for 28 days in which the type of carbohydrate was varied and serum cholesterol and beta-lipoproteins were determined. 1. Rats fed diets containing sucrose as the carbohydrate and with added cholesterol and cholic acid had higher serum cholesterol and serum beta-lipoprotein concentrations than did control rats fed diets with corn starch substituted for sucrose. Liver cholesterol concentrations were not significantly different. 2. Feeding of sucrose, glucose, and fructose as the dietary carbohydrate had essentially equal effects on cholesterol-cholic acid induced hypercholesteremia. 3. Rats which were fed cholesterol containing diets without cholic acid and with corn starch as the carbohydrate had somewhat lower serum cholesterol values than did the controls which were fed sucrose. 4. When sulfasuxidine was added to sucrose containing diets, there was no change in serum cholesterol values; however, addition of sulfasuxidine to starch containing diets resulted in elevation of serum cholesterol values to the level of that in rats fed sucrose.

1. Portman, O. W., Mann, G. V., and Wysocki, A. P., *Arch. Biochem.*, 1955, v59, 224.

2. Siperstein, M. D., and Chaikoff, I. L., *Fed. Proc.*, 1955, v14, 760.

3. Siperstein, M. D., and Murray, A. W., *J. Clin. Invest.*, 1955, v34, 1449.

4. Page, I. H., and Brown, H. B., *Circulation*, 1952, v6, 681.

5. Byers, S. O., Biggs, M. W., and Gunning, B., *J. Exp. Med.*, 1953, v97, 511.

6. Frantz, I. D., and Hinkelman, B. T., *ibid.*, 1955, v101, 225.

7. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, v138, 459.

8. Portman, O. W., and Mann, G. V., *ibid.*, 1955, v213, 733.

9. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *ibid.*, 1950, v187, 97.

10. Gofman, J. W., Lindgren, F. T., and Elliott, H., *ibid.*, 1949, v179, 973.

11. Norman, A., *Acta physiol. scand.*, 1955, v33, 99.

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Isolation of Partially Purified Human Plasmin (Fibrinolysin). (22251)

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Although many methods for the preparation of plasmin from blood have been described, the utilization of highly purified plasminogen as a starting material appeared to offer the possibility of providing much purer plasmin than any previously reported.

This paper describes the preparation of plasmin from purified plasminogen, and the alcoholic fractionation of the resulting solution to obtain a plasmin product which is stable at low temperature and, in terms of fibrinolytic activity, is far more potent than any previously described fibrinolysin.

Materials and methods. Plasminogen. Lyophilized powder prepared by the method developed in this laboratory(1) from human plasma fraction III†. *Streptokinase* (SK). Commercial Varidase (Lederle).‡ Lots Nos. 7-1089-165A and 7-1089-142A were used. These contained 4,700 and 3,000 SK units per mg, respectively. *Fibrinogen.* Bovine Fraction I (Armour) was reprecipitated three times by the method of Laki(2) and was lyophilized. The critical assays were rechecked with fibrinogen which was reprecipitated seven times to eliminate the possibility that contaminating bo-

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vine plasminogen might have contributed to the results obtained(3,4). The quantity of fibrinogen in the lyophilized powder was calculated from nitrogen determinations, and a suitable amount was dissolved in phosphate buffer to a concentration of 0.4%. Fresh solutions were prepared for each series of assays. *Phosphate buffer.* 0.05 M, pH 7.8 for assays using 3 times purified fibrinogen; 0.01 M, pH 7.8 for 7 times reprecipitated fibrinogen. *Thrombin.* Commercial thrombin (Upjohn) was dissolved in an equal mixture of saline and glycerol to a concentration of 100 units per ml. *Assay of Fibrinolytic Activity.* A slight modification of the method of Ferguson(5) gave reproducible results. In one tube (8x70 mm), 0.5 ml of diluted enzyme solution, 0.5 ml of buffer and 0.04 ml of thrombin solution were mixed. To a second tube were added 0.5 ml of fibrinogen solution and 0.5 ml of buffer. The tubes were mixed immediately by pouring back and forth 3 times, placed in a water bath at 37.5°C, and the time of clot lysis determined. This tube contained a dilution of enzyme solution which had previously been found to dissolve the clot in the range of 270 to 330 seconds and was designated as 100%. Similar tubes containing 0.2 and 0.1 ml of diluted enzyme solution were made up to volume with buffer and were designated as 40 and 20%, respectively.

Units of Activity. The logarithm of the lysis time in seconds was plotted against the logarithm of the concentration (100, 40 or 20) and a straight line was drawn through the points. For a given fibrinogen solution, the slope of the line was constant. The activity of the 100% tube was arbitrarily assigned the value of 100 units. To obtain the activity per ml of original enzyme solution, the units obtained in each assay were multiplied by 2, since 0.5 ml is used in the assay, and then by the dilution factor. The specific activity is the activity per mg of nitrogen. *Activation.* The plasminogen powder was suspended in distilled water and dissolved by the addition of a few drops of N HCl. The concentration of enzyme was then adjusted to 4 mg/ml by the further addition of water. This solution was adjusted to pH 6.8 by the careful addition of dilute NaOH solution and 5,425 units of streptokinase per mg of plasminogen was added. The mixture was incubated at 37.5°C for 10 minutes after which the solution was adjusted to pH 8.7 and centrifuged. The pH was then lowered to 7.6 and again centrifuged, leaving a clear solution of plasmin. *Alcohol Fractionation.* The plasmin solution was chilled in an ice and salt bath to about 0°C and cold 95% ethyl alcohol was added dropwise with mechanical stirring until the desired alcohol concentration by volume was reached. The precipitate was permitted to settle overnight at 4°C, collected by centrifugation and dissolved in distilled water. A solution of plasmin so obtained has retained its original activity after seven months in the frozen state.

TABLE I. Alcohol Fractionation of 100 mg of Plasminogen after Streptokinase Activation.

% alc.	Nitrogen, mg/ml	Activity, units/ml (×1000)	Spec. activity, units/mg N (×1000)	Total activity in fraction,* units ($\times 10^6$)
0	.67	40	60	1
10	.29	123	423	.6
21	.21	43.6	207	.2
Ppt. at pH 5.3	.89	4	4.5	.02
0	.85	40	47.06	1
10	.26	86	330.8	.4
0	.84	40	57.5	1
10	.46	70	151	.35
21	.32	21	66	.1
Ppt. at pH 5.3	.97	1.68	1.73	.01
0	.82	40	48.78	1
10	.37	100	294	.5
15	.21	35.6	170	.2
21	.14	17.8	127	.09
0	.76	40	53	1
21	.58	192	330	1
0	1.03	40	39	1
21	.76	155.2	203	.8

* Unfractionated solutions were 25 ml each. 0.5 ml of a 1:200 dilution was used in each assay. Total activity, therefore, was: 100 × 2 × 200 × 25 as described in the text. Alcohol fractions were dissolved in 5 ml of water which was then diluted 1:400 for assay.

Results. Table I shows the total and specific activities of the activated solutions, of the alcohol fractions, and of the remainder of the active material which precipitated at pH 5.3. It will be observed that the precipitate

obtained from 10% alcohol is the most active per mg of nitrogen, and that the fractionation is sharp, little activity remaining in the supernatant solution after removal of the 21% alcohol fraction. The combined activities recovered averaged about 75% of that present in the unfractionated solution, and the specific activity of the combined active fractions was about 3.5 times that of the starting solution.

To insure that these results did not represent the presence or absence of SK in any of the fractions, additional SK, 1000 units per assay, was added to each fraction with no effect upon the activity. In addition, to control SK to plasminogen ratios, 1000 units of SK per mg of enzyme nitrogen was added to each fraction assayed, again without affecting the activity appreciably.

Discussion. Although the plasmin obtained by streptokinase activation of purified plasminogen followed by alcoholic fractionation is the most potent fibrinolytic preparation thus far reported, the total yield and specific activity obtained may possibly be increased by varying the conditions of activation and fractionation. It is recognized that the amount of SK used in this procedure is quite large. In our laboratory, such amounts of SK alone or after mixing with plasminogen have regularly produced severe toxic effects and even death in the dog. Studies in dogs which are being carried out in the laboratory by Messrs. Fishbein and Newman, in collaboration with Dr. Louis Nahum, will be reported in detail elsewhere. Preliminary results indicate that the intravenous infusion of up to 1 mg/kg of purified plasmin is tolerated by the dog with no significant alterations of body temperature, blood pressure or electrocardiogram. Incoagulability of the dogs' blood appeared within 5 minutes of the injection. The following day, clotting times had returned to normal. The absence of toxic reactions in the dog of the new plasmin preparation would indicate that the toxic materials originally present in the SK(6) are largely eliminated by the fractionation procedure. Streptokinase itself is completely soluble in 21% alcohol. It is possible that a non-toxic SK-plasmin complex is the active form of the enzyme.

The dissolving of clots made from seven times reprecipitated fibrinogen would appear to rule out bovine plasminogen as an essential element of the clot-lysing system. The addition of 2 ml of human blood to 0.1 mg of purified plasmin resulted in immediate incoagulability. This experiment also eliminates the possibility that the release of heparin from the liver of the dog was responsible for the incoagulability observed. In preliminary experiments being carried out in association with Dr. W. Glenn the *in vivo* dissolving of a radiochromium labeled clot in the dog has been observed after the intravenous injection of 0.5 mg of plasmin per kg. The dissolving of the clot was confirmed by autopsy.

With the availability of this water-soluble, fibrinolytic enzyme preparation, preliminary clinical studies of the efficacy of this material in thrombotic conditions is now brought much closer. New light may also be thrown upon the physiology of blood coagulation by studies of the action of plasmin. Work is now in progress for the further purification and characterization of this protein.

Summary. A method for preparation and isolation of plasmin from purified human plasminogen after activation with streptokinase is presented. The product is water soluble, stable in the frozen state and produced no toxic manifestations in the dog after intravenous infusion of 1 mg/kg.

1. Kline, D. L., *J. Biol. Chem.*, 1953, v204, 949.

2. Laki, K., *Arch. Biochem. and Biophys.*, 1951, v32, 317.

3. Sherry, S., *J. Clin. Inv.*, 1954, v33, 1054.

4. Mullertz, S., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 291.

5. Lewis, J. H., and Ferguson, J., *Am. J. Physiol.*, 1952, v170, 636.

6. Tillett, W. S., Johnson, A. J., and McCarty, W. R., *J. Clin. Inv.*, 1955, v34, 169.

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