Isolation of Human Thrombin.* (27840)

KENT D. MILLER AND WILLIAM H. COPELAND (Introduced by Rachel Brown)
Division of Laboratories and Research, New York State Department of Health and Department of Pathology, Albany Medical College, Albany, N. Y.

Extensive molecular data have been gathered with highly purified bovine thrombin (TH) preparations(1-9). Human TH has escaped detailed study, however, probably due to limited supplies of its source, human prothrombin (PTH). The present report describes the isolation of human TH by high-yield methods. Specific activities of human TH are, respectively, 2 and 3 times greater than those of bovine TH and equine TH, indicating either a smaller or more active enzyme.

Methods and procedures. PTH and TH were measured by the 2-stage technics(10) and standardized against a frozen pool of oxalated bovine plasma containing 262 "Iowa" units per ml. Protein nitrogen and tyrosine were determined by micro-Kjeldahl and Folin-phenol-reagent technics, respectively. IRC-50-Rivanol is prepared by adding 1 g of Rivanol to each 100 g of IRC-50-Na^+ (XE-64) (>200 mesh), stirring for 30 min in 0.1 M sodium acetate, then washing 3-4 times with the 0.1 M acetate.

Human TH was isolated from the PTH present in Cohn Fraction III of human plasma, either prepared in this laboratory or obtained from E. R. Squibb & Sons, New Brunswick, N. J. Fractions III-1 and III-2, 3 were somewhat more difficult to process. Generally, procedures involved (a) 2 adsorptions of PTH on barium citrate(11), each precipitate being dissolved by stirring with a cation exchange resin; (b) reduction of salt content by ethanol precipitation from dilute solution at pH 4.7; (c) rapid activation of PTH to TH; (d) Rivanol precipitation of some impurities(7); (e) reduction of salt content by ethanol or acetone precipitation followed by dialysis or Sephadex filtration; and, (f) chromatography on IRC-50-Rivanol(7). Several of the steps provide quantitative recoveries. Details of the methods are presented below. Yields and specific activities of fractions and final products from several batch sizes are recorded in Table I.

Up to 500 g of Fraction III (or subfraction) are suspended in a 5% concentration in 0.02 M sodium citrate, adjusted to pH 7.5, and stirred for 15-30 min at room temperature. Insoluble material is removed by centrifugation and the supernatant (*original extract*, Table I) cooled to 5-8°C by one of several means, including addition of chipped ice. One molar $BaCl_2$ is added dropwise to 0.1 M, following which the barium citrate precipitate is collected by centrifugation, washed with 0.1 M $BaCl_2$, and blended in an amount of cold water equal to one-half the *original extract* volume (OV). The precipitate is dissolved by adding the weak cation exchanger IRC-50-Na^+ (>200 mesh) in sufficient quantity to raise the pH to 7.5-8.0. The amount of resin required varies with its water and sodium ion content, the pH and disappearance of the barium citrate being the best criteria for proper dissolution. Ten to 15 min are usually sufficient elution time. The resin is then quickly separated by filtration through S. & S. 40-cm fluted No. 520-B-½ papers, with the resulting filtrate called *first eluate* (Table I). One molar $BaCl_2$ is again added to the cooled filtrate to a final concentration of 0.08-0.10 M. The precipitate is recovered by centrifugation, suspended in 1/10 the OV of cold water, and dissolved by adding IRC-50-Na^+ (>200 mesh) as before to pH 7.5-8.0. Filtration yields the *second eluate* (Table I).

The second eluate is diluted 2-4 times with cold distilled water, the pH adjusted to 4.7 with HCl, and ethanol added to a final 15% at 0-5°C. The precipitate, recovered by centrifugation, is suspended in sufficient water to give 1500-3000 "Iowa" units per ml and the pH raised to 7.2 with dilute NaOH (*first*

* This work was financed, in part, with funds and material provided under an agreement between the American National Red Cross and the Division of Laboratories and Research, New York State Department of Health.

TABLE I. Specific Activities* and Yields† of Fractions in Human Thrombin Preparations.

F III to start, g	Prothrombin				Thrombin	
	Original extract	1st eluate	2nd eluate	1st ethanol PPT	2nd ethanol PPT	Final product
200	11.2*(100)†	280	1,080 (61.5)	955 (56.5)	900	9,300
100	45 (100)	330 (55)	1,020 (54)	1,020 (48)	1,100 (40)	10,900 (40)
500	22 (100)	394 (66.7)	890 (61.3)	890 (53)	1,440 (44)	11,000 (38)
500	20.5 (100)	340 (87)	790 (83.5)	— (83)	1,770 (68.5)	10,000 (52)

* Specific activities recorded as "Iowa" units per mg protein.
† Yields in parentheses express % of original extract.

ethanol precipitate, Table I). One-fifth volume of a thromboplastin suspension(7) in 0.15 M $CaCl_2$ and 0.15 M cacodylate (pH 7.2) is added. Activation at room temperature is followed by thrombin analysis; a quantitative conversion is ideally complete in 30-60 min or less.

The activated solution is made 0.05 M with respect to sodium citrate and cooled to 0-5°C. Two per cent Rivanol is added to a final 0.03-0.05% concentration. After being stirred 5 min, the thromboplastin and Rivanol-precipitable proteins are removed by centrifugation at 3000 × *g* for 10-15 min. The supernatant TH is precipitated with 2 volumes of cold ethanol or acetone at a temperature of 0-4°C. The precipitate is dissolved in about 1/50 of the OV, then dialyzed overnight against several changes of cold, distilled water. Before use Visking dialysis casings should be soaked 15 min in boiling 0.1 M Versene buffer (pH 7.2). Alternatively, the solution can be desalted by passage through an appropriate sized column of Sephadex G-25. This fraction is termed the *second ethanol (or acetone) precipitate* (Table I). Maximum yields are obtained by completing the procedure to the dialysis or Sephadex step in one day.

The TH is reprecipitated with 2 volumes of cold ethanol or acetone, then dissolved in 0.1 M sodium acetate. This solution is placed on a column of IRC-50-Rivanol and inert proteins are washed from the adsorbent with 0.1 M sodium acetate. Subsequently, the TH is quantitatively eluted with 0.15 or 0.20 M $CaCl_2$. The TH may be freed of Rivanol and $CaCl_2$ by 1-2 precipitations from solution with cold ethanol or acetone yielding the *final product* (Table I). The final product is dissolved either in water or a buffer of choice.

The average specific activities of the human TH products in Table I were 10,300 units per mg protein, 69,000 units per mg nitrogen, and 99,400 units per mg tyrosine. These preparations were free of plasminogen and plasmin in analyses by Dr. Alan Johnson, New York University Medical Center. Two preparations examined in the analytical ultracentrifuge were homogeneous.

Discussion. The high over-all yields (40-50%) of human TH from PTH in Fraction III resulted from adherence to the fractionation conditions described. Continuous first-day fractionation up to the dialysis (or Sephadex-passage) step prevents significant (20-30%) storage losses in the earlier fractions. The reported instability of TH to dialysis(12) has been corroborated but is prevented by soaking Visking dialysis casings in hot EDTA solution. After dialysis the enzyme is stable and yields from subsequent procedures are frequently quantitative.

In this laboratory's experience, quantitative TH yields are obtained from the IRC-50-Rivanol columns described, whereas about 70% yields are eluted from phosphate-buffered IRC-50 columns(2).

Gross differences exist between molecular weights of bovine (62,700) and equine (130,000) PTH(1,13). Also, remarkable species differences exist in specific activities of thrombins. Human TH (10,300 units per mg protein; 99,400 units per mg tyrosine) is about twice as active as bovine TH (4,100 units per mg protein; 45,000 units per mg tyrosine) and 3 times as active as equine TH (30,800 units per mg tyrosine)(3,7). If smaller than other species of TH, the human enzyme will have considerable value in some types of detailed molecular study such as amino acid sequence work.

Summary. Detailed procedures are de-

scribed for isolation of human thrombin from plasma Fraction III in 40-50% yield. Average specific activities of these preparations are 10,300 "Iowa" units per mg protein, 69,000 units per mg nitrogen, and 99,400 units per mg tyrosine. The higher activity of human thrombin indicates that it is either a smaller molecule or more active than bovine or equine thrombin.

1. Lamy, F., Waugh, D. F., *Physiol. Rev.,* 1954, v34, 722.

2. Rasmussen, P., *Biochim. Biophys. Acta,* 1954, v16, 157.

3. Seegers, W. H., Levine, W. G., Shepard, R. S., *Can. J. Biochem. Physiol.,* 1958, v36, 603.

4. Magnusson, S., *Acta Chem. Scand.,* 1958, v12, 355.

5. Gladner, J. A., Laki, K., *J. Am. Chem. Soc.,* 1958, v80, 1263.

6. Seegers, W. H., Casillas, G., Shepard, R. S., Thomas, W. R., Halick, P., *Can. J. Biochem. Physiol.,* 1959, v37, 775.

7. Miller, K. D., *Nature,* 1959, v184, 450.

8. Seegers, W. H., Landaburu, R. H., *Canad. J. Biochem. Physiol.,* 1960, v38, 1405.

9. Cohly, M. A., Scheraga, H. A., *Arch. Biochem. Biophys.,* 1961, v95, 428.

10. Ware, A. G., Seegers, W. H., *Am. J. Clin. Path.,* 1949, v19, 471.

11. Lewis, M. L., Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 636.

12. Steichele, D. E., Mammen, E. F., Hatta, Y., Seegers, W. H., *Thromb. Diath. Haem.,* 1960, v4, 365.

13. Miller, K. D., McGarrahan, J., *Annual Report of Division of Laboratories and Research,* N. Y. State Dept. Health, Albany, 1958, p69.

Received September 14, 1962. P.S.E.B.M., 1962, v111.

In vitro Cell Migration as a Model for Delayed Hypersensitivity.* (27841)

MARIAM GEORGE AND JOHN H. VAUGHAN (Introduced by W. F. Bale)
Department of Microbiology and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

One of the most pressing problems in the study of delayed hypersensitivity is whether there is an adequate *in vitro* model of this state of altered reactivity. Rich and Lewis (1) reported that explants of spleens and lymph nodes from tuberculin sensitive animals grow out poorly in the presence of tuberculin, but do quite well in its absence. Similar observations have been made(2) on the outgrowth of cells of animals with delayed hypersensitivity to other bacteria. Inhibition was not seen with cells from animals anaphylactically sensitive to soluble antigens(3). Chase(4) noted that tuberculin hypersensitivity, though not transferred with serum, could be transferred with spleen or lymph node cells. Miller and Favour(5) attempted to provide a simple *in vitro* model of tuberculin sensitivity with a system of peripheral blood or splenic sensitive leukocytes, serum, and complement, in which lysis of cells was noted when the cells were made to tumble for relatively short times in a rotating drum at 37°C. Evidence accrued(6), however, that Favour's "cytolytic" effect could be seen with normal cells in the presence of added antigen, antibody, and complement, and doubt has consequently been expressed that the model really represents delayed sensitivity.

Interest in macrophages as cells implicated in delayed hypersensitivity has gradually developed. Rich(7) pointed to the disposition for macrophages to collect in the lesions of tuberculous animals, and commented upon the ability of the wax of the tubercle bacilli to evoke this type of cellular response. Injection of tuberculoprotein together with tuberculowax has been reported by Raffel(8) to result in development of delayed hypersensitivity to the protein, while immunization with tuberculoprotein alone evoked only anaphylactic hypersensitivity. Gangarosa *et al.*(9) pointed out that addition of antigen to spleen cultures from animals with delayed sensitivity to tuberculin or to mumps virus had a demon-

* Supported by grant from Nat. Inst. Health, U.S.P.H.S.