cyte suspensions were incubated with uremic plasma and the studies of Markson and Moore (13) which demonstrate that hemoglobin synthesis by normoblasts is not inhibited by uremic serum suggest that diminished iron incorporation into erythrocytic precursors is not the explanation for the altered ferrokinetics noted in these experiments.

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## Activities Associated with Thrombokinase Derived from Bovine Plasma.* (30306)

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Thrombokinase has been isolated from bovine plasma in a state approaching electrophoretic homogeneity(1), and it has given a single boundary in the ultracentrifuge(2). Yet this evidence does not prove that such preparations are free from significant impurities. The purest preparations have displayed the following activities: 1. activation of prothrombin in the presence of oxalate; 2. rapid activation of prothrombin in the presence of ionic calcium, phospholipid and accelerator (factor V); 3. hydrolysis of p-toluenesulfonyl-L-arginine methylester (TAMe); 4. activation of chymotrypsinogen(3).

The TAMe esterase activity has already been found to parallel thrombokinase closely (1,4). One purpose of the present study was to learn whether gel filtration easily separates the other activities. Since it was also desirable to explore the usefulness of gel filtration in purification, thrombokinase was used after the seventh step in the 8-step procedure now used for its isolation(2). That is, it had been chromatographed on DEAE-cellulose, but it had not been fractionated electrophoretically.

*Materials and methods. Thrombokinase concentrate.* 86 ml of Step-7 thrombokinase (2), the product from about 200 liters of bovine plasma, was brought to 0.7 saturation with ammonium sulfate, and the resulting precipitate was dissolved in 10 ml 0.4 M phosphate buffer, pH 8.13. A small amount of undissolved light brown material was removed by centrifugation. The solution had 2849 TAMe units and 9.01 mg protein per ml.

*Barium carbonate-treated bovine serum.* Frozen bovine serum was thawed and mixed with 50 mg barium carbonate per ml for 15 minutes at room temperature. After removal of the barium carbonate by centrifugation, the treated serum was stored at —23°C.

*Prothrombin.* Prepared by a procedure involving adsorption on barium sulfate, elution with 0.3 M phosphate buffer, pH 6.6 and passage through a flat column of Standard Super-Cel(5).

*Chymotrpysinogen.* Chymotrypsinogen A. 5× crystallized, chromatographically homo-

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geneous. Worthington Biochemical Corp.

*BTEE.* Benzoyl-L-Tyrosine Ethylester. Mann Research Laboratories, Inc.

*TAMe.* p-Toluenesulfonyl-L-Arginine Methylester HCl. H. M. Chemical Co. Ltd.

*Tris.* Tris (hydroxymethyl) aminomethane.

*Dextran Gel.* Sephadex G-200, 140-400 mesh. Pharmacia. Allowed to swell in 0.9% sodium chloride, and fines removed by repeated decantation.

*Thrombokinase assay.* Measurement of number of NIH units of thrombin produced in 1 hour at 28°C per 0.1 ml of thrombokinase fraction in 1.0 ml of activation mixture containing 256 units of prothrombin and 0.01 M potassium oxalate.

*Working assay.* Production of thrombin in a 5-reagent system containing prothrombin, phospholipid, accelerator, calcium chloride and variable thrombokinase, as previously described(2,6). Activity is expressed in terms of a working standard of thrombokinase tested concurrently.

*Accelerator assay.* Production of thrombin in a 5-reagent system similar to the foregoing, except that there was a constant ample supply of thrombokinase, and the accelerator was the variable. Results are expressed in per cent of input concentration.

*Chymotrypsinogen activator.* 0.2 ml of thrombokinase fraction was incubated with 0.8 ml of 0.05 M tris buffer, pH 7.8, containing 200 $\mu$g chymotrypsinogen, for 120 minutes at 30°C. At that time the mixture was assayed for chymotrypsin by hydrolysis of BTEE, as followed by increase in optical density at 256 m$\mu$(7). Under the conditions defined, the amount of chymotrypsin produced in 120 minutes, was in turn a measure of chymotrypsinogen activator. The data plotted in the Figure represent O.D.$_{256}$ per minute $\times$ $10^3$.

*TAMe esterase.* Method of Sherry and Troll(8), with minor modifications as described(2).

*Results and discussion.* A column of the dextran gel, 2.54 $\times$ 36 cm, was equilibrated with 0.72 M sodium chloride:0.05 M tris buffer, pH 7.3 and operated at 5°C. Enough sodium chloride was added to a portion of barium carbonate-treated serum to make the

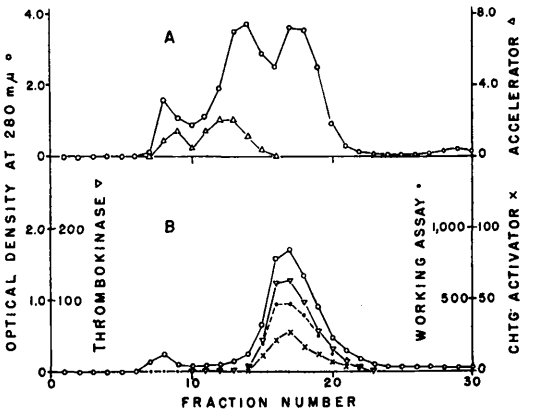


FIG. 1. Gel filtration on a column of Sephadex G-200. A. Barium carbonate-treated bovine serum. B. Step-7 thrombokinase. CHTG,-chymotrypsinogen. See text for procedures.

added salt 0.72 M; and 3 ml of this serum was layered on top of the gel and beneath a layer of buffer. Eluant was fed to the column from a reservoir bottle, and the hydrostatic pressure was adjusted to make the flow rate about 10 ml per hour. Fractions of 75 drops each (approximately 6 ml) were collected automatically.

As seen in Fig. 1A, accelerator activity was found under the first protein peak and under the ascending portion of the second protein peak, in accordance with the previous observations of others on 2-day-old bovine serum (9).

After the same column had been used for a second, similar run, it was equilibrated with 0.1 M sodium chloride:0.02 M tris buffer, pH 8.60, the eluant to be used for thrombokinase. 6 ml of thrombokinase concentrate was layered on top of the gel. The flow rate was about 8 ml per hour; and fractions of 75 drops each were collected automatically. At the end of the run the height of the gel was 33.5 cm.

As seen in Fig. 1B, almost all of the protein appeared in the position occupied by the third peak in the earlier fractionation of serum on the same column, and the protein was closely paralleled by the 3 assays of activity. There was, however, the usual sharp contrast in the concentrations required for the different assays. To activate prothrombin in the presence of oxalate, Fraction 17 was

used at a concentration of 8.1 μg protein per ml. To activate chymotrypsinogen, it was used at 324 μg per ml. But when complemented by calcium, phospholipid and accelerator in the working assay for thrombokinase, it was used at a concentration of 0.0016 μg per ml.

The defining characteristic of thrombokinase is its capacity to convert prothrombin to thrombin. This it can do, albeit slowly, without the help of accelerator. To test this further, the same column of Sephadex G-200 was used to fractionate a preparation of prothrombin that was already poor in accelerator. Prothrombin Fraction 17, from the after side of the prothrombin wave (not illustrated), was then activated by thrombokinase Fraction 18, from the after side of the thrombokinase wave of Fig. 1B. Neither of these fractions should have contained appreciable accelerator, since any accelerator present should have emerged in the earlier fractions, as it did in the run of Fig. 1A. Yet together they produced thrombin.

Moreover, it was then found that thrombokinase Fraction 19 of Fig. 1B could activate a prothrombin reagent in the presence of 0.01 M oxalate, after both reagents had been heated separately at 53°C for 2 hours. Such heat treatment has been reported to destroy accelerator globulin practically completely in neutral distilled water(10). In preparation for heating, thrombokinase Fraction 19 had been diluted 1:10 with distilled water, which brought the pH to 8.2; and the prothrombin reagent had been dialyzed against distilled water and brought to pH 7.1 by addition of sodium hydroxide. After heating, 56 to 78% of the thrombokinase activity remained, and 83% of the prothrombin. The thrombokinase had been heated at pH 8.2, because it rapidly loses activity a little below pH 7.0, even at 37°C(4).

Nevertheless, it was desirable to heat thrombokinase under conditions more closely approximating those known to be destructive for accelerator globulin. A portion of thrombokinase Fraction 17 (Fig. 1B) was dialyzed against 0.001 M veronal buffer, pH 7.4, after which a spot-plate test with phenol red indicated pH 7.1. It had 486 TAMe units per ml and 1.48 mg protein per ml. Heating this solution at 53°C for 2 hours destroyed about 97% of its TAMe esterase, as well as 99% of its activity, as estimated by the working assay, and 98 to 99% as measured in the presence of oxalate. It could still activate heated prothrombin in the presence of oxalate, but only very slowly.

A different approach was to take fractions 21 and 22 from the trailing end of the thrombokinase wave of Fig. 1B. These thrombokinase fractions, the ones least likely to contain accelerator, readily activated heated prothrombin in the presence of oxalate. Conceivably, a yet unrecognized form of accelerator globulin, differing either in its behavior on gel filtration or heat stability, might remain in one of the reagents. It would then have to differ further in its degree of dependence on calcium ions, in order to function effectively in the presence of oxalate. This,too, is conceivable.

Under conditions yet tried, neither accelerator nor phospholipid is effective in the presence of oxalate, and accumulating evidence indicates that thrombokinase can activate prothrombin without the help of these factors. However, production of thrombin is much faster when accelerator, phospholipid and ionic calcium are included(11). This 5-reagent system yields an assay value which parallels the assay of thrombokinase performed by activation of prothrombin in the presence of oxalate. These assay values have run parallel in a study of electrophoretic subfractions(12), and now again in subfractions obtained by gel filtration. This suggests that the same factor is being measured in both assays but it does not prove it. Nor does this prove that it is the only thrombokinase, *i.e.*, the only prothrombin-activating enzyme, obtainable from blood.

The Stuart-Prower defect, also called congenital factor X deficiency, was first described in 1956-7(13-15). The similarity between activated factor X and thrombokinase was first pointed out in 1961(16). Further studies have encouraged this idea(17-20). Nemerson (personal communication, 1963), and Spaet (personal communication, 1964) have each found that electrophoretically isolated thrombokinase, obtained from us, showed high activity in their tests for activated fac-

tor X. Recently, Ratnoff has also tested electrophoretically isolated thrombokinase and concluded (personal communication, 1964) that it is a rich source of Stuart factor, as determined by tests with plasma from a patient congenitally deficient in Stuart factor. Thus, correction of the Stuart defect is a fifth measurable effect of thrombokinase preparations. Are they all due to a single molecular species?

*Summary.* Bovine thrombokinase, incompletely purified, was subjected to gel filtration on Sephadex G-200. Almost all of the protein emerged from the column in the position corresponding to the third wave of serum proteins. The thrombokinase subfractions had the capacity to activate prothrombin in the presence of oxalate, to cause prothrombin activation in a system containing calcium, phospholipid and accelerator (factor V), and to activate chymotrypsinogen. No separation of these activities was evident. This makes it desirable to enquire further whether these activities, as well as the TAMe esterase and the power to correct the Stuart defect, are all properties of the thrombokinase molecule. Accumulating evidence continues to favor the view that thrombokinase can, under proper circumstances, activate prothrombin without the help of presently known forms of accelerator (factor V).

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## Nicotinamide Adenine Dinucleotide Synthesis by *Mycobacterium tuberculosis* var. bovis.* (30307)

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Scientists have long studied the various species and strains of *Mycobacterium tuberculosis* in the hope of obtaining simple methods for distinguishing virulent from avirulent strains, and of elucidating the nature of their virulence. Most of these investigations were attempts to ascertain the nutritional requirements of the microbe or to discover morphological and biochemical differences between strains. Only in recent years have studies been undertaken to delineate basic metabolic differences between strains. Pope and Smith (1) observed that both the human H37 and bovine Ravanel strains of *M. tuberculosis,* when grown on synthetic media devoid of preformed vitamins, were capable of *de novo* synthesis of nicotinic acid. Moreover, they demonstrated that the culture filtrate of the

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