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A New Method for the Preparation of Thrombin.

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In the preparation of thrombin (or prothrombin) 4 different methods have been followed. The method of Schmidt[1] starts with fresh serum which is precipitated by a large excess of alcohol, the precipitate subsequently being extracted with water or saline solution. The method of Buchanan as modified subsequently by Gamgee[2] and by Howell[3] starts with the fibrin of the clot from which the thrombin is extracted by a strong saline solution and purified by acetone precipitation. The method of Mellanby,[4] as modified by Bleibtreu,[5] and by Mellanby,[6] starts with oxalated plasma, from which, after strong dilution, the fibrinogen and prothrombin are precipitated together by the addition of weak acid. The separation of the prothrombin from the fibrinogen is effected in different ways. The method of Bordet[7] also starts with oxalated plasma from which the prothrombin (serozyme) is precipitated by adsorption upon a suspension of tricalcium phosphate, or, as suggested by Fuchs, by adsorption upon magnesium hydroxide. It is well known that the original method of Schmidt usually gives a feeble preparation of thrombin which is not wholly satisfactory for purposes of experiment or demonstration. The methods described by Howell, Mellanby, Bleibtreu and Bordet, on the other hand, give, under favorable conditions, very potent preparations which cause rapid and strong coagulation of oxalated plasma or fibrinogen solutions. Experience in this laboratory has shown that the method of Howell does not always give a satisfactory product. With some specimens of fibrin, the yield is excellent, while with others it is poor. The principal object of the work here reported was to restudy and improve this method so as to obtain a more uniform preparation of thrombin and

[1] Schmidt, Alexander, *Zur Blutlehre*, F. C. W. Vogel, Leipzig, 1892.

[2] Gamgee, A., *J. Physiol.*, 1879, **2**, 145; Physiological Chemistry, 1880, Macmillan and Co., London.

[3] Howell, W. H., *Am. J. Physiol.*, 1913, **32**, 264.

[4] Mellanby, J., *J. Physiol.*, 1909, **38**, 28.

[5] Bleibtreu, M., *Pflüger's Arch.*, 1929, **213**, 642.

[6] Mellanby, J., *Proc. Roy. Soc. B.*, 1930, **107**, 271; 1933, **113**, 93.

[7] Bordet, J., et Delange, L., *Bull. Soc. Roy. Sc. Méd. et Nat. Bruxelles*, 1914, **72**, 87.

if possible a preparation free from admixture with impurities. The attempt to improve the method has proved to be a more difficult task than was anticipated. After many unsuccessful attempts, modifications have been developed which it is believed, are actual improvements and insure a good yield of very active thrombin in a state of considerable purity. In the absence of definite knowledge of the chemical nature of thrombin it is not possible to establish a wholly satisfactory criterion of its purity. The criterion that has been used in this work is the absence of any detectable amount of heat coagulable protein in the solutions of the thrombin as finally obtained.

As a basis for this work Howell's second method for the preparation of thrombin was followed.

Three principal changes were made in this method of which the first was the choice of fibrin. Experience with the blood of the different slaughter house animals that could be obtained readily for such work, pig, ox, and calf, showed that fibrin from very young calves gives by far the best yield of thrombin. The second change was the omission of the precipitation of the saline extract of the fibrin by acetone since this procedure appeared to inactivate a part of the thrombin, subsequent extracts being less effective or less potent than the original saline extract. In place of the method of precipitation by acetone as a means of removing other proteins there was substituted the method of prolonged dialysis against a buffer mixture of phosphates as described in detail below. The third change was the preliminary extraction of the washed fibrin with ether for 24 hours or more prior to the extraction of thrombin by strong saline. The resulting saline extract was less syrupy in character and contained less foreign matter than when the ether extraction was omitted.

Fresh fibrin from whipped calves blood is washed free of hemoglobin by squeezing it repeatedly in cold, running water. This material is then run through a food chopper to free it further from any trace of hemoglobin and to place it in suitable form for extraction. It is now placed in ether at room temperature and allowed to remain at least 24 hours or more in a closed container, or until it is ready for use. At that time the ether is filtered off, the fibrin washed with clean ether and finally dried before an electric fan to the point where the odor of ether disappears. It is now extracted with a volume of 8% sodium chloride equal to its own volume, for 12 hours in the ice chest. The extract is filtered off through 2 thicknesses of filter paper.

This filtrate is then dialyzed in cellophane tubing for several hours against distilled water to remove the excess of sodium chloride. It is advisable at this point to test the thrombic action of the extract upon some oxalated plasma to ascertain whether a sufficiently active preparation has been obtained. The solution is next dialyzed for 72 hours against a Sørensen buffer mixture pH 7.38 (95 gm. Na_2HPO_4 and 19 gm. KH_2PO_4 in 20 liters of water). During this period the dialyzing liquid is renewed either by a continuous slow stream or by changing the outside liquid half a dozen or more times. The material is finally dialyzed for 5 hours against running, distilled water to remove the phosphates. The solution is filtered without pressure through a No. 5 Whatman filter paper and the now clear filtrate is dried at room temperature before an electric fan. The material thus obtained is soluble in water and the solutions show strong thrombic action. Two drops of a 0.7% solution will cause clotting of 3 drops of oxalated plasma in approximately 30 seconds with most specimens. In dry form the potency of the preparation is retained apparently indefinitely. Saline solutions give neither precipitate nor opalescence on boiling, indicating the absence of heat coagulable protein. Positive reactions for protein are given by the biuret, xanthoproteic, Adamkiewicz, ninhydrin, and Folin-Denis reagents. Positive reactions are given also for the presence of sulfur (cystine) and phosphorus. Tests with the Molisch reagent and for purine bases were negative. Prolonged dialysis of the solutions against distilled water inactivates this thrombin indicating, possibly, that it is a protein of the globulin group.

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Number of Thrombocytes and Leucocytes in Blood of Adrenalectomized Rats.

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Peripheral blood was obtained from each of a group of 15 normal rats for determining the total number of thrombocytes. Each blood sample was collected in a Trenner automatic red cell pipette and diluted one part to 200 with Ringer's solution (Casey and Helmer) and a small amount of cresyl blue added. After being shaken for 5 minutes in an automatic shaker, the platelets in 240 small squares