α_1 and α_2 that produce the same retardation (Table II) are identical is not clear, since there is no other clarifying physical or chemical evidence.

We do not know what portion or constituent of the red cell membrane or possible extraneous coats is acted upon or modified by the slowing factors. Eylar *et al.*(5) report that modification of the sialic acid component of the cell membrane by neuramidase may result in as much as a 94% retardation of the red cells of humans. Our experiments furnish no evidence either for or against operation of a sialic acid mechanism. Also the physiological role played by the SF-89 factor is obscure, but it is invariably present. The slowing factor in cancer that travels with the α_2 protein fraction, differs in activity as well as in mobility from the factor that travels with the α_1 fraction.

Conclusion. The red blood cells of persons with malignant neoplasm move more slowly in an electric field than do cells of persons in health. They do so because their surface electrical charge has been altered by a factor present in the blood serum, called for convenience the SF-89 factor. By paper electrophoresis it has been demonstrated that this factor is located in the α_1 protein fraction of the serum.

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Studies on Fibrinolysis: I. A Circulating Substance Capable of Converting Plasmin-Sensitive to Plasmin-Resistant Clots.* (27896)

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It has been observed that clots produced *in vivo* can develop resistance to lysis by fibrinolytic agents(1). This phenomenon has been attributed to clot organization. The object of this study is to investigate the mechanism of acquired clot resistance to fibrinolysis.

Materials and methods. Fibrinogen. Crude human fibrinogen, Fraction I (Cohn), was obtained from American Red Cross. Purified "Profibrinolysin-Free" human fibrinogen was obtained from Cutter Laboratories, Berkeley, Calif. 0.6% Solutions of fibrinogen were prepared in either 0.05 M imidazole buffer, pH 7.35(2), or a 1:25 dilution (v/v) of this buffer in normal saline. The method of Back *et al.*(3) was utilized for iodination of fibrinogen I^{131}.

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Bovine topical thrombin (Parke Davis & Co.) was rendered plasminogen-free by the following procedure: 2.0 g crude thrombin was suspended in 200 ml cold 0.15 M phosphate buffer pH 7.3. The solution was stirred slowly and 4.0 g calcium phosphate (anhydrous) was added slowly at 4°C. The suspension was then brought to 20% (w/v) with ammonium sulfate and allowed to stand for 30 minutes. Precipitate was separated by centrifugation and supernatant dialyzed against 16 liters of water. Siliconized or plastic equipment was employed for preparation and storage of thrombin. The final product was diluted to give a 15-second clotting time when 0.1 ml was added to 0.5 ml of 0.6% fibrinogen. The solution was stored at −20°C. *Streptokinase* was obtained from Lederle Laboratories *(Varidase). Urokinase* was obtained from Leo Pharmaceutical Prod-

ucts, Denmark. *Plasmin and plasminogen.* Plasminogen was prepared from human plasma Fraction III (Cohn) by a modification of the Kline method(4). Plasmin was prepared by activation of plasminogen with either urokinase or streptokinase at pH 7.4 in 25% glycerol at room temperature. The activation system contained 0.1 M glycine and 0.01 M phosphate buffer, pH 7.4. The ratio of urokinase to plasminogen was one Ploug unit to 2 Remmert and Cohen casein units, while the ratio of streptokinase to plasminogen was one Christensen unit to one Remmert and Cohen casein unit. After completion of activation, usually 24 hours, the mixture was adjusted to pH 3.7 with hydrochloric acid. The plasmin solution contained 25-75 Remmert and Cohen casein, or 75-225 RPMI (Roswell Park Memorial Inst.) units per ml and was stable for at least 6 months at 4°C.

Plasmin was also prepared from sterile Human Plasminogen-B Dry Powder, obtained from Parke Davis & Co. 124 mg of this powder was dissolved in 50 ml imidazole buffer pH 7.35. 200 Units of streptokinase were added and the mixture incubated at 28°C for 24 hours. Final plasmin activity was 1.0 RPMI units per ml. Solution was stored in aliquots at –20°C.

Serum. Approximately 5 ml of blood from normal subjects was gently agitated during coagulation with several glass beads, then incubated at 37°C for 3 hours. Serum was separated by centrifugation and either used immediately or stored at –20°C. Later experiments utilized pooled, freshly frozen, normal serum, collected from at least 140 healthy donors. Similar results were obtained with serum from either source.

Serum fractionations. To normal human pooled serum was added an equal volume of saturated ammonium sulfate. The "globulin" precipitate was separated after 24 hours by centrifugation and dissolved in water. Solid ammonium sulfate was added to the supernatant to full saturation and the mixture permitted to stand for several days at 4°C. The "albumin" precipitate was filtered and dissolved in water. Both fractions were individually dialyzed against 0.15 tris buffer, pH 7.4, and reconstituted to starting volume. Cellulose acetate electrophoresis was performed on these fractions at pH 8.6 in 0.05 M barbital buffer. Strips were stained with amido-black dye.

Plasma. Blood was added to 1/9 volume 0.1 M sodium oxalate. Barium sulfate adsorbed plasma was prepared by incubating 1.0 ml oxalated plasma for 15 minutes at room temperature in the washed precipitate formed from mixture of 0.1 ml 1.0 M sodium sulfate and 0.1 ml 1.0 M barium chloride. After mixing with wooden applicators, the adsorbed plasma was separated by centrifugation.

Assay system for fibrinolysis inhibition. Clots were produced in a series of 12 × 100 mm plastic test tubes by addition of 0.05-0.1 ml thrombin to 0.5 ml fibrinogen. After clots were well formed, 0.5 ml of reagents such as serum or 0.0025 M calcium chloride in 0.05 M imidazole buffer pH 7.35 (calcium reagent) were added and clots gently rimmed, thus permitting them to float in surrounding fluid for stated length of time (usually 2 hours). Clots were then washed 3 times by adding isotonic saline and then decanting the saline, using a wooden applicator stick to avoid losing the clot. After drying the inside of the test tube, the fibrinolytic agent, or 8 M urea, was added. Lysis or solubility of treated clots (preincubated in serum, etc.) was compared with controls preincubated in calcium reagent or heated serum. When radioactive clots were employed, aliquots of supernatant fluid (fibrinolytic agent or urea) were transferred at indicated time to another plastic tube and radioactivity determined in a well-type scintillation detector, using a Packard Auto-Gamma Spectrometer (Model 410A). The control for elution of radioactivity was a clot prepared in identical manner using buffer rather than fibrinolytic preparation.

Assays for plasminogen, plasmin, activator, and antiplasmin were performed by methods of Ambrus, employing plasminogen-free clots (5).

Results. Inhibition of fibrinolysis occurred when thrombin-fibrinogen clots were incubated in serum prior to exposure to plas-

TABLE I. Inhibition of Fibrinolysis by Preincubation of Clot in Serum: Effect of Duration of Preincubation on Fibrinolysis and on Urea Solubility.

Preincubation time (min)	Lysis time in plasmin (hr)		Dissolution time in 8 M urea (hr)	
	Clots preincubated in		Clots preincubated in	
	Serum	Calcium reagent†	Serum	Calcium reagent†
0	1.75	1.75	.5	.25
5	1.50	2.25	.5	.5
30	6.5 *	3.25	.5	.5
60	9.5 *	2.5	.75	.5
120	16.0 *	2.0	.5	.5

* Small clot remaining.

† .0025 M calcium chloride in .05 M imidazole buffer, pH 7.35.

min (Table I). Two series of test tubes containing identical clots were produced. One series of clots was incubated in 0.5 ml serum, the other in calcium reagent. At indicated intervals, 2 tubes from each series were removed and clots washed. Plasmin was then added to one tube of each series and 8.0 M urea to the other pair of tubes. There was no change in lysis time with increasing periods of preincubation in calcium reagent whereas definite prolongation of lysis time occurred following preincubation in serum. Urea solubility, on the other hand, remained unchanged.

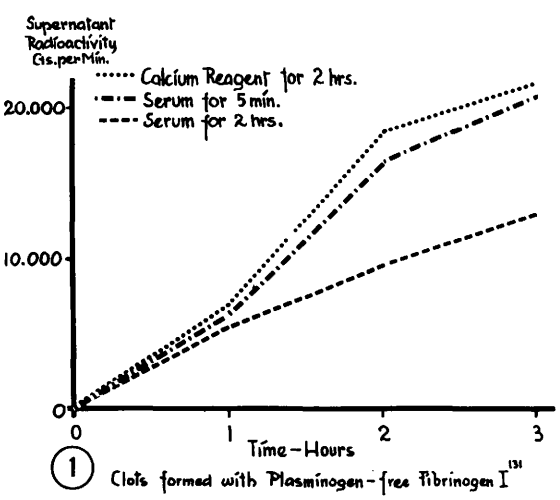
Inhibition of fibrinolysis was also demonstrated with clots produced with I^{131} labeled plasminogen-free fibrinogen (Fig. 1). Following addition of plasmin, aliquots of supernatant were removed hourly for measurement of radioactivity. The inhibition produced by preincubation in serum was reflected by decreased radioactivity. Similar results were obtained with clots produced with crude, plasminogen-contaminated fibrinogen.

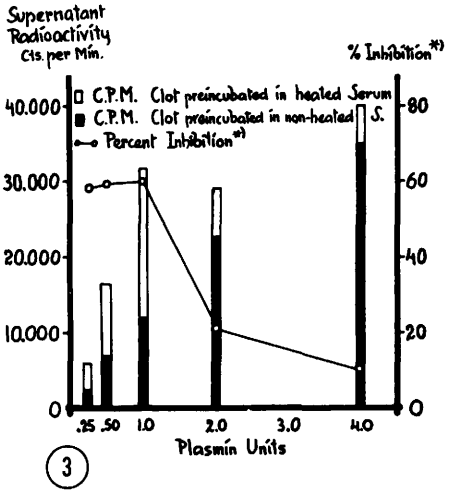
Samples of serum heated at 56°C for various periods were employed for preincubation of clots. Indicated in Fig. 2 is the per cent of fibrinolysis obtained when clots preincubated with serum are compared to maximum lysis obtained with a clot preincubated in calcium reagent. This was determined by radioactivity of supernatants removed after both 2 and 3 hours in contact with clots. Preincubation of clots in nonheated serum was followed by a reduction in fibrinolysis of 40% (60% of maximum). After serum was heated for 60 minutes at 56°C, it no longer had the ability to alter clot sensitivity to lysis and no inhibition was demonstrable (100% lysis).

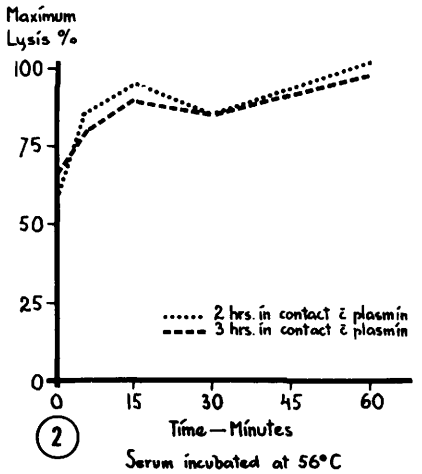
In the experiment depicted in Fig. 3, a series of clots was preincubated for 2 hours, using heated serum rather than calcium reagent as control. Varying concentrations of plasmin were employed as indicated. The inhibitory effect of serum progressively decreased as the activity of plasmin employed was increased over 1.0 RPMI unit/ml. With higher plasmin concentrations (Fig. 4), the inhibitory effect produced by preincubation in serum was further decreased and was completely lost when 10 units/ml or more were employed, despite prolonged 24-hour preincubation.

The possibility was entertained that the inhibitory effect observed in these experiments was due to adsorption of serum antiplasmin onto the clot. Although there is no evidence that this takes place, the following experiments were carried out to distinguish inhibitory from antiplasmin activity. Suggestive, but not conclusive, was the fact that plasmin assays performed on supernatant plasmin at intervals during incubation of pretreated and control clots revealed no increase in plasmin destruction in tubes containing serum-treated clots. Similarities were found in that antiplasmin and inhibitory activity were both: destroyed by heating, inactivated by reducing pH to 2.0 for 15 minutes, absent from euglobulin fraction of serum, and nondialyzable. The inhibitory and antiplasmin activity of serum could be separated, however, by employing albumin and globulin fractions of normal serum, prepared by ammonium sulfate precipitation (Table II). Although these fractions were far from pure, since cross contamination was demonstrable by cellulose acetate electrophoresis, most of the inhibitory activity was found in globulin fraction, while antiplasmin was mainly in albumin fraction.

Other studies revealed that activity necessary to alter clot sensitivity to plasmin was present in oxalated and barium sulfate adsorbed plasma as well as serum. It was sta-







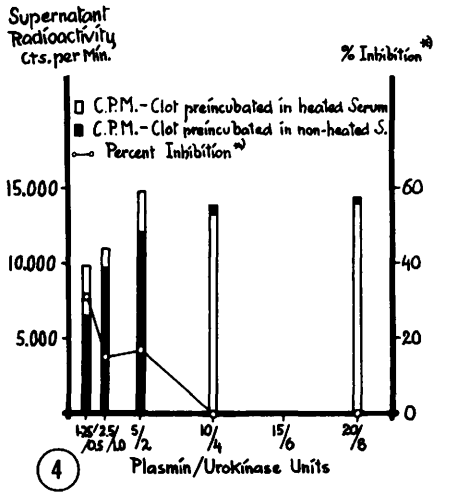


FIG. 1. Inhibition of fibrinolysis: Effect of preincubation of clot in serum. Clots preincubated in calcium reagent for 2 hr or in serum for 5 min or 2 hr. Urokinase activated plasmin 2 RPMI units/ml employed.

FIG. 2. Inhibition of fibrinolysis: Effect of heat on serum activity. Clots formed with plasminogen-free fibrinogen I^{131} clotted with thrombin and preincubated in serum which was previously heated at 56°C for length of time indicated. 0.5 ml of urokinase activated plasmin containing 2 RPMI units plasmin/ml was employed.

FIG. 3. Inhibition of fibrinolysis: Effect of plasmin concentration. Clots preincubated for 2 hr. White bars indicate supernatant radioactivity in counts/min (C.P.M.) of tubes containing clots preincubated in heated serum. Black bars indicate supernatant radioactivity (C.P.M.) of tubes containing clots preincubated in non-heated serum. U.K. activated plasmin (RPMI units) used. Final concentration of urokinase was minute. Per cent inhibition calculated from following formula:

$$\% \text{ inhibition} = 100 - \left[\frac{\begin{matrix}\text{Supernatant radioactivity (C.P.M.)} \\ \text{Clots preincubated in non-heated serum}\end{matrix}}{\begin{matrix}\text{Supernatant radioactivity (C.P.M.)} \\ \text{Clots preincubated in heated serum}\end{matrix}} \right] \times 100.$$

FIG. 4. Inhibition of fibrinolysis: U.K. activated plasmin (RPMI units) used containing minute concentration of urokinase as indicated (Ploug units). See legend Fig. 3.

TABLE II. Inhibition of Fibrinolysis by Preincubation of Clot in Fractions of Human Serum: Comparison with Antiplasmin Activity.

Reagent	Supernatant radioactivity (cpm) after 3 hr	% inhibition by preincubation in reagent	Reagent antiplasmin conc. (RPMI/ml)
Serum	1721	86.4	22.5
Albumin	9465	25.0	14.5
Globulin	2709	78.5	4.9
Calcium	12610		

Clots formed with .5 ml pure (plasminogen-free) fibrinogen I^{131} + .05 ml thrombin. Streptokinase activated plasmin—.5 ml—4 cu S.K./ml was employed. Clots preincubated for 2 hr.

ble at –20°C for at least one year, and at 4°C for at least 4 days.

Discussion. Clots produced by addition of a small amount of thrombin to fibrinogen (thrombin-fibrinogen• clots) are frequently employed for studies of *in vivo* fibrinolysis. When fibrinogen is isotopically tagged with I^{131} (radioactive fibrinogen), the degree of lysis is said to be related to fall in radioactivity over the venous segment containing the thrombus. With this method, it has been demonstrated that fresh clots lyse more readily than those permitted to remain *in situ* for 24 hours prior to plasmin administration(1). Furthermore, with fresh thrombi, maximum fall in radioactivity occurs during the first 12-24 hours after infusion of fibrinolytic agent. This acquired *in vivo* resistance to fibrinolysis has been attributed to clot organization.

An alternate explanation is suggested by the present study. The data clearly indicate that *in vitro* resistance to fibrinolysis can be produced by incubation of thrombin-fibrinogen clots in serum, plasma, barium sulfate adsorbed plasma, or the globulin fraction of serum. The activity responsible for clot resistance to fibrinolysis has also been shown to be non-dialyzable, destroyed by acidification or heat, and separate and distinct from antiplasmin. Furthermore the disappearance rate of plasmin activity in test tubes containing serum-treated and control clots was the same. It is therefore probable that the lack of fibrinolysis is the result of an alteration of the clot itself.

It is known that thrombin-fibrinogen clots differ from clots produced from whole blood or recalcified plasma. The former are soluble and the latter insoluble in weak acids(6) or urea(7). A factor, called fibrin-stabilizing factor(7), or fibrinase(8), has been isolated which can convert soluble to insoluble clots. Although with development of clot-resistance to fibrinolysis there was no change in urea solubility, further work is indicated to definitely separate these mechanisms. It remains possible that fibrin-stabilizing-factor can alter clot-resistance to plasmin in concentrations too small to be easily detected by changes in solubility.

It is tempting to attribute the property of plasma or serum associated with clot-susceptibility to lysis to a distinct factor. Although this may indeed be the case, further work is indicated before this can or cannot be established. At the present, one can only emphasize the importance of recognizing the existence of this phenomenon.

Summary and conclusions. *In vitro* resistance to fibrinolysis was produced by incubation of thrombin-fibrinogen clots in either serum, plasma, barium sulfate adsorbed plasma or the globulin fraction of serum. The activity responsible for clot-resistance to fibrinolysis was non-dialyzable, destroyed by acidification or heat, and distinguishable from serum antiplasmin. Tht data presented suggest that the above effect is mediated by a change in the clot itself rather than an alteration in fibrinolytic mechanism.

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Influence of 2α-Methyl-17β-Hydroxy-5α-Androstan-3-one on Incorporation of Glycine-2-C^{14} into Proteins of a Rat Mammary Fibroadenoma. A Possible Bioassay Method.* (27897)

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Huggins(1,2) and Glenn(3,4,5) and their coworkers suggested a test involving the action of steroids on growth of a transplanted rat mammary fibroadenoma as a means of finding possible useful therapeutic agents for human mammary tumors. Huggins(2) suggested the use of the effective steroid 2α-methyl-17β-hydroxy-5α-androstan-3-one on the basis of this rat test and Glenn *et al.*(5) were able to suggest the active anti-tumor steroid 9α-fluoro-11β, 17α-acetoxy-6α-methylpregna-1, 4-diene-3, 20-dione for human trial at least partly on the basis of its activity against the rat mammary fibroadenoma. This communication reports an assay procedure both more rapid and more efficient than those previously available. This new test uses the end-point of glycine-2-C^{14} incorporation into the tumor proteins.

Method. Aged Sprague-Dawley rats bearing spontaneous mammary fibroadenomas obtained from the Sprague-Dawley Co. (Madison, Wisc.) and a transplanted tumor kindly furnished by Dr. E. Myles Glenn, Upjohn Co., Kalamazoo, Mich., served as the starting tumors.

The transplantation technic of Glenn *et al.* (3,4) was followed with few modifications. Female Sprague-Dawley rats, 51 ± 2 days old and weighing 140 ± 15 g were anesthetized with ether and through a dorsal incision, channels were made in the subcutaneous connective tissue with surgical forceps about ½ inch caudal to each front leg. Seeds weighing 70 ± 20 mg were prepared with a nasal cutting forceps from a parent tumor, which would afford enough seed tumors for a complete experiment. Transplants soaked in either saline or Eagle's Medium were quickly inserted into previously made channels and the incision closed with surgical wound clips. The animals were kept at constant temperature and fed Purina Lab Chow *ad libitum* and tap water.

Rats with suitable sized tumors, usually 30 or 35 days after transplantation, were randomized and divided into experimental groups. The test compounds were suspended in an aqueous suspending medium consisting of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethyl cellulose (0.5%), and benzyl alcohol (0.9%) and injected subcutaneously once daily usually for 14 days. The daily dose was contained in 0.5 ml of medium. Control animals received only the vehicle and were run in each experiment in parallel with the test compound. On the final day of treatment, glycine-2-C^{14} in 0.5 ml of saline per rat was injected intraperitoneally and food was removed. Twenty-four hours later tumors were removed, weighed, and processed for the isolation and counting of the protein.

Tumors were frozen in liquid nitrogen and pulverized in a stainless steel mortar. The crushed tissue was washed successively with 5% trichloracetic acid at 90°C for 15 minutes, 5% trichloracetic acid at room temperature, 80% ethanol, 95% ethanol, 95% etha-

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