Biochemistry VOLUME 3, NUMBER 3

March 13, 1964

Native Plasminogen*

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Received September 10, 1963

Native plasminogen is a glyco euglobulin isolated from Cohn's fraction III by adsorption on Sephadex or tricalcium phosphate and eluted with basic buffers containing lysine or ε-aminocaproic acid. Acid treatment converts it without loss of activity into a pseudoglobulin, apparently affecting only the secondary and tertiary structures. Both forms have the same high caseinolytic activity, but the fibrinolytic activity of the native plasminogen is 50% higher than that of the pseudoglobulin. Native plasminogen consists of about 92% protein, containing —S—S— groups but no —SH groups, 5% hexose, 2.8% hexosamines, and 0.6% sialic acid. The hexose and hexosamines seem to be essential for the activity, but the sialic acid does not.

Native plasminogen with the solubility characteristics of a euglobulin was isolated from Cohn's fraction III by chromatography on Sephadex, employing lysine-containing basic buffers (Slotta *et al.*, 1962). If this native plasminogen is treated with acid (Kline, 1953), a typical pseudoglobulin is formed which is insoluble in isotonic salt solutions in the physiological *p*H range. Neither the euglobulin nor the pseudoglobulin form of the plasminogen prepared in this way was very active, nor was there enough material available for us to carry out all necessary analyses.

Fortunately, however, sufficient quantities of both plasminogens have become available to us since the end of 1961. They were prepared from large amounts of plasma proteins by more elaborate adsorption and elution techniques (Hink and McDonald, 1962a) and showed a steady increase in purity. The ultracentrifuge sedimentation patterns indicated a single component. The best preparations of native plasminogen give only one band in electrophoresis on acetate-cellulose paper. Notwithstanding these facts, the presence of traces of other proteins cannot be excluded. It is also possible that plasminogen consists of a protein complex, formed by subunits. Nevertheless, we compared this native euglobulin plasminogen with its corresponding pseudoglobulin form and determined its chemical composition with regard to its protein and carbohydrate components.

METHODS AND RESULTS

Preparation of Euglobulin and Pseudoglobulin.—Each kg of human plasma fraction III-2,3 paste (Oncley *et al.*, 1949) was washed with 20 liters of 0.02 M sodium acetate at *p*H 6.25. A similar procedure for the removal of prothrombin and lipoproteins had been previously used by others (Sgouris *et al.*, 1960). To the solution of 1 kg of the washed protein paste in 0.3 M glycine buffer at *p*H 7.9, 5 kg tricalcium phosphate (Mallinckrodt) was added; this resulted in the adsorption of less than 20% of the proteins including, however, the total content of plasminogen. The native euglobulin plasminogen was eluted with a solution of 0.2 M sodium chloride and 0.2 M ε-aminocaproic acid at *p*H 9. Then it was precipitated with 2.6 M ammonium sulfate at *p*H 8 and the occluded ammonium sulfate was removed by dialysis against 0.05 M ammonium carbonate at *p*H 7.6. Traces of accessory proteins could be removed by chromatography on DEAE-cellulose. After freeze-drying, about two-thirds of the total plasminogen was obtained as euglobulin.

Even though the whole process of isolation was carried out very carefully at +4°, part of the native proenzyme was always transformed into the pseudoglobulin form during the washing and adsorption process. The pseudoglobulin plasminogen was finally eluted from the calcium phosphate with 0.02 M phosphoric acid. This solution was brought to *p*H 2.1, made 1.2 M sodium chloride, and dialyzed against water at *p*H 3.7. By freeze-drying the pseudoglobulin, plasminogen was obtained. Figure 1 summarizes the whole process.

Activity of Plasminogen Preparations.—The method for the determination of the caseinolytic activity described earlier (Slotta *et al.*, 1962) has been further modified so that it can be employed accurately with small quantities of plasminogen. Our best results were obtained by using casein washed with water, alcohol, and ether (Norman, 1957). The 5% solution in 0.1 M Tris buffer, *p*H 8.6, was tested by dilution 2:5 with buffer and precipitation with the same volume of 10% trichloroacetic acid solution. The supernatant should not give a higher OD than 0.100 at 280 mμ.

Different amounts of plasminogen solution in Tris buffer, *p*H 8.6, were brought to 1.0 ml with the same buffer; then 0.2 ml streptokinase (=2000 units) and after 3 minutes 0.8 ml 5% casein solution were added and the mixture was kept in a water bath of 37° for exactly 30 minutes. The casein thus precipitated with 2 ml 10% trichloroacetic acid solution was removed by centrifugation and the OD of the supernatant measured in a 1.2-ml cuvet in the Beckman spectrophotom-

* This investigation was supported by a grant (HE-04889-04) from the National Institutes of Health, U. S. Public Health Service.

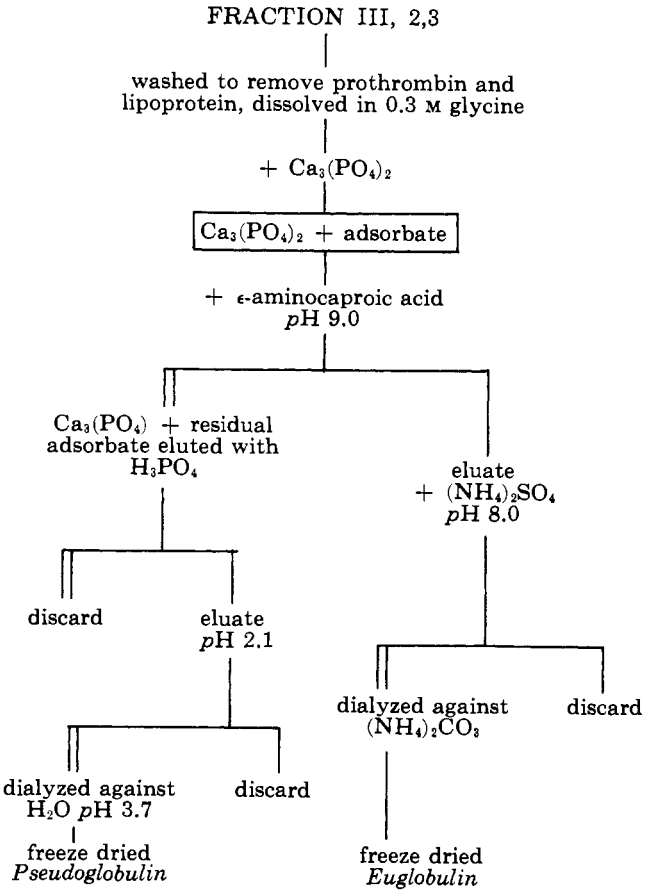

Fig. 1.—Preparation of plasminogen. Work done by J. H. Hink, Jr. and J. K. McDonald at the Cutter Laboratories, Berkeley, Calif., 1962a.

eter at 280 mμ. A blank was prepared in the same manner, but precipitated immediately after addition of casein. Under these conditions, the concentrations of streptokinase (=2000 units) and casein (0.80 ml 5% casein solution = 40 mg) are constant; the only variable is the amount of plasminogen. By definition, one unit of activity is the quantity of plasminogen or plasmin which provides an increase of 10^{-3} absorbancy unit under the conditions described above.

$$OD_{30\ min} - OD_{0\ min} = OD\ activity$$

$$\frac{OD_{activity} \times 1{,}000}{mg\ plasminogen} = Miami\ units/mg = specific\ activity$$

This type of unit will be designated as "Miami unit" in cases where specification is advisable.

The same test but without addition of streptokinase can be applied to determine the plasmin content. It is known that plasminogen undergoes slow, spontaneous activation in 50% glycerol at neutral *p*H and that both plasminogen and plasmin are stabilized in this solution (Alkjaersig *et al.*, 1958a). When a solution of plasminogen (608-52)[1] was left standing in Tris buffer, *p*H 8.5, at room temperature for 3 days, the total activity decreased from 1370 units to 1000 after 1 day, and to 125 after 3 days. In the same buffer, but mixed with glycerol 1:1, the following values were found:

After 6 hours	Plasmin	55	Total 1,255 units
29		100	1,270
120		1,175	1,210
191		1,020	1,045

[1] The numbers represent the original numbers of different preparations of plasminogen obtained by the technique described above.

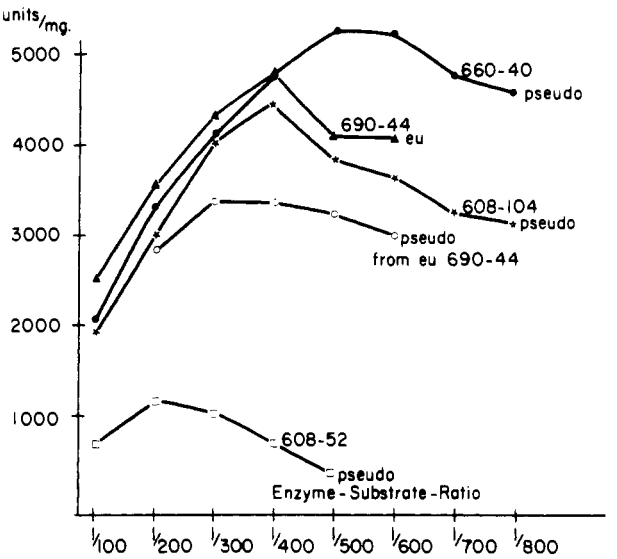

FIG. 2.—Activities of euglobulin and pseudoglobulin plasminogen.

Thus the autocatalytic transformation of plasminogen also takes place at *p*H 8.5, and plasmin is surprisingly stable in this buffer-glycerol mixture.

Some of the plasminogen preparations were not completely dissolved or remained in the form of very light suspensions. In these cases the determination of the activity was more successful after the substance had been dissolved in 3 M urea. This was particularly striking in one of the pseudoglobulins (608-52), whose activity rose from 1200 to 1800 units/mg. One euglobulin (690-33), dissolved in 0.15 M sodium chloride, left a residue with only 3500 units/mg equally active as the plasminogen contained in the supernatant. When the substance was dissolved in 3 M urea, however, its activity rose to 5320 units/mg. Not even after the material had stood in the cold for 27 hours did an appreciable decrease of activity occur; 5000 units/mg were found. Thus urea does not destroy plasminogen, but on the contrary increases its solubility, thereby allowing plasminogen preparations that are difficult to dissolve to become more active.

According to our previous findings (Slotta *et al.*, 1962) the specific activity of a plasminogen preparation depends definitely on the ratio of proenzyme to substrate (plasminogen to casein). Since we always use 0.8 ml 5% casein solution, containing 40 mg of casein, the ratio is *x* mg plasminogen to 40 mg casein; e.g., we obtained the units for the ratio 1:200 by using 0.2 mg plasminogen and those for the ratio 1:500 by using 0.08 mg plasminogen per tube.

With increasing activity of the preparations the specific activity not only increases but the maxima of the activities shift to smaller ratios. Figure 2 shows the activity curves of pseudoglobulin and euglobulin plasminogen preparations. For preparation 608-52 the maximum of activity lies at 1:200, for 608-104 at 1:400, and for 660-40 at 1:500–1:600.

If a fibrin clot prepared from plasminogen-free fibrinogen (Hink and McDonald, 1962b), rather than casein, is used for the substrate determination of the activity of pseudoglobulin and euglobulin, a considerable difference appears: the native plasminogen is much more active than the pseudoglobulin. Pseudoglobulin had only 158 RPMI[2] units/mg nitrogen whereas

[2] One RPMI unit is the fibrinolytic activity which dissolves a standard clot made from plasminogen-free human fibrinogen in two minutes at 45° and under the conditions specified by Ambrus *et al.*, 1960.

TABLE I
ANALYTICAL DATA OF PLASMINOGENS

	Percentage of Components	
	Pseudoglobulin 660-40	Euglobulin 690-44
Water (60°)	6.1–6.3	
Water (110°)	8.5	10.5
N (dry)	14.5	14.9
Protein (% N × 6.25)	91	93
Hexoses	5.0	5.2
Hexosamines	2.8	
Sialic acid	0.6	0.6
S	1.76	1.76
Activity in Caseinolytic units:		
Miami (per mg protein)	5000	4750
Remmert-Cohen (per mg N)	75	78
Fibrinolytic units:		
RPMI (per mg N)	158	247

the best euglobulin plasminogen preparations had 247 and 278 RPMI units per mg nitrogen (Table I).

Chemical Analyses.—The preparations earlier obtained by Kline's method (Kline, 1953) contained varying amounts of *phosphorus*. We found 0.02%, 0.12%, and in one instance even 1.69% phosphorus. This is not, however, a component of plasminogen but a contamination due to dialysis against phosphate buffer as the last step of this technique.

To determine the *sulfur* content of pseudoglobulin and euglobulin plasminogen about 5 mg substance was combusted in the Schöniger oxygen flask and the sulfate was titrated with barium perchlorate (Alicino, 1958). The different preparations all had similar sulfur contents. In the most active plasminogen preparations 1.8, 1.8, 1.7, 1.8, and 1.7% were found (Table I).

The sulfur determinations became even more important because the amino acid analyses of hydrolysates resulted in varying values for half-cystine.

For every analysis the preparations had to be dried over phosphorus pentoxide at 110°. By this procedure they lost not only 8.5–10.5% *water* but also their solubility and activity. When the very active pseudoglobulin preparation 660-40 was dried at 60° it lost 6.1–6.29% water but retained about half its activity.

Nitrogen determinations (micro-Kjeldahl) gave 14.5% nitrogen for dry pseudoglobulin plasminogen 660-40 and 14.9% nitrogen for euglobulin plasminogen 690-44.

To determine the *amino acid* composition of the protein part, 5-mg amounts of the various plasminogen preparations were hydrolyzed in 5 ml constant-boiling hydrochloric acid for 24 hours at 110°. If the solution and the tube were not free of oxygen, 27 moles cysteic acid per 10^5 g plasminogen were found. Therefore the solutions were freed of oxygen by evacuation, and the hydrolysis was carried out *in vacuo*. Even then 6 moles cysteic acid were found. However, after the solution had been frozen and thawed three times in high vacuum and hydrolysis had been carried out *in vacuo*, no trace of cysteic acid could be detected. Thus we found no thiol groups. This also corresponds to all other experiments with plasminogen solutions with and without urea.

Table II shows average values of amino acid analyses of pseudoglobulin and euglobulin plasminogen preparations (Moore *et al.*, 1958). Only about five values

TABLE II
MOLES AMINO ACID PER 10^5 g PLASMINOGEN

	Pseudoglobulin 660-40	Euglobulin 690-44	Oxidized 690-44
Lys	43	43	41
His	22	21	19
Arg	39	36	34
Asp	77	77	76
Thr	55	53	52
Ser	54	54	49
Glu	77	82	80
Pro	70	63	61
Gly	60	53	45
Ala	36	36	26
½ Cys[a]	42	40	0
½ Cys[b]	46	46	0
Val	47	41	45
Met	9	9	0
Iso	22	21	22
Leu	50	50	43
Tyr	32	26	21
Phe	23	24	22
Cysteic acid			35
Methionine sulfone			7

[a] Widely varying. [b] Calculated by subtracting methionine sulfur from total sulfur on the assumption that the difference represents the cystine sulfur.

deviate considerably; all others are equal or nearly so.

As was to be expected, we only found small amounts of tryptophan. Minimal quantities of galactosamine and slightly higher ones of glucosamine had escaped destruction every time. Aside from the usual amino acids only a small amount of alloisoleucine was found. Since the cystine curves were not conclusive, euglobulin plasminogen was oxidized with performic acid (Schram *et al.*, 1954), converting methionine into its sulfone and cystine into cysteic acid. However, hydrobromic acid was not applied to remove the excess performic acid (Moore, 1963) and therefore only 35 moles cysteic acid and 7 moles methionine-sulfone were found. This means that over 10% of the sulfur-containing substances had deen destroyed.

Carbohydrate Content.—It had been previously established (Alkjaersig *et al.*, 1958b) that 1.1% carbohydrate, estimated as glucose, is present in plasminogen. Seven per cent of this carbohydrate was found to be in the trichloroacetic acid–soluble fraction of plasminogen. We also found 1.0 and 1.2% carbohydrate in both plasminogen forms with the anthrone reaction. We found, however, considerably higher values after heating 3-mg samples in 2 ml 0.1 N HCl in sealed tubes in a boiling water bath for varying periods of time. Pseudoglobulin as well as euglobulin plasminogen proved to contain 4–5% hexose after heating for 4–6 hours with the anthrone and the Folin-Wu reactions. These experiments with the two plasminogen forms were checked with the orcinol-H_2SO_4 reaction (Winzler, 1955). After the samples were heated for 3 hours, 5.0 and 5.1% hexose were found for the pseudoglobulin plasminogen 660-40 and 5.24% for the euglobulin plasminogen 690-44.

To determine the *hexosamine* content, 10 mg of plasminogen 660-40 in 10 ml 2 N HCl was hydrolyzed for 4 hours in a boiling water bath, the solution was evaporated to dryness, and the residue was dissolved in 2 ml water. The hexosamine content was 2.8% with the method of Elson-Morgan (Winzler, 1955). Next we determined the *sialic acid* content, using the extraction method with butylacetate (Miettinen and Takki-Luukkainen, 1959); it gave a value of 0.6%.

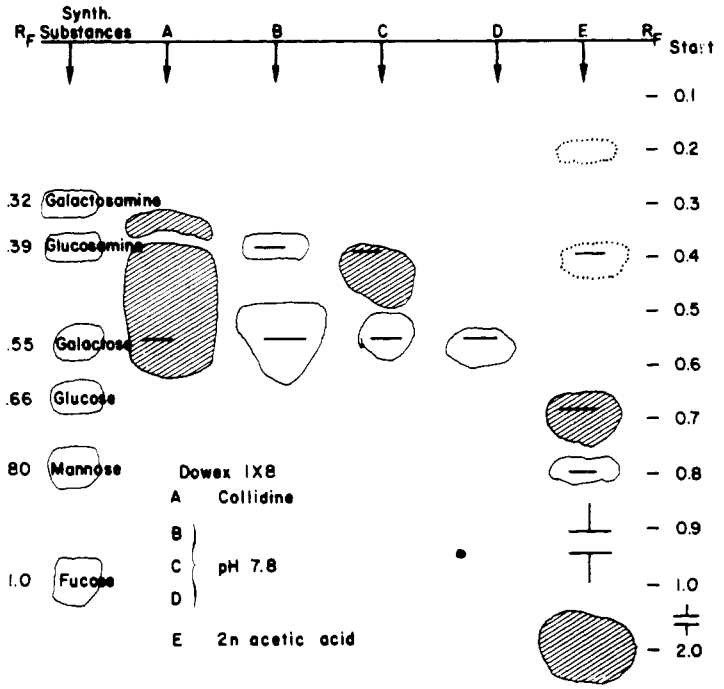

FIG. 3.—Chromatogram according to Bourrillon and Michon. Carbohydrates from euglobulin plasminogen 690-44 hydrolysate, separated on a Dowex 1 × 8 column. A = collidine; B, C, D = buffer, *p*H 7.8; E = 2 N acetic acid.

Thus plasminogen contained, aside from 91% protein, a total of 8.6% carbohydrate.

In another experiment 10 mg pseudoglobulin plasminogen 660-40 with an activity of 5000 Miami units/mg was stirred in 2 ml of an aqueous solution of 10 mg potassium periodate at room temperature. After dialysis against water and freeze-drying the plasminogen preparation lost some of its water solubility and became absolutely inactive. However it is difficult to decide whether this loss of activity is due only to the destruction of the carbohydrates.

Euglobulin plasminogen 690-44, 25 mg, was heated for 1 hour at 80° in 10 ml 0.025 N H_2SO_4 and was then precipitated with 75% alcohol. Supernatant and washings were evaporated, the residue was dissolved in 4 ml water, and sialic acid was determined. As before, we found 0.6%, which shows that all sialic acid had been already split off with 0.025 N H_2SO_4 (Spiro, 1960). This corresponds to the fact that we did not find any sialic acid in the precipitated plasminogen when we repeated the hydrolysis with 0.1 N H_2SO_4. The activity of sialic acid–free plasminogen was the same as that of the starting material, namely 4500 units/mg at the ratio of 1:400.

The neuraminidase at our disposal contained activity of 100 units/ml in 0.05 M sodium acetate buffer, *p*H 5.5, with 0.9 g NaCl and 0.1 g $CaCl_2$ per 100 ml. We therefore tried to determine whether plasminogen would lose any activity in this buffer when heated at 37° for 30 minutes. This was not the case. Thereupon, 2 mg euglobulin plasminogen 690-44 was incubated for 30 minutes at 37° with 0.2 ml neuraminidase solution. Plasminogen kept its full activity also after being exposed to neuraminidase.

In the course of oxidation with performic acid the hexosamines are almost completely destroyed. With the Elson-Morgan reaction we did not find any hexosamine in the hydrolysate of the oxidation product of plasminogen. The hexoses, however, were still present at 4.1%. When plasminogen, oxidized by performic acid, was extracted with water several times, the solution contained after evaporation only half the hexoses. The residue dissolved in 0.01 N NaOH contained the other half. These experiments with oxidized plasminogen showed that the undestroyed carbohydrates were only partly free. About half of them remained firmly bound to acidic polypeptides. This is why we hydrolyzed plasminogen with acid and in another experiment with pepsin, followed by trypsin. The carbohydrates thus freed as well as those bound to peptides were investigated.

Pseudoglobulin plasminogen 660-40, 30 mg, was hydrolyzed with 10 ml 1 N HCl for 3 hours in a boiling water bath. After removal of the acid by distillation, the residue was dissolved in 2 ml of a 0.1 N NH_4OH solution containing 1% collidine. The solution was applied to a column of 10 ml Dowex 1 × 8 (200–400 mesh) and eluted with 40 ml of the collidine solution. This solution was evaporated and the residue was dissolved in 1 ml water. The solution contained *0.9 mg hexoses* (orcinol-H_2SO_4) = *3%* and *0.83% hexosamines* (Elson-Morgan). The polypeptides retained in the column were eluted with 1% collidine, collidine–acetic acid buffer, *p*H 7.8, 0.1 N acetic acid, 2 N acetic, then glacial acetic acid. Only those fractions which had been collected at *p*H 7.8 (neutral peptides) and those with acetic acid (acidic peptides) gave positive reactions with aniline phthalate.

Euglobulin plasminogen 690-44, 30 mg, was dissolved in 3 ml 0.2 N HCl and digested with 0.3 mg twice-crystallized pepsin at 52° for 2 hours. The solution was heated for 10 minutes to above 90°. After the solution was cooled and the *p*H was adjusted to 8.6, 0.3 mg twice-crystallized trypsin was added and the solution was kept at 37° for 16 hours. The solution was evaporated and the residue was separated in the same manner as in the preceding experiment on the anion-exchange resin column. Two-ml fractions were jointly evaporated and the carbohydrates contained therein were determined by paper chromatography (Bourrillon and Michon, 1959); (see Table III and Figure 3).

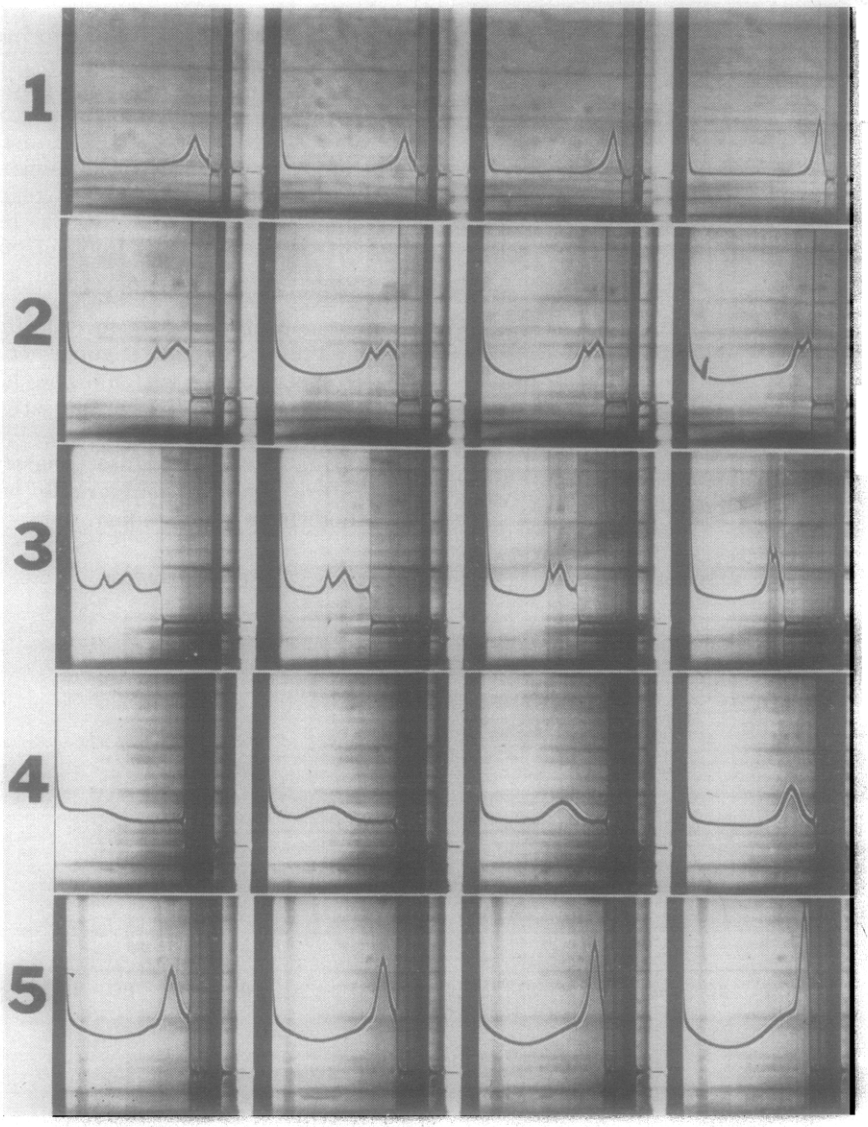

FIG. 4.—Ultracentrifugal pattern of pseudoglobulin plasminogen (608-52) and euglobulin plasminogen (690-33). (1) Pseudoglobulin in water; (2) in 8 M urea; (3) in 15% glycerol; (4) euglobulin in 0.15 M NaCl; (5) in 8 M urea.

TABLE III

SEPARATION OF CARBOHYDRATES AND PEPTIDES ON AN ANION-EXCHANGE COLUMN

Fraction	Tubes	Eluted by	Carbohydrate Found
A	1–28	0.1 N NH_4OH + 1% collidine	galactosamine + glucosamine + galactose + glucose
B	29–36	*p*H 7.8	glucosamine + galactose
C	38–44	*p*H 7.8	much glucosamine, some galactose
D	49–72	*p*H 7.8	some galactose
E	89–115	0.1 N acetic acid	much glucose, trace of mannose, and unidentified sugar
	116–142	0.1 N acetic acid	
	148–187	2 N acetic acid	

Transformation of Euglobulin Plasminogen into Pseudoglobulin Plasminogen.—According to Kline's method (Kline, 1953) 25 mg euglobulin plasminogen 690-44 was stirred with 2 ml 0.05 N H_2SO_4 for 10 minutes. The clear solution was brought to *p*H 11 with 1 N NaOH and immediately to *p*H 5.3 with 1 N HCl. After standing for 3 hours in the refrigerator the solution, together with the precipitate, was brought to *p*H 2 with 1 N HCl. The clear solution was dialyzed overnight against water. Plasminogen did not precipitate from the neutral solution. After freeze-drying the solution, 15 mg pseudoglobulin plasminogen was obtained. Figure 2 shows the activity curve of the transformation product compared to the curve obtained with the starting material. It is interesting to note that the activity curve of the transformation product is the same whether it has been dissolved in water or in 3 M urea.

Physical Chemical Characterization of the Two Plasminogen Forms.—Figure 4 shows ultracentrifugal patterns of pseudoglobulin and euglobulin plasminogen in water, urea, glycerol, and saline; Figure 5 shows that of euglobulin 690-44. While in the curves of the previous euglobulin plasminogen preparations 690-33 a very small secondary peak beside the main peak can be distinguished (Figure 4, no. 5), the patterns of euglobulin plasminogen 690-44 show only one component with a sedimentation constant of 4.1.

Measurements of viscosity and analytical sedimentation on solutions of pseudoglobulin plasminogen

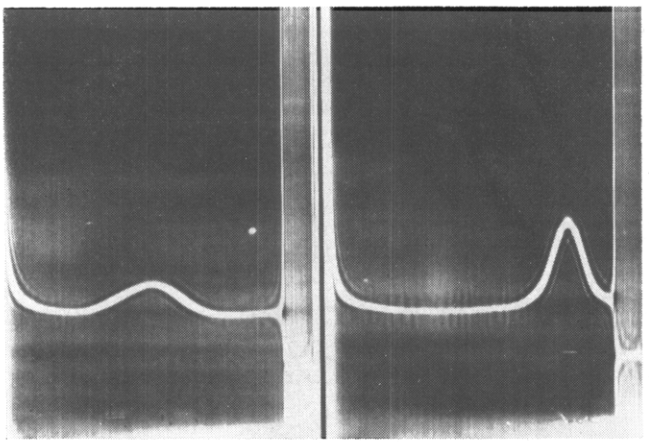

FIG. 5.—Ultracentrifugal pattern of euglobulin plasminogen (690-44) in 1% solution in 0.15 M NaCl. Pattern at 32 min (left) and 96 min (right) after the ultracentrifuge reached full speed of 52,640 rpm.

Using these "Miami units" we found about 5000 units/mg protein for both forms. The 14.5% and 14.9% nitrogen, which they contain, thus corresponds to 72.5 and 75 units/mg nitrogen. The same preparations gave 75 and 78 units/mg nitrogen by another caseinolytic test method (Remmert and Cohen, 1949). For the "crystalline plasminogen" an activity of 56 units/mg nitrogen has been reported (Kline, 1953). Others (Alkjaersig *et al.*, 1958a) refer to 60–100 units/mg nitrogen and in still another publication (Alkjaersig *et al.*, 1958c) to 100–150 casein units/mg of tyrosine. Recently, methods to obtain preparations of much higher activity have been described (Kline and Fishman, 1961). Others (Sgouris *et al.*, 1960) also prepared plasminogen with a higher activity, but preferred to carry out their further investigations with pseudoglobulin preparations of an average activity of only 76.2 units/mg nitrogen. Unfortunately, many data in the literature are not comparable because different test methods have been applied.

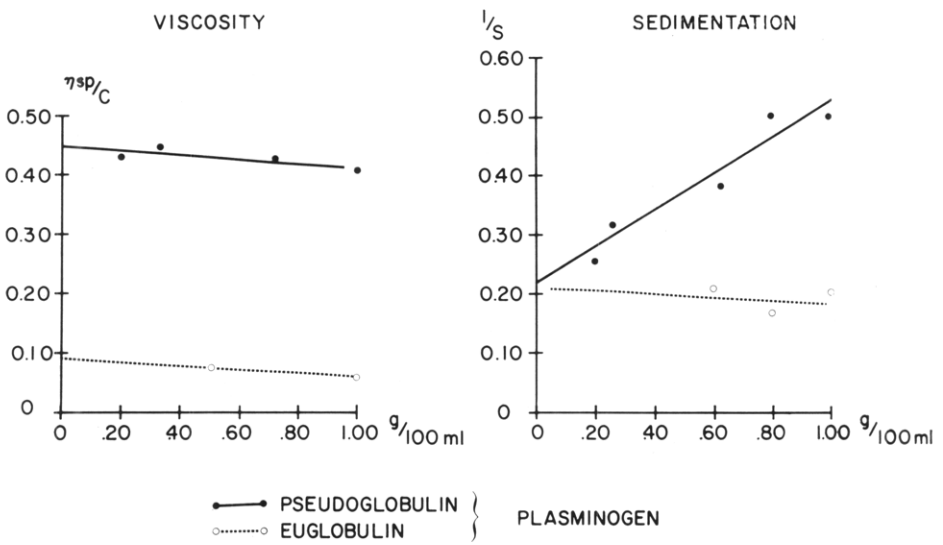

FIG. 6.—Intrinsic viscosity and sedimentation of pseudoglobulin plasminogen (660-40) and euglobulin plasminogen (690-44).

660-40 in water and of euglobulin plasminogen 690-44 in 0.15 M NaCl resulted in the values compiled in Figure 6.

DISCUSSION

The plasminogen preparations studied by us correspond in activity very closely to those prepared in other laboratories. This is particularly the case for the pseudoglobulin form. The test method employed for the determination of the caseinolytic activity of the plasminogen has been described before (Slotta *et al.* 1962). We did not preincubate plasminogen with streptokinase, because the reaction requires less than 2 minutes. We limited the reaction time to 30 minutes because we found that the rate of enzyme action against digestion time is linear under the chosen conditions, as long as the reaction does not exceed 30 minutes. We relate the activity directly to the increase of absorbancy (Hagen *et al.*, 1960) because the latter is not produced solely by the tyrosine content of the supernatant. One advantage of our method is that by measuring the activity of each preparation at different enzyme-substrate ratios we obtain distinctive curves (Figure 2). Their maxima were found in the case of the more active plasminogens at lower enzyme-substrate ratios and vice versa.

The fact that euglobulin plasminogen has a higher specific activity in the RPMI test than its pseudoglobulin form was at first a surprise. On the other hand this is understandable considering that fibrin is the natural substrate for the plasminogen-plasmin system. The reason we prefer the caseinolytic to the fibrinolytic test, even for the native plasminogen, is a purely practical one. These determinations in Miami units are reproducible within 5%, an accuracy which we could not achieve with any other technique.

Neither pseudoglobulin nor euglobulin contain thiol groups and the sulfur is only cystine and methionine sulfur. The plasminogen contains about 23 cystine residues in 10^5 g and a minimum molecular weight of 11,000 can be deduced from the methionine content.

We first noticed the relatively high carbohydrate content when we performed electrophoresis on cellulose acetate paper. In parallel experiments one strip was stained with Ponceau S, the other, after periodate oxidation, with Schiff's reagent. A Schiff positive band and the protein band appeared always in identical positions. Several experiments prove that the carbohydrates are bound to the protein in very different ways and on different sites. The small amount of sialic acid could be split off with acid or by enzymic means without any decrease in activity. At the same time, considerable amounts of glucose and glucosamine

remained bound to the polypeptides after acidic and enzymatic hydrolysis.

We are now able to say more about the difference between the two plasminogen forms. They are not only different in regard to their solubility but also in their activity against fibrin. Native plasminogen represents the labile form which can be easily and irreversibly transformed into the stabile pseudoglobulin form. This probably happens partially during the process of preparation. Each of the forms behaves as a uniform protein in electrophoresis and column chromatography.

The sedimentation data show that pseudoglobulin is present as a molecular aggregate at higher concentrations. At infinitely low concentrations both curves of sedimentation values extrapolate to the same point. This means that under these conditions the molecular weight of both forms is the same.

The viscosity curves do not extrapolate to the same value because the two forms, notwithstanding their identical molecular weight, have different shapes. The transformation of euglobulin to pseudoglobulin by acid treatment can be visualized as an opening of the structure in such a way that shape and size of the molecule are modified.

Acknowledgments

We are greatly indebted to Cutter Laboratories, Berkeley, Calif., for these valuable substances. We are further obliged to Behring Werke, Marburg, Germany for neuraminidase and to Lederle Laboratories, Pearl River, N. Y. for streptokinase (Varidase). We are very grateful to Dr. H. R. Elden (Howard Hughes Institute for Medical Research, Miami, Florida) for the determination of viscosity and sedimentation of the plasminogens and to Mr. R. F. Wehr for the amino acid determination by column chromatography of many plasminogen hydrolysates.

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The Dissociation of Hemoglobin by Inorganic Salts*

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Received September 30, 1963

The dissociation of hemoglobin into half-molecules by NaCl, $CaCl_2$, $MgCl_2$, and $(NH_4)_2SO_4$ has been studied by measurement of the sedimentation velocity as a function of salt concentration. It is established that dissociation is in fact responsible for the observed decrease in sedimentation velocity, and that the reaction shows the expected dependence on protein concentration. The dissociation is apparently unaffected by changes in the nature of the heme iron atom, in contrast to unfolding of the protein, which depends strongly on the state of the heme group. Thermodynamic data indicate that stabilization of the native four-chain structure in dilute salt solutions is predominantly entropic rather than energetic.

Hemoglobin may be dissociated into half-molecules by many reagents. A study of the relative effectiveness of some of these reagents has been initiated in this laboratory. The results of this study will be compared with the relative effectiveness of the same or similar reagents in producing unfolding of globular proteins and in solubilizing amino acids and small peptides. In this way it is hoped to determine what chemical groups are exposed in the dissociation of hemoglobin, and to determine what forces hold it together in its native state. This information can then be compared with similar knowledge gained from the study of the unfolding of a variety of proteins. It is to be expected that the forces which hold the folded polypeptide chains of hemoglobin together will not be unique, but that the same forces will be found to be important in maintaining the native structure of many globular proteins.

* Abstracted from the dissertation of A. G. Kirshner, Duke University, 1963. A preliminary report has been published (Grossman and Tanford, 1962). This investigation was supported by a Public Health Service research career program award (GM-K6-14,222), by a research grant (AM-04576) from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service, and by a research grant (G-17477) from the National Science Foundation.