11. Billingham, R. E., Silvers, W. K., *Science,* 1958, v128, No. 3327, 780.

12. Mariani, T., Martinez, C., Smith, J. M., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 287.

13. ———, *ibid.,* 1959, v101, 596.

14. ———, *Proc. Am. Assn. Cancer Research,* 1959, v3, 40.

15. Martinez, C., Smith, J. M., Aust, J. B., Good, R. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v98, 736.

Received July 22, 1959. P.S.E.B.M., 1959, v102.

Mechanism of Bovine Plasminogen Activation by Human Plasmin and Streptokinase. (25388)

JOHN ZYLBER, WILLIAM F. BLATT AND H. JENSEN*

Biochemistry Division, U.S. Army Medical Research Laboratory, Fort Knox, Ky.

It has been known for some time that conversion of bovine plasminogen to plasmin can be effected by addition of streptokinase in presence of trace amounts of either human plasminogen or plasmin. This is in contrast to activation of human plasminogen, elicited by addition of streptokinase alone. In both systems, the formation of plasmin has been ascribed by some investigators(1,2,3) to conversion of a specific proactivator by streptokinase to a singular activator which then acts on plasminogen to produce plasmin. Other investigators(4,5,6), however, have expressed the possibility that the proactivator of bovine plasminogen, when activation mixtures of streptokinase and human plasminogen or plasmin are used, may be identical with either human plasminogen or plasmin. The following studies were performed to examine the mechanism of bovine plasminogen conversion by human plasmin and streptokinase under various experimental conditions, and to determine whether this conversion is brought about by a specific activator principle, formed by action of streptokinase on a separate distinct proactivator, or whether streptokinase may form a complex with human plasmin which can directly convert bovine plasminogen to plasmin.

Materials and methods. Human plasmin. This enzyme was obtained from human plasma Fraction III† by a method previously described(7). *Bovine plasminogen.* Twenty-four liters of freshly collected bovine blood were permitted to clot at room temperature; after 2 hours standing, the clots were dispersed and strained through gauze. Final serum clarification was obtained by centrifugation for 30 minutes at 2,000 rpm at 0°C. The serum obtained (approximately 8 liters) was cooled to 0°C and brought to 25% ammonium sulfate saturation by addition of pre-cooled saturated ammonium sulfate solution, stirred for 30 minutes, then left for 1 to 2 hours at 0°C. The precipitate obtained following centrifugation was discarded and the supernatant solution adjusted to 33% saturated ammonium sulfate by further addition of pre-cooled saturated ammonium sulfate solution at 0°C. After remaining overnight at 4°C, the precipitate was collected by centrifugation, dispersed in distilled water, and dialyzed against running tap water for 18 hours at 4°C. The precipitate formed on dialysis was collected by centrifugation, dispersed in 100 to 150 ml of 0.9% sodium chloride and then diluted 1:8 with distilled water at room temperature. To achieve maximum solubilization, pH was adjusted to 9.4 with 1 N sodium hydroxide, and the solution was then brought to pH 5.3 with 1% acetic acid. The precipitate that formed was allowed to settle overnight at 4°C. After centrifugation, the precipitate was dissolved in 1 liter of phosphate buffer pH 7.0, ionic strength 0.15. The solution was cooled to 0°C and brought to 25% saturated ammonium sulfate by addition of pre-cooled satu-

* Died Sept. 30, 1959.

† Obtained through courtesy of Am. Nat. Red Cross, Washington, D.C., and kindly supplied us by Cutter Labs., Berkeley, Calif., as well as by E. R. Squibb and Sons, New Brunswick, N. J.

rated ammonium sulfate solution. After centrifugation, the supernatant solution was adjusted to 33% ammonium sulfate saturation. The precipitate obtained after centrifugation was dispersed in distilled water, dialyzed against running tap water and then against distilled water at 4°C; the water insoluble material was removed by centrifugation, dispersed in distilled water and lyophilized. In all experiments, a freshly prepared solution of 5 mg of bovine plasminogen in 1 ml of phosphate-sodium chloride buffer, pH 7.0, ionic strength 0.15, was utilized. *Streptokinase.*‡ Solutions of desired concentrations were made with 0.9% sodium chloride, pH 7.0. All stock solutions were stored at 4°C and kept no longer than one week. *Casein.* One per cent stock solutions of casein were prepared (7), either in saline-phosphate buffer (0.1 M phosphate, 0.9% saline), pH 7.4 or in 0.1 M phosphate buffer, pH 7.6. *Crystalline pancreatic trypsin inhibitor.*§ Stock solutions of 4, 6, 8, and 12 μg/ml were prepared in 0.1 M phosphate buffer, pH 7.0. *Standard test procedure.* On the basis of preliminary studies, the following standard procedure was devised. A 1 ml incubation mixture containing 0.05 mg of human plasmin and 250 units of streptokinase was obtained by addition of 0.5 ml of 0.1 mg/ml plasmin solution in 0.001 N hydrochloric acid to 0.5 ml of 500 units/ml streptokinase solution prepared in 0.9% saline adjusted to pH 7.0. The reaction at pH 6.6 proceeded at room temperature 5 to 10 minutes prior to addition to 1 ml of a 5 mg/ml bovine plasminogen solution freshly prepared in saline-phosphate buffer (pH 7.0, ionic strength 0.15). Following 10 minute activation at room temperature for the mixture of human plasmin, streptokinase and bovine plasminogen, 2 ml of a 1% casein solution in saline-phosphate buffer, pH 7.4, prewarmed to 35°C were added to the mixture, and proteolysis of casein proceeded at 35°C for 30 minutes prior to addition of 5 ml of 5% trichloroacetic acid. Blanks were prepared by immediate addition of trichloroacetic acid prior to addition of casein. Extent of proteolysis was determined by reading optical densities of the trichloroacetic acid filtrates relative to the blanks at 280 mμ. Proteolytic units were determined from standard calibration curves(7). Schematically, the details of activation, conversion, and proteolysis can be summarized as follows:

(1) Activation mixture: (1 ml; 5 to 10 min.)	Human plasmin (0.05 mg) (0.5 ml)	+	Streptokinase (250 units) (0.5 ml)
↓			
(2) Conversion mixture: (2 ml; 10 min.)	Bovine plasminogen (5 mg/ml) (1 ml)	+	Activation mixture (1 ml)
↓			
(3) Digestion mixture: (4 ml; 30 min.)	1% casein (2 ml)	+	Conversion mixture (2 ml)

Effect of acid pH on the plasminogen activation. Four 80 ml stock solutions (0.9% sodium chloride, pH 6.6) were prepared, containing 0.05 mg human plasmin/ml and the following units of streptokinase/ml, 1) 31.25; 2) 62.5; 3) 125; and 4) 250. Ten ml aliquots were withdrawn from each stock solution and quadruplicate assays at each streptokinase level were performed for activator potency by addition to bovine plasminogen under standard test conditions. The remaining solutions were adjusted to pH 2.0 by addition of 4 N hydrochloric acid, were permitted to equilibrate for 30 minutes at room temperature, then readjusted to pH 6.6 by addition of 4 N sodium hydroxide. Ten ml aliquots were removed for activator assays. Streptokinase, contained in 0.1 ml of 0.9% saline, pH 7.0, was added to each stock solution in initial units of streptokinase employed for that series. Aliquots were removed for assay. Once again the pH was reduced, readjusted, and an aliquot removed for assay. The entire procedure was repeated 3 times, utilizing original stock solutions throughout. The protocol has been summarized at top of Fig. 1, in which results have been graphically recorded. *Influence of pancreatic trypsin inhibitor on formation of activator.* Stock solutions in the following volume relationships were prepared: 1 ml of human plasmin : 0.5 ml of crystalline pancreatic trypsin inhibitor solution : 0.5 ml

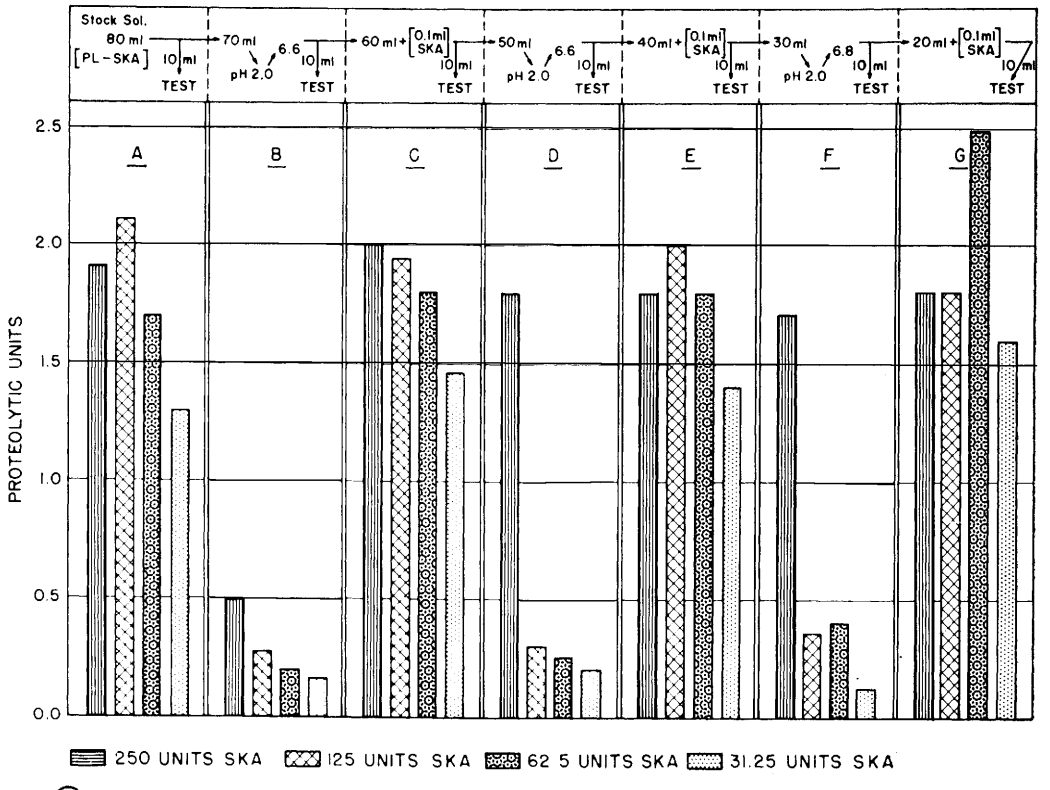
‡ Streptokinase-streptodornase (Varidase) was supplied through the generosity of Lederle Labs. Division, Amer. Cyanamid Co., Pearl River, N. Y.

§ Obtained from Worthington Biochemical Corp., Freehold, N. J.

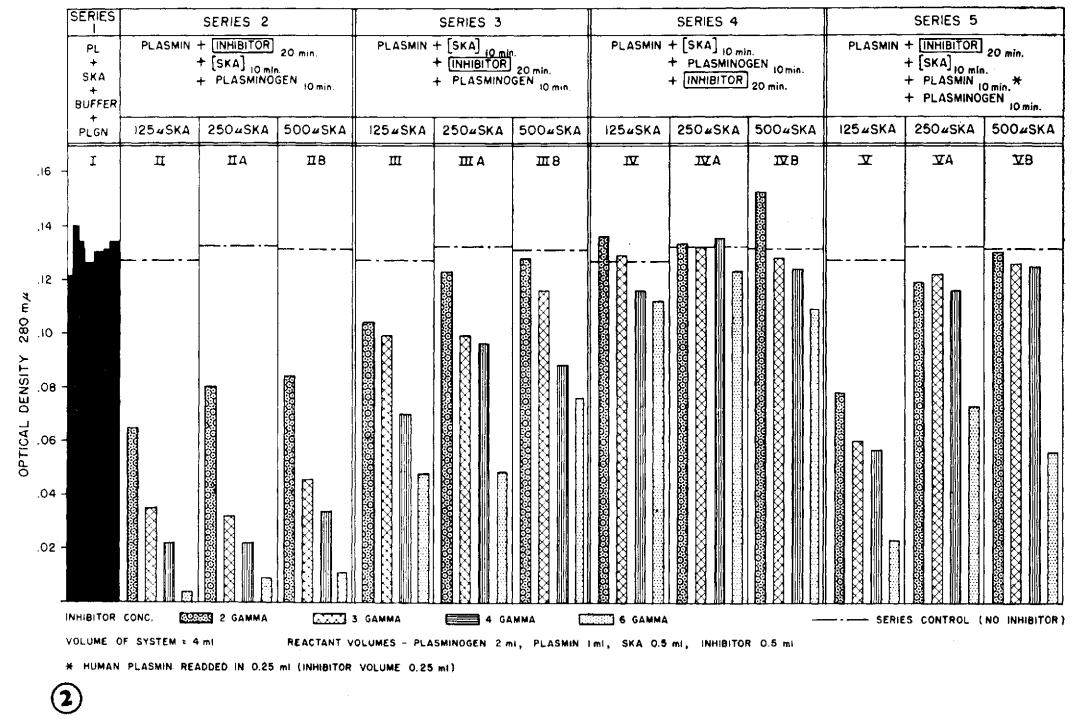
streptokinase solution : 2 ml of bovine plasminogen solution (5 mg/ml). Final concentrations of reactants in the 4 ml stock solutions were as follows: 0.2 mg human plasmin; 2, 3, 4, or 6 μg of inhibitor 125, 250, or 500 units of streptokinase; and 10 mg of bovine plasminogen. Streptokinase, inhibitor, and plasminogen were prepared in 0.1 M phosphate buffer, pH 7.0, while plasmin solutions were made with 0.0025 N hydrochloric acid. In all series careful consideration was given to any volume changes so that all series would be comparable. Similarly, accurate control of equilibration times for each series was maintained. Briefly, this was as follows: 20 minutes equilibration for inhibitor addition; 10 minutes each for the reaction with streptokinase and with plasminogen. The inhibitor was added at various stages of the reaction system as follows: Series 1: Controls; no inhibitor added. Series 2: Inhibitor added to human plasmin prior to addition of streptokinase and then of plasminogen. Series 3: Inhibitor added after human plasmin and streptokinase were allowed to react, and preceding addition to plasminogen. Series 4: Inhibitor added after human plasmin plus streptokinase had reacted with plasminogen but prior to addition to casein. Series 5: An additional series was devised in which human plasmin plus inhibitor and streptokinase were allowed to react, then additional human plasmin was added prior to mixing with plasminogen. Casein proteolysis was carried out according to Kunitz's procedure. One ml aliquots of varying reaction mixtures were added to 1 ml of 1% casein solution prepared in 0.1 M phosphate buffer, pH 7.6, prewarmed to 35°C and incubated at 35°C for 20 minutes prior to addition of 3 ml of 5% trichloroacetic acid. Blanks were prepared by immediate addition of trichloroacetic acid prior to casein addition. Extent of proteolysis was determined by reading optical densities of the trichloroacetic acid filtrates relative to blanks at 280 mμ. Four inhibitor concentrations of 2, 3, 4, or 6 μg, were employed for each of the 3 different streptokinase concentrations. The reactants were assayed for intrinsic proteolytic activity, but since no significant contributions to proteolytic activity by individual reactants were evident, the results are not listed. Fig. 2 contains both the protocol and results of this experiment. The protocol for each series is listed on top and the demonstrable proteolytic activity, as expressed in O.D. units, is listed on side of chart.

Results. In the standard test procedure, a solution of human plasmin plus streptokinase following preliminary incubation was added to a solution of bovine plasminogen, the mixture allowed to react and then casein added. Three reactions proceed: Formation of an activator, conversion of bovine plasminogen by the activator, and proteolysis of casein by bovine plasmin formed. Addition of incubated solution of bovine plasminogen, containing human plasmin and streptokinase, to a casein substrate has been used to follow the activating effect of human plasmin plus streptokinase on bovine plasminogen. The amount of human plasmin employed was relatively small and produced no significant proteolysis when tested alone against casein. There was no conversion of bovine plasminogen to plasmin in either the presence of human plasmin or streptokinase alone; activation of bovine plasminogen was observed only in presence of both human plasmin and streptokinase.

Fig. 1 summarizes results of the effect of pH 2 on bovine plasminogen conversion activity. The values represent an average of duplicate determinations for at least 4 experiments. Generally, there was a corresponding increase in activator potency with increasing streptokinase concentration (column A). Following low pH treatment, there was a striking decrease in activator capacity (column B). Readdition of streptokinase returned activator levels to the pre-acid treatment values (compare column C with column A). With exception of 250 units streptokinase level, subsequent low pH equilibrations reduced activator potency again, while readdition of streptokinase returned the activator levels to original values (columns E and G). At a concentration of 250 units of streptokinase, the second and subsequent low pH treatments did not result in an apparent loss of activator potency (columns D and F). While there may have been destruction of ac-


Stock Sol.
80 ml [PL-SKA] 10 ml TEST
70 ml pH 2.0 6.6 10 ml TEST
60 ml + [0.1 ml SKA] 10 ml TEST
50 ml pH 2.0 6.6 10 ml TEST
40 ml + [0.1 ml SKA] 10 ml TEST
30 ml pH 2.0 6.8 10 ml TEST
20 ml + [0.1 ml SKA] 10 ml TEST
PROTEOLYTIC UNITS
2.5
2.0
1.5
1.0
0.5
0.0
A
B
C
D
E
F
G
250 UNITS SKA
125 UNITS SKA
62 5 UNITS SKA
31.25 UNITS SKA

①


SERIES I
PL + SKA + BUFFER + PLGN
SERIES 2
PLASMIN + [INHIBITOR] 20 min. + [SKA] 10 min. + PLASMINOGEN 10 min.
SERIES 3
PLASMIN + [SKA] 10 min. + [INHIBITOR] 20 min. + PLASMINOGEN 10 min.
SERIES 4
PLASMIN + [SKA] 10 min. + PLASMINOGEN 10 min. + [INHIBITOR] 20 min.
SERIES 5
PLASMIN + [INHIBITOR] 20 min. + [SKA] 10 min. + PLASMIN 10 min. * + PLASMINOGEN 10 min.
125 u SKA
250 u SKA
500 u SKA
I
II
IIA
IIB
III
IIIA
IIIB
IV
IVA
IVB
V
VA
VB
OPTICAL DENSITY 280 mμ
.16
.14
.12
.10
.08
.06
.04
.02
INHIBITOR CONC. 2 GAMMA 3 GAMMA 4 GAMMA 6 GAMMA SERIES CONTROL (NO INHIBITOR)
VOLUME OF SYSTEM = 4 ml
REACTANT VOLUMES - PLASMINOGEN 2 ml, PLASMIN 1 ml, SKA 0.5 ml, INHIBITOR 0.5 ml
* HUMAN PLASMIN READDED IN 0.25 ml (INHIBITOR VOLUME 0.25 ml)

②

tivator, higher concentration of streptokinase in excess of non-reacted streptokinase could probably re-form activator after alkaline adjustment of the solution.

Plasmin as well as streptokinase were quite stable when incubated separately at low pH for 2 to 3 hours, since no decrease in activator activity was observed upon mixing after adjustment to pH 6.6 and addition to bovine plasminogen. The destruction of plasminogen activator activity following low pH treatment is in agreement with similar observations by others(5,8,9). However, the present results do not seem to support the concept that streptokinase interacts with a specific proactivator resulting in formation of a distinct singular plasminogen activator(1,2,3), since one would expect that repeated acid treatments would eventually lead to a decrease in activator activity, which was not observed.

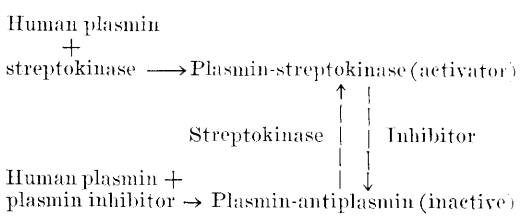
Fig. 2 summarizes results of 4 duplicate determinations, graphically illustrating the influence of a plasmin inhibitor on formation of plasminogen activator activity. Preliminary experiments showed that the pancreatic trypsin inhibitor had the capacity to inhibit proteolytic activity of human plasmin and that this inhibition for the amount of plasmin used was complete at higher concentrations of inhibitor employed. Column I indicates plasmin activity formed (as indicated by O.D. of the trichloroacetic acid soluble filtrates) when no inhibitor had been introduced at any stage in the test system. Columns II, II-A, and II-B demonstrate the marked decrease in activator potency when the inhibitor was added to plasmin prior to addition of streptokinase at the 3 levels employed. As the inhibitor concentration was increased, the decrease in activator formation became more evident. These results seem in agreement with the assumption that human plasmin may interact with streptokinase forming the bovine plasminogen activator. However, it was possible that the inhibitor may have interacted with the plasminogen activator rather than with plasmin. Experiments in which the inhibitor was added after interaction of human plasmin and streptokinase examine this aspect (columns III, III-A, and III-B). Although evidence of some decreased bovine plasminogen activation was manifested, this decrease in activator activity was definitely less than that observed when plasmin was first interacted with the inhibitor (columns II, II-A, and II-B). Once again, the decrease in activator activity was more marked as inhibitor concentration was increased. The decrease in activator activity under these experimental conditions may be due to an inhibition of the activator by the pancreatic inhibitor or to a replacement of some streptokinase in a plasmin-streptokinase complex. In the latter case, streptokinase apparently competes with the inhibitor in combining with human plasmin. If this were so, more pronounced activator activity might be expected as the ratio of streptokinase to inhibitor is increased. The data (columns III and III-B) seem to support this possibility. To determine that there was actually a decrease in activator activity and in resultant bovine plasminogen conversion in presence of inhibitor rather than any inhibition of plasmin formed from bovine plasminogen, the inhibitor was added after activation of bovine plasminogen. As can be seen from the data, illustrated in columns IV, IV-A, and IV-B, there was only a slight decrease in plasmin activity (more pronounced at 6 μg level of inhibitor) when compared with control series (column I). In the final experiments, human plasmin was readded after incubation of plasmin and inhibitor followed by streptokinase addition. At the level of 125 units of streptokinase (column V) there was more reactivation in the presence of 2 μg of inhibitor as compared with 3, 4, or 6 μg of inhibitor. At the levels of 250 (column V-A) or 500 (column V-B) units of streptokinase, with the exception of the 6 μg inhibitor concentration, reactivation was essentially complete. These findings would indicate that as long as there is no great excess of inhibitor, complete reactivation of the system is possible upon readdi-

FIG. 1. Effect of low pH on conversion of bovine plasminogen to plasmin by human plasmin and streptokinase.

FIG. 2. Effect of a plasmin inhibitor on conversion of bovine plasminogen to plasmin by human plasmin and streptokinase.

tion of human plasmin. Apparently streptokinase will compete with the inhibitor in combining with human plasmin.

Our results may suggest that human plasmin and streptokinase combine to form a complex which has the capacity to convert bovine plasminogen to plasmin. The following scheme may serve to illustrate the formation of activator and the influence of the plasmin inhibitor on this reaction:



It is assumed that in the presence of plasmin inhibitor, streptokinase will compete with the inhibitor in combining with plasmin. The amounts of the plasmin-streptokinase or plasmin-antiplasmin complexes formed are dependent upon concentrations of streptokinase and of plasmin inhibitor.

Discussion. Our data do not seem to support the concept of a separate proactivator which under the influence of streptokinase is converted to a specific singular plasminogen activator(1,2,3). It may be pointed out that the postulated proactivator has not as yet been definitely demonstrated to be an entity, except by indirect inference. Our attempts to separate proactivator activity from human plasmin or plasminogen have thus far been unsuccessful. The data also do not substantiate the assumption that proactivator and human plasminogen are identical(6). Our results are in agreement with the postulation that human plasmin reacts with streptokinase perhaps proportionally, to form a plasmin-streptokinase complex which has the ability to convert bovine plasminogen to plasmin. The complex may contain streptokinase in a form so altered (possibly by preliminary reaction of plasmin on the streptokinase prior to complexing) that upon dissociation by acid, the bound streptokinase has become acid labile. This concept would be in agreement with the finding that activator activity can be destroyed by acid treatment and can be restored by subsequent addition of streptokinase after pH adjustment to 7.2.

Studies on the influence of a plasmin inhibitor, added to various stages involved in bovine plasmin formation, on final proteolytic activity indicate that apparently streptokinase can compete with the plasmin inhibitor in combining with human plasmin. Amounts of plasmin-streptokinase or plasmin-antiplasmin complexes formed seem to depend upon concentrations of streptokinase and plasmin inhibitor.

The formulation of a plasmin-streptokinase complex as the bovine plasminogen activator is in accord with the observation of Kline and Fishman(5) that there was no significant change in the ratio of proactivator to plasminogen (human) during purification of plasminogen as well as with the finding of Ablondi and Hagan(6) that there was no difference in heat stability between proactivator and human plasminogen. The proposed scheme of bovine plasminogen activation by human plasmin in the presence of streptokinase is in accord with similar schemes proposed by Kline and Fishman(5) as well as by Sherry and his associates(4). Meyers and Burdon(10) have shown that plasminogen in sera from a large number of different animal species could be activated with human plasminogen-streptokinase mixtures.

Summary. Equilibration of human plasmin-streptokinase mixtures at pH 2 led to a loss in ability of mixture to convert bovine plasminogen to plasmin. Plasminogen conversion activity could be re-established upon readdition of streptokinase. Results of repeated low pH treatments and readditions of streptokinase do not seem to support the hypothesis of the existence of a specific proactivator for formation of a singular plasminogen activator. Studies on the influence of a plasmin inhibitor, added at various stages of the interactions, on formation of plasminogen converting activity yielded results in agreement with the hypothesis that plasmin reacts, probably proportionally, with streptokinase to form a plasmin-streptokinase complex which has the ability to convert bovine plasminogen to plasmin.

1. Astrup, T., *Blood,* 1956, v77, 781.

2. Mullertz, S., *Acta physiol. scandinav.,* 1956, v38, Suppl. 130.

3. Siegel, M., Cliffton, E. E., *J. Appl. Physiol.*, 1957, v10, 289.

4. Alkjaersig, N., Fletcher, A. P., Sherry, S., *J. Biol. Chem.*, 1958, v233, 86.

5. Kline, D. L., Fishman, J. B., *Ann. N. Y. Acad. Sci.*, 1957, v68, 25.

6. Ablondi, F. B., Hagan, J. J., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 195.

7. Sale, E. E., Priest, S. G., Jensen, H., *J. Biol. Chem.*, 1957, v227, 83.

8. Mullertz, S., *Biochem. J.*, 1955, v61, 424.

9. Troll, W., Sherry, S., *J. Biol. Chem.*, 1955, v213, 881.

10. Meyers, W. M., Burdon, K. L., *Am. J. Physiol.*, 1957, v190, 303.

Received August 24, 1959. P.S.E.B.M., 1959, v102.

Serum Lipoproteins and Glycoproteins in Aminopterin Treated Rats.* (25389)

KRISHNASUDHA ROHATGI AND SACHCHIDANANDA BANERJEE†
Dept. of Physiology, Presidency College, Calcutta, India

We observed(1) alterations in serum protein patterns of aminopterin treated folic acid deficient rats. Folic acid is involved in lipid metabolism(2-4). Lipids and the bulk of carbohydrates in blood are transported in association with proteins. The nature and composition of these lipoproteins and glycoproteins might reflect basic changes in metabolism and thereby give information on the nature of these changes. In the present investigation different electrophoretic components of serum glycoproteins and lipoproteins were determined in aminopterin-treated folic acid deficient and pair-fed normal rats. Total lipid, free cholesterol, ester cholesterol, phospholipid and neutral fat were also estimated in the serum of these animals.

Methods. Selection of rats, production of folic acid deficiency by intraperitoneal injection of aminopterin and collection of blood samples from carotid artery were the same as described previously(1). Only clear serum was used. Total glycoproteins in serum were determined by the method of Weimer and Moshin(5) as modified by Winzler(6). Cholesterol was estimated by the method of Sobel and Meyer(7). Phospholipid was estimated by determination of P of the serum extract(8) and multiplying the value by 25(9). Total serum lipid was determined by the method of Bragdon(10). Neutral fat was calculated by subtracting the figure for total cholesterol and phospholipid from the value for total lipid. Ionographic separation of lipoprotein and glycoprotein fractions of sera were carried out by the paper electrophoretic technic described earlier(1,11). For glycoproteins .01 cc and .04 cc serum were applied on different parallel paper strips and electrophoresis continued for 16 hours with a current of 4 ma and 200 volts in a barbiturate-barbituric acid buffer of pH 8.6 and ionic strength .05. The strip with .01 cc serum was stained with bromophenol blue for proteins, and used as reference strip in subsequent measurement of polysaccharides. Strips with .04 cc serum were stained for glycoproteins by the method of Koiw and Gronwall(12), modified by Roboz *et al.*(13), and based on histochemical technic described by Hotchkiss(14). Polysaccharides were indicated by violet bands. Densitometric evaluation of strips was performed immediately after staining, as the color was not permanent and the unstained portion of the paper turned pink after exposure for several hours in air. The densitometric curves of the different glycoprotein bands were measured by planimeter. For lipoproteins, the prestaining procedure of McDonald and Bermes(15) was followed. .05 cc of stained serum for lipoprotein and .005 cc of serum for protein determination were applied to parallel adjacent strips and electro-

* We are indebted to Lederle Laboratories (India) Ltd. for gift of aminopterin. Ministry of Scientific Research & Cultural Affairs, Govt. of India, granted a National Research Fellowship to KR.

† Present address: Prof. of Physiology, Medical College, Bikaner, India.