine methyl ester (TAMe) as substrate(5). The formation of free carboxyl groups was titrated with sodium hydroxide by the pH stat technique at 37°C, pH 7.0 with a Radiometer type TTT1a, automatic titrater. When measuring activity of the insoluble preparation, it was necessary to stir vigorously to maintain dispersion of the insoluble enzyme. Results are reported as the amount of NaOH necessary to keep the pH constant and hence equivalent to the acid released by the hydrolysis.

Protein was measured at 280 mμ in a Beckman DU spectrophotometer and by Winzler's modification of the method of Folin and Ciocalteu(6).

Platelet-rich plasma was prepared by centrifuging human blood collected in acid-citrate-dextrose, NIH solution B, (1 part ACD to 3 parts blood) at 170 *g* for 15 minutes. The platelets were separated from this plasma by centrifugation for 30 minutes at 1500 *g* and then washed in a solution consisting of 6 parts tris buffer, pH 7.3, ionic strength 0.154, 2 parts ACD, and 1 part platelet-poor plasma. The washed platelets were resuspended in a standard, separately prepared, platelet-poor plasma to a concentration of 1 million platelets/mm^3. One drop of this suspension was mixed with approximately 0.037 mg of insoluble thrombin on a microscope slide, incubated at 37°C and examined under the phase microscope.

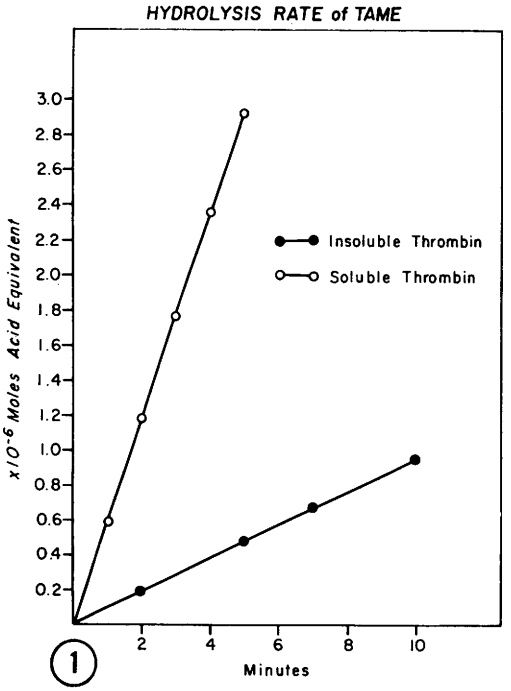
Commercial human *fibrinogen* (Merck Sharp & Dohme) was diluted in tris buffer to give a 0.1% solution.

A control preparation designated as *inactive copolymer* was prepared by boiling insoluble papain(2) for 3 hours.

Results. Fig. 1 shows the esterase activity of 10 NIH units of soluble thrombin compared to insoluble thrombin derived from .0255 mg of copolymer. The latter represents the equivalent amount of thrombin (calculated as 11 NIH units) if 100% yield had been effected. Fig. 2 shows the esterase activity over a range of substrate concentration from 0.9 mM to 8 mM. Use of the double reciprocal plot permits the conclusion that the Michaelis constant for soluble and insoluble thrombin under these circumstances is nearly identical. This was calculated(7) to be 3.23 mM for soluble and 3.22 mM for insoluble thrombin.

The stability of insoluble thrombin was evaluated by measurement of its esterase ac-

† We acknowledge with thanks the advice and help of Dr. John Cebra, without which this preparation would have been impossible.



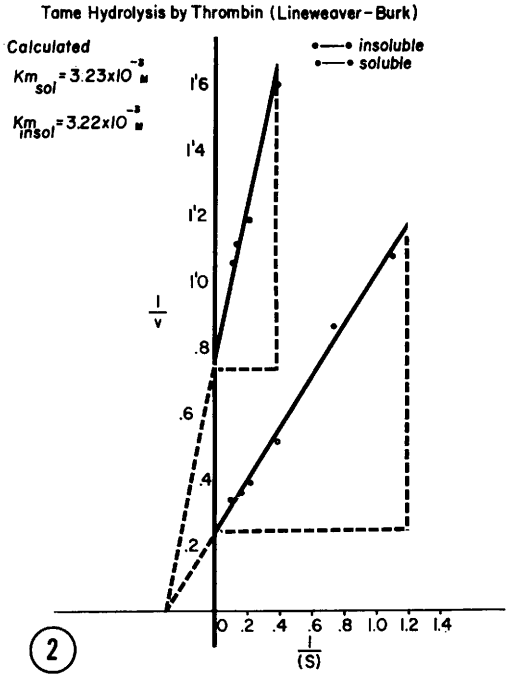


FIG. 1. Soluble thrombin, 10 NIH units in 0.1 ml, was added to a reaction mixture containing 9 × 10^{-3} M TAMe in 6 ml. Insoluble thrombin was added in an amount calculated as 11 NIH units assuming 100% yield.

FIG. 2. Calculation of Km for soluble and insoluble thrombin where S = substrate concentration and V = initial velocity of TAMe hydrolysis.

tivity. The stability is considerably enhanced by addition of glycerol to a final concentration of 50% (Table I). This stabilization by glycerol is similar to that seen with soluble thrombin.

If insoluble thrombin is added to fibrinogen at 37°C, no macroscopic clotting occurs. However, the thrombin particles agglutinate after a period varying from 5 minutes to 2 hours depending on the preparation. If the particles are removed before agglutination and examined microscopically, no fibrin is seen. If examined microscopically after agglutination fibrin is found adherent to the particle. If the supernatant solution (either before or after agglutination) is decanted and placed at 4°C, a solid gel forms. Serial exposure of successive tubes of fibrinogen to the same insoluble thrombin particles results in gel formation in the cold in each tube until the particles become agglutinated. This gel is most likely cryoprofibrin which was described by Shainoff and Page(8) and results from the action of small amounts of thrombin on large amounts of fibrinogen.

Neither esterase activity, gel formation, nor fibrin formation is observed with the control preparation of inactive copolymer.

Addition of insoluble thrombin to platelet-rich plasma causes clumping of the platelets with each other and about the particles, followed by the formation of fibrin strands on and between the particles after 15 minutes

TABLE I. Stability of Insoluble Thrombin at 4°C Assayed by TAMe Hydrolysis.
(Moles × 10^{-6}/10 min)

Day	Activity with glycerol	Activity without glycerol	
0	8.1		
3	—	3.65	
		With glycerol	Without glycerol
20	7.96	3.56	1.23

The preparation was stored with and without glycerol from day 0. On day 3, the aliquot without glycerol was assayed and again divided and stored with or without glycerol added. All values are corrected for dilution.

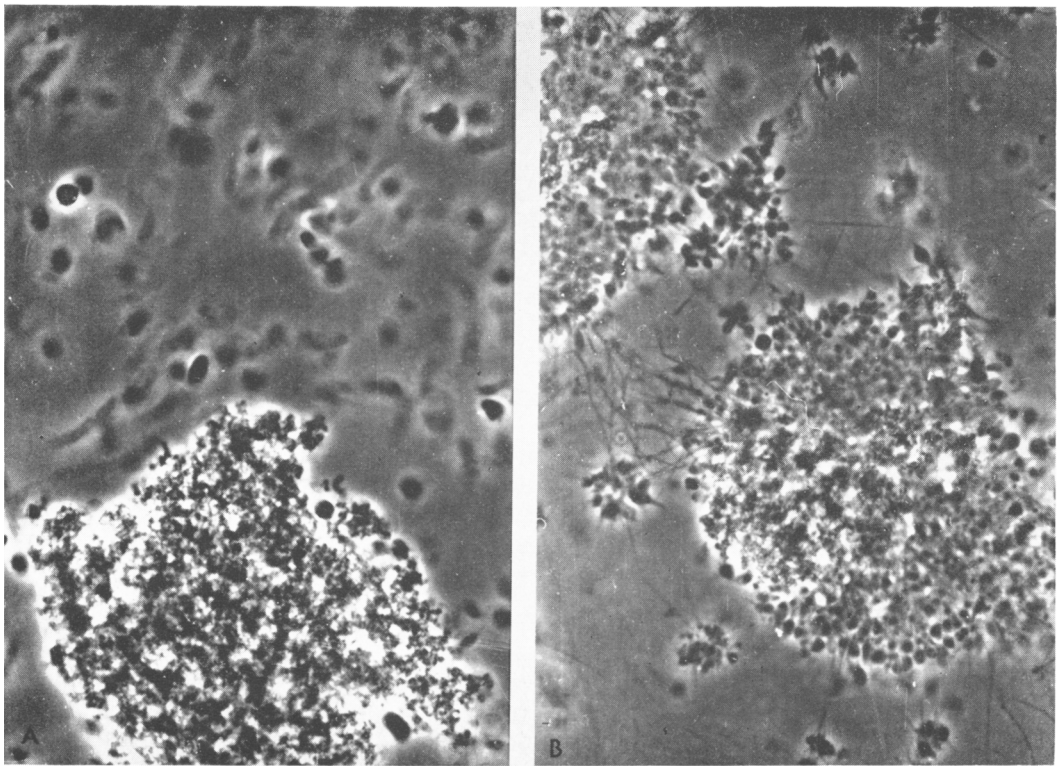


FIG. 3. A. Platelet rich plasma and control particle. Note platelets have settled out of focus and lack spicules. There is no fibrin. B. Platelet rich plasma and insoluble thrombin particles. Platelets have clumped and fibrin has formed.

to 2 hours. This is not observed when the control material (boiled insoluble papain) is added in identical fashion (Fig. 3).

Discussion. In addition to demonstrating the feasibility of preparing an insoluble thrombin, these experiments raise new questions. The Km reported for soluble thrombin is consistent with that previously reported by Sherry and Troll(9) if one considers the difference in pH involved. No comparable data have been published for insoluble thrombin. Insoluble thrombin resembles acetylated thrombin(10) in that it shows excellent esterase activity, but decreased clotting activity. It might seem therefore that the coupling procedure blocks amino groups and perhaps hydroxyl groups of thrombin as acetylation does. Diazotization is more likely to involve aromatic groups, however(11), therefore an alternative explanation of the preferential hydrolysis of TAMe over fibrinogen is needed. Bar-Eli and Katchalski(1) have shown that insoluble trypsin can hydrolyze a low molecular weight substrate such as benzoyl-L-arginine methyl ester at a rate 6 times faster than that seen with larger molecules such as casein. It seems reasonable that the physical properties of the insoluble enzyme interfere in the latter reaction and that this explanation should be investigated further with regard to the insoluble thrombin-fibrinogin reaction. It may be concluded, however, that the preparation has the ability to cleave fibrinogen and can initiate the complex processes which result in clumping of platelets and fibrin formation in plasma. Thus we believe it highly likely that insoluble thrombin will be a research tool which can be used to explore the intermediate steps of clotting once its preparation is optimal and its properties defined completely.

Summary. The preparation of an insoluble particle having some of the enzymatic properties of thrombin has been accomplished by coupling thrombin to a copolymer of p-amino-DL-phenylalanine and L-leucine. Esterase

thrombin activity is easily demonstrable and some clot forming activity is preserved.

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Acid-Soluble Nucleotides and Peptides of Skin.* (28898)

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Numerous reports have appeared concerning the presence of "peptide-nucleotides" in acidic and alcoholic extracts of mammalian liver(1-3) and microorganisms(4-7), and in one instance(8) the structure of such a compound has been determined. Although the function of these compounds has not been established, we decided to study perchloric acid-soluble preparations of neonatal animals to determine the extent and nature of the peptide-nucleotide pool, and whether proline and hydroxyproline were present in such a pool as possible intermediates in the metabolism of collagen.

Materials and methods. Animals and tissues. Rabbits less than one week of age were decapitated and their skins, less that of head and limbs, removed. Subsequent handling of tissues, including preparation and fractionation of extracts, was carried out at 0 to 5°C.

Extracts were prepared by homogenizing the skins in cold 0.25 M perchloric acid to yield a 20% suspension of the tissue; the mixture was centrifuged at 1500 × *g* for 10 minutes and the first extract was decanted. The residue was reextracted with a second equivalent volume of 0.25 M perchloric acid and centrifuged; the combined extracts were then filtered.

Extracts were examined at 260 mμ in a Beckman DU spectrophotometer using 1 cm cells. The total absorbancy at this wavelength, determined from the volume of extract and the optical density, was expressed as A_{260} units. In sub-fractions of the extract, prepared subsequently, measurements were also made at 275 mμ, and A_{275} units determined.

Adsorption of nucleotides on Norit and elution. Norit A was washed with 10^{-3} M Versene (pH 7), water, 4 N acetic acid and finally again with water until washings were neutral. The Norit was then dried at 110°, and a 10% suspension prepared in water.

The suspension was added to the perchloric acid extract (40 mg Norit for each 100 optical density units measured at 260 mμ in a 1 cm cell (A_{260} units)). The mixture was stirred for 2 hours and then filtered through a coarse sintered-glass funnel layered with

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