off, and the moist precipitate reduced as before by using one-fifth the quantity of reagents. Filtrates from the two reductions were combined, extracted with one 300-cc. and three 200-cc. portions of ether, and the ether extract washed with two 25-cc. portions of water. The ether was distilled off and the residue crystallized from 75 cc. of boiling water. The yield was 0.90 g. of pale-yellow, lens-shaped, thin plates, m. p. 228–232° (dec.), which gave a deep purple-red color on addition of zinc and hydrochloric acid to their alcoholic solution. The yellow substance (possibly the corresponding chalcone) was difficult to remove, and several recrystallizations from water and aqueous alcohol were necessary. The purified material crystallized in elongated hexagonal plates or in needles (the two crystalline forms were interconvertible and probably were two different hydrates), m. p. 238–241° and mixed m. p. with the racemized natural flavanone 238–241° (dec.).

Anal. Calculated for $C_{15}H_{12}O_7$: C, 59.21; H, 3.95. Found: C, 58.85; H, 4.04.

Coigue Flavanones.—Coigue heartwood (*Nothofagus dombeyi*) when processed by the same general methods as described for Douglas-fir, gave a mixture of two flavanones. One, separated by its very low solubility in water, crystallized from aqueous alcohol in colorless needles, m. p. 252–253° (dec.) (literature[27] for naringenin 251°). Mixed m. p. with naringenin 252–253°.

An alcoholic solution gave a cherry-red color with magnesium and hydrochloric acid, but not with zinc and hydrochloric acid.

Anal. Calculated for naringenin $C_{15}H_{12}O_5$: C, 66.18; H, 4.41. Found: C, 65.46; H, 4.62.

The second more soluble flavanone (the 3-hydroxyflavanones appear to be considerably more soluble in water and alcohol than the corresponding 3-desoxy compounds) crystallized from 300 parts of water as a hydrate in the form of colorless needles, m. p. 237–241° (dec.), $[\alpha]^{20}D$ +45° (*c*, 4 in equal volumes acetone and water).

Anal. Calculated for $C_{15}H_{12}O_6$: C, 62.50; H, 4.17. Found: C, 62.60; H, 4.18. Calculated for the hydrate $C_{15}H_{12}O_6 \cdot 1.5H_2O$: H_2O, 8.57. Found: H_2O, 8.37.

Zinc and hydrochloric acid gave a cherry-red color with an alcoholic solution; and ferric chloride, either in aqueous or alcoholic solution, produced a dull orange color. When reduced with zinc dust and hydrochloric acid, as described with Douglas-fir flavanone, the purified crystals, m. p. 249–251° (dec.), showed no depression in m. p. when mixed with an authentic sample of naringenin. When oxidized with air in sulfuric acid solution, yellow spherocrystals, m. p. 270° softens, 279° melts (literature[28] for kaempferol 276–278°), resulted.

Cherry Flavanones.—With cherry heartwood (*Prunus serotina*) two flavanones were isolated that gave undepressed mixed melting points with the respective compounds described under coigue. The whole extract, however, gave a more purplish tint with zinc and hydrochloric acid than the isolated flavanone, indicating one or more additional 3-hydroxyflavanones to be present.

A crystalline material was recovered along with the flavanones that gave no reduction color, but produced an emerald green with ferric chloride, gave a phlobaphene-like substance with boiling mineral acids, and the wood-splint reaction with hydrochloric acid. The hydrate (needles containing 19.8 % water) had a m. p. of 94–96°; anhydrous material m. p. 173–176°, $[\alpha]^{27}D$ +14 (approximately) (*c*, 3 in equal volumes of acetone and water). These properties correspond to *d*-catechin, with which the compound gave an undepressed mixed melting point.

Pulping Studies

Small-scale sulfite pulping experiments were made, using unextracted and extracted Douglas-fir heartwood, Douglas-fir sapwood, spruce with the Douglas-fir flavanone added to the cooking liquor, and spruce impregnated with the Douglas-fir flavanone. The results indicated that the flavanone in Douglas-fir heartwood is an important factor in the resistance of this species to sulfite pulping.

Summary

1. A new flavanone, the 3,3′,4′,5,7-pentahydroxy derivative, was isolated from Douglas-fir heartwood.

2. The flavanone was oxidized to quercetin by air and reduced to eriodictyol with zinc dust and hydrochloric acid. Preparation of the racemic flavanone was accomplished by reduction of quercetin with sodium hydrosulfite.

3. Naringenin and another new flavanone, 3-hydroxynaringenin, were found in coigue and black cherry heartwoods.

(27) F. Mayer, "The Chemistry of the Natural Organic Coloring Matters," A. C. S. Monograph No. 89, 1943, p. 176.

(28) F. Mayer, *ibid.*, p. 182.

MADISON, WISCONSIN RECEIVED MAY 16, 1948

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, MEDICAL RESEARCH DIVISION, SHARP AND DOHME, INC.]

Isolation of a Crystalline Trypsin Inhibitor–Anticoagulant Protein from Pancreas[1a]

BY LOUIS A. KAZAL, DANIEL S. SPICER AND ROSE A. BRAHINSKY

The isolation of a crystalline trypsin inhibitor from pancreas was reported by Kunitz and Northrop[1] in 1936. According to their method minced pancreas was treated with dilute sulfuric acid, crystalline chymotrypsinogen and trypsinogen then were separated by fractional precipitation with ammonium sulfate, and trypsin, by precipitation with trichloroacetic acid. The inhibitor, a polypeptide, was crystallized from approximately 0.7 saturated ammonium sulfate solution at *p*H 5.5 and 20° in the form of long hexagonal prisms.

The crystalline inhibitor from pancreas was shown to be an anticoagulant by Ferguson[2] and by Grob[3] inasmuch as the coagulation of recalcified plasma was inhibited *in vitro*.

In the course of other studies in this Laboratory the observation was made that a substance with anticoagulant properties was obtainable from the 15% sodium chloride filtrate that was discarded in the process for the preparation of insulin from

(1a) Presented at the meeting of the American Chemical Society, Division of Biological Chemistry, in New York City, September 16, 1947.

(1) Kunitz and Northrop, *J. Gen. Physiol.*, **19**, 991 (1936).

(2) Ferguson, *Proc. Soc. Exptl. Biol. Med.*, **51**, 373 (1942).

(3) Grob, *J. Gen. Physiol.*, **26**, 423 (1943).

beef pancreas. This substance, which was precipitated from the filtrate by 31% sodium chloride[4] and then freed of salt by dialysis, was capable of inhibiting the coagulation of whole blood, recalcified citrated plasma, and the action of thrombin, *in vitro*. After purification by fractional precipitation with alcohol, it was crystallized from 2.5% trichloroacetic acid solution at *p*H 3.25 and 5° as long, slender needles. The method of preparation and some of the chemical and physical properties of the crystalline substance, which proved to have trypsin–inhibitor as well as anticoagulant properties, are presented here.

Experimental Details and Results

An anticoagulant assay based on the inhibition of the clotting action of thrombin on plasma was used to follow the isolation of the inhibitor.

Assay.—0.5 ml. of a standardized thrombin solution (6 units) was incubated at 37° with 0.5 ml. of inhibitor solution of varying concentration (0.25% to 1.0% in 0.05 *M* imidazole buffer[5] (*p*H 7.4) or in distilled water solution at *p*H 7.2–7.4) for fifteen minutes; 0.2 ml. aliquot portions of each mixture then forcibly were blown into 0.2 ml. of citrated human plasma, and the time in seconds for a clot to form was determined. A control containing no inhibitor was included with each group of inhibitor preparations tested. The average clotting time of a minimum of three individual determinations was obtained for each dilution tested. The relationship between concentration of the inhibitor and the clotting time was essentially linear for most preparations (Fig. 6), but occasionally proved to be curvilinear; for that reason activity arbitrarily was expressed as the increase in the average clotting time produced by 1 mg. of the inhibitor (1% solution in the test) over that of the control. In the text below this activity will be referred to as seconds per mg.

Thrombin was prepared from citrated human plasma by a combination of the methods of Eagle[6] and of Mellanby.[7] The prothrombin–fibrinogen complex was precipitated from chilled, ten-fold diluted plasma with 1% acetic acid at *p*H 5.3; prothrombin was converted to thrombin, and fibrinogen to fibrin, at *p*H 7.4 by the addition of thromboplastin and calcium chloride solutions. The thrombin solution which was pressed out of the fibrin clot was dried from the frozen state under vacuum. Aliquot portions were reconstituted with water and standardized against citrated plasma. One unit arbitrarily was defined as the quantity of dried thrombin dissolved in 0.1 ml. of distilled water that coagulated 0.2 ml. of citrated (9 vol. of blood + 1 vol. 4% aqueous solution of sodium citrate + $2H_2O$) human plasma in ten seconds. In these experiments 91 micrograms contained 1 unit of thrombin. Our unit was equivalent to approximately 1.2 Iowa units, when standardized against a N. I. H. standard thrombin. The relationship between the concentration of thrombin (units) and clotting time was similar to that obtained by Quick[8] and Jaques.[9]

The plasma and standard thrombin solutions used in the assay were filled into small vials (1 to 5 ml. per vial), rapidly frozen in methyl-cellosolve and Dry Ice mixture, and stored at −20°. No deterioration was observed in these reagents throughout the period of investigation. Rapid freezing and rapid thawing (at 37°) of the reagents were essential for satisfactory results. This method of handling reagent solutions insured a reproducible assay procedure.

Preparation of the Inhibitor.—The initial steps of the procedure for obtaining the inhibitor were essentially the same as those of Romans, Scott and Fisher[10] for the preparation of insulin from beef pancreas. The crude inhibitor was obtained from the 15% sodium chloride brine that was discarded in their process after separation of the reprecipitated insulin.

Preparation of Crude Inhibitor.—Sodium chloride was added to the 15% sodium chloride brine to a final concentration of 31%, and the resultant dark brown precipitate was skimmed off the surface of the brine and drained on filter paper. Eighty-seven to 275 g. (wet weight) of precipitate was obtained from 816.5 kg. (1800 lb.) of frozen pancreas. The precipitates from 8165 kg. were pooled, dissolved in approximately 6 liters of tap water, dialyzed in 2.25-in. diameter Visking cellophane tubing for three to four days against running water, filtered through Hormann D-2 pads with suction to remove an insoluble residue, and then dried from the frozen state under vacuum: yield, 350 g. (Preparation A); activity, 5.6 seconds per mg.

This substance was a light tan, water-soluble powder, which gave typical tests for proteins. It was recovered in yields of 43 to 88 mg./kg. of fresh pancreas (av. 66). As an anticoagulant, it was not very active (Fig. 6). Precipitation experiments with various concentrations of ethanol and ammonium sulfate, and with 2.5% trichloroacetic acid indicated that the greatest concentration of activity was obtained in a fraction soluble in 80% ethanol but insoluble in 90% ethanol.

Purification of Crude Inhibitor.—To a 5% solution of 350 g. of preparation A (*p*H 5.5) at room temperature absolute ethanol was added to 80% concentration. The voluminous precipitate that separated was collected by decantation and vacuum filtration through Hormann D-2 pads, and discarded. (This fraction after drying from the frozen state weighed 134 g. and was slightly active—4.0 seconds per mg.) The 80% ethanol filtrate then was adjusted to 90% ethanol and the resulting precipitate allowed to settle out overnight at room temperature. It was collected and dried (as a precipitate) under vacuum from the frozen state: yield, 35 g. of tan-colored powder (preparation B); activity, 21.5 seconds per mg.

Preparation B was roughly four times as active as preparation A. This fraction was recovered in yields of 2 to 4.4 mg. per kg. (average 3.5 mg.). It contained insulin. Attempts to crystallize the inhibitor of Kunitz and Northrop from this fraction by their ammonium sulfate technique were unsuccessful. However, in the course of these attempts a crystalline substance was obtained at *p*H 3.25 from a chilled 2.5% trichloroacetic acid extract. The crystallization of this substance, which proved to be an anticoagulant and a trypsin inhibitor, was observed over the *p*H range of 2.6 to 3.5.

(4) Percentage concentrations refer to g. of sodium chloride added to 100 ml. of solution of the crude inhibitor. We are indebted to Mr. Henry Rock for kindly collecting all the 31% sodium chloride precipitates used in this investigation.

(5) Mertz and Owens, *Proc. Soc. Exptl. Biol. Med.*, **43**, 204 (1940).

(6) Eagle, *J. Gen. Physiol.*, **18**, 531 (1935).

(7) Mellanby, *Proc. Roy. Soc. (London)*, **107B**, 271 (1930).

(8) A. J., Quick, "The Hemorrhagic Diseases and the Physiology of Hemostasis," Chas. C. Thomas, Springfield, Ill., 1942, p. 16.

(9) Jaques, *J. Gen. Physiol.*, **100**, 275 (1941).

(10) *Ind. Eng. Chem.*, **32**, 908 (1940). These steps included acid-ethanol treatment, separation of insoluble residues at *p*H 2 and 8.5, concentration and removal of alcohol by vacuum distillation, precipitation of crude insulin by 31% sodium chloride and partial purification by reprecipitation from acid solution by 15% sodium chloride.

Crystallization.—21.5 g. of preparation B representing 4899 kg. (10,800 lb.) of frozen pancreas was dissolved in 215 ml. of distilled water, and added to an equal volume of 5% trichloroacetic acid. After thirty minutes, the precipitate[11] that formed was centrifuged and discarded (2.7 g. dry weight, activity 2.0 seconds per mg.). The supernatant was heated for five minutes at 80°, and centrifuged. The resulting precipitate (0.1 g. dry weight, 3.9 seconds per mg.) also was discarded. The supernatant then was adjusted to *p*H 3.25 with 5 *N* sodium hydroxide solution, and the cloudy solution chilled to 5°. In approximately two hours, occasionally overnight, needle-crystals were formed, which settled out as a white precipitate.

Crystallization was allowed to proceed for three to four days, during which the crystals formed sheafs or bundles. The crystalline mixture then was collected at 5° by centrifugation (or vacuum filtration with E. & D. #576 filter paper): activity, 30.8 seconds per mg. (Preparation C).

Additional small crops of crystals were obtained by reworking the mother liquor, after dialysis and drying, starting with the 80% ethanol precipitation step as described for preparation B.

These crystals (Preparation C) were admixed with much amorphous material; a small portion of this amorphous fraction appeared to change to crystals on standing. On centrifugation the white precipitate separated into two layers; the upper, white layer appeared to consist chiefly of crystals, and the small, lower, yellow layer, of amorphous material.

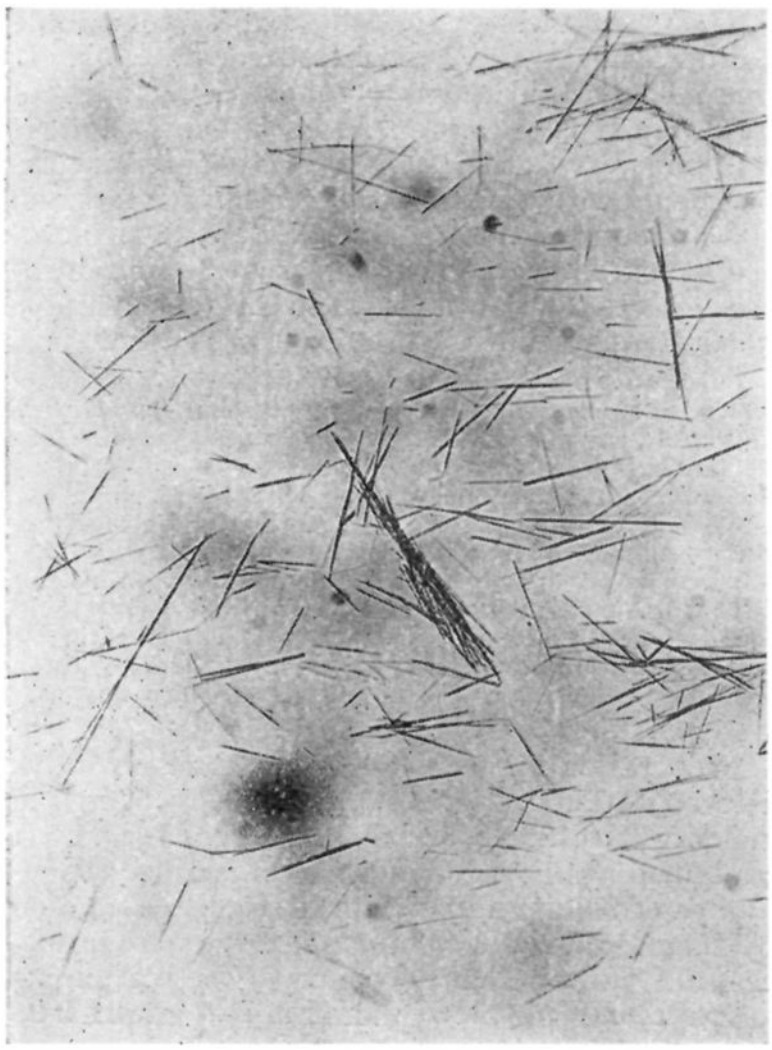

Fig. 1.—Photomicrograph (90 ×) of the crystalline inhibitor, recrystallized once from trichloroacetic acid at *p*H 3.25. Except for the size of the crystals, which varied unpredictably, the original as well as first and second recrystallized preparations exhibited the same crystalline shape.

(11) In one instance this precipitate did not separate until the mixture was heated at 80° for five minutes, as in the following step.

The crude crystals (Preparation C) were not stable at room temperature in the presence of the mother liquor. When placed on a microscope slide, disintegration into smaller irregularly shaped particles occurred in a few minutes. In fact, unless microscopic observation was made rapidly at room temperature or at 5°, where the needle-crystals retained their shapes until the slide dried, the presence of crystals might easily be overlooked. This phenomenon did not appear to be due to a solubility effect at the higher temperature, since the formation of discrete and permanent particles from individual crystals was observed. This property of crystal instability was lost with recrystallization.

Recrystallization.—The crystalline–amorphous mixture (Preparation C) was recrystallized by extraction with 2.5% trichloroacetic acid at *p*H 3.25 and room temperature (or 37°), followed by chilling to refrigerator temperature. A crop of needle-crystals (Fig. 1) that microscopically were essentially free of the amorphous fraction were formed. This method appeared to be the one of choice for obtaining a preparation free of amorphous matter. A description follows:

Preparation C, derived from 21.5 g. of Preparation B, was extracted at room temperature with 170 ml. of a 2.5% trichloroacetic acid solution previously adjusted to *p*H 3.25 with 5 *N* sodium hydroxide solution. The insoluble residue[12] was centrifuged and reextracted with 75 ml. of the *p*H 3.25 trichloroacetic acid solution. Both extracts were chilled, and the crystals recovered several days later by filtration: activity, 43 seconds per mg.

A second recrystallization was carried out in the same manner as the first recrystallization except that two 50-ml. extractions were made. The crystalline precipitate then was dissolved in water, dialyzed in a cellophane bag against distilled water until free of acid, and then dried from the frozen state. The *p*H of the dialyzed solution was 5.7: yield, 843 mg. white powder, activity, 47.6 seconds/mg.

The inhibitor, twice recrystallized from *p*H 3.25 trichloroacetic acid solution, appeared to be free of amorphous material. Yields of 12.7 to 172 micrograms (average 75.3) per kg. of frozen pancreas were obtained. Additional small crops of crystals that sometimes separated on long standing in the refrigerator or that could be recovered be reworking mother liquors have not been included in the yields reported above.

It was possible to recover and recrystallize the inhibitor in Preparation C by two other procedures.

One was based on the solubility of the crystalline (white sedimented fraction referred to above) and the insolubility of the amorphous fractions (yellow sedimented fraction) of Preparation C in

(12) The residue after two extractions was quite active, 44.8 seconds per mg. However only small crops of crystals, and sometimes none, were obtained by making additional extracts. Such residues plus the mother liquors of all recrystallizations were pooled, dialyzed to remove the acid, dried from the frozen state and then reworked through the recrystallization steps. The small quantity recovered in this manner was not included in the final yield.

acid alcohol. Several extractions with 95% ethanol acidified with dilute hydrochloric acid dissolved the crystalline fraction. The addition of an equal volume of ether to the ethanol extract produced a white amorphous precipitate. The ether dried powder, which was as active as the inhibitor twice recrystallized from *p*H 3.25 trichloroacetic acid solution, could be crystallized from the latter solution in the form of needles that, however, were not quite as well-defined.

By the other procedure, recrystallization was obtained in aqueous solution essentially free of trichloroacetic acid with the formation of two kinds of crystals. When a concentrated solution of Preparation C in distilled water at *p*H 6 or higher was readjusted to *p*H 3.25 with 5 *N* sodium hydroxide, an amorphous and gummy noncrystallizable (by procedure 1) but active precipitate (twenty-five seconds per mg.) was formed. When this precipitate was filtered off, the supernatant on chilling yielded a mixture of crystals consisting of needles and hexagonal platelets, as well as much amorphous material (Fig. 2). Preliminary experiments have shown that it is possible to extract this crystalline mixture with *p*H 3.25 trichloroacetic acid solution and to obtain needle-crystals on chilling to 5°. However, attempts to crystallize the remaining residue in aqueous solution at *p*H 3.25, conditions under which needle and platelet crystals previously were formed, were unsuccessful, even though the residue was essentially as active as the crystalline inhibitor. Whether or not the platelet and needle crystals represented different forms of the same substance was not entirely clear from these experiments.

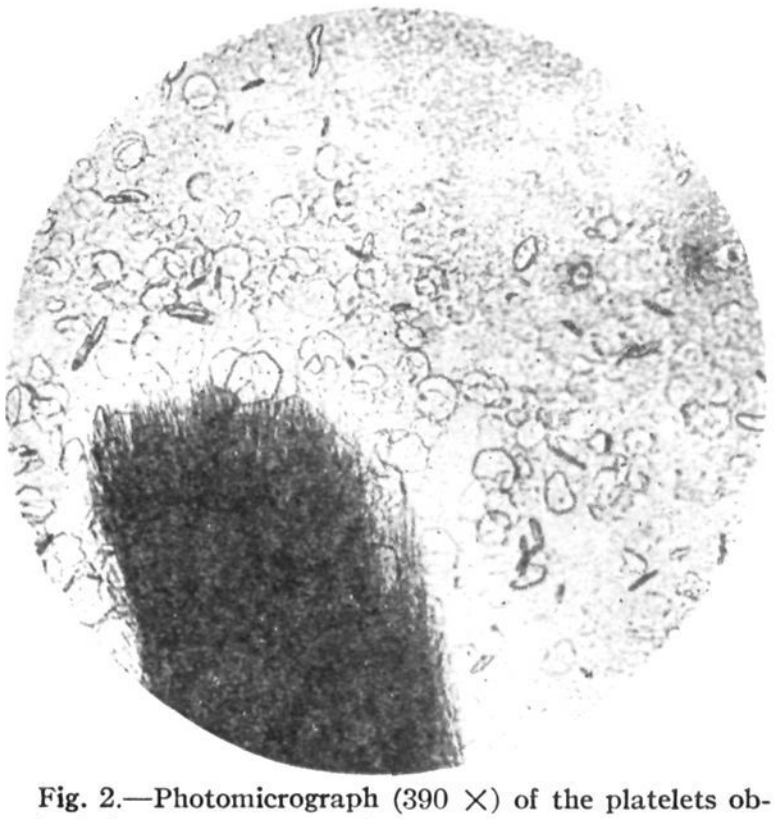

Fig. 2.—Photomicrograph (390 ×) of the platelets obtained following recrystallization of the first crystalline product from aqueous solution at *p*H 3.25 instead of from trichloroacetic acid. The fragile platelets became well-defined after a few weeks storage at 5°. The dark object in the field was the end of a large sheaf of the needle crystals that also were formed in aqueous solution at this *p*H.

Chemical Properties.—The investigation of the properties of the trypsin inhibitor–anticoagulant substance was limited to preparations twice recrystallized by the *p*H 3.25 trichloroacetic acid extraction procedure. An extensive analysis was not attempted since the three-times crystallized substance proved to be inhomogeneous, as shown below.

The dialyzed and dried inhibitor was a light, white and very weakly hygroscopic powder. A 1% solution gave a positive (violet) biuret test. Precipitation occurred with picric acid, and in saturated magnesium sulfate, saturated sodium chloride, and half-saturated ammonium sulfate. From 1% solution, 50% of the inhibitor (on the basis of Kjeldahl nitrogen analyses) was precipitated in 5% trichloroacetic acid, and 96.2% in 15% trichloroacetic acid solutions. The inhibitor was not precipitated from 10% sulfosalicylic acid or 2.5% lead acetate. No precipitation occurred on boiling a 1% distilled water solution of the inhibitor for five minutes.

The elementary analyses, except for the lower value for carbon, were somewhat typical of a protein: C, 43.36; H, 7.25[13]; N, 16.78[14]; ash, 0.22. Tests for sulfur were positive; for phosphorus, negative. The Molisch test for carbohydrate was negative. Amino nitrogen determinations (Van Slyke, manometric) were variable, averaging 7.0%; reproducible readings were difficult to obtain due to coagulation of the protein in the acid solution.

The value for specific rotation, $[\alpha]^{30}D$, was −92 when the substance was dissolved in distilled water; the *p*H was 5.7. Ultraviolet absorption of an aqueous solution was typical of proteins (Fig. 3).

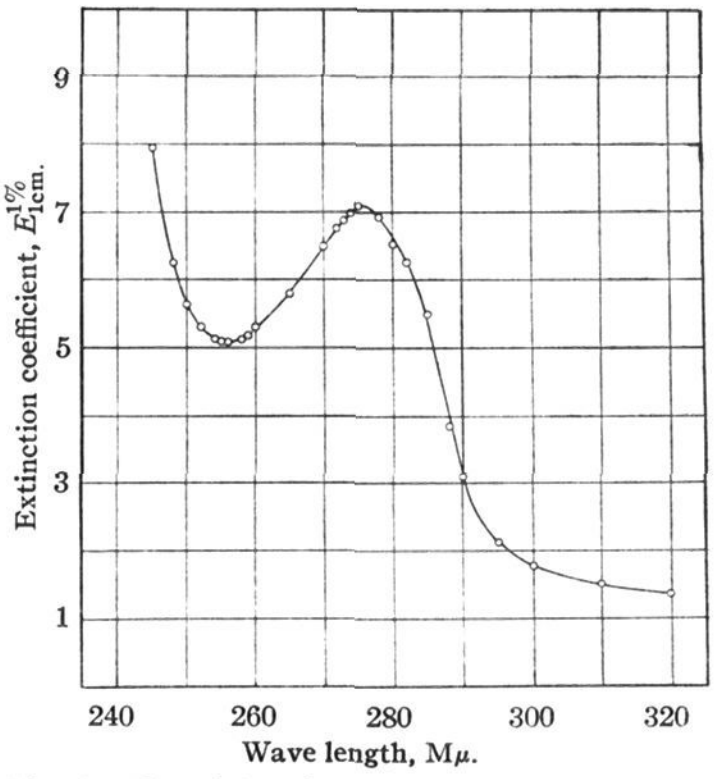

Fig. 3.—Ultraviolet absorption spectrum of twice recrystallized inhibitor. A Model DU Beckman Spectrophotometer was employed for the determination of the optical density of a distilled water solution of the inhibitor (*p*H 5.7). Extinction coefficients were calculated from the equation $E^{1\%}_{1cm.} = (\log I_0/I)/cd$, where c = 1.0 g. inhibitor/100 ml., d = 1 cm.

(13) Microanalyses by J. B. Leibee, Wilmington, Delaware.

(14) We are indebted to K. B. Streeter, Organic Chemistry Department, Sharp and Dohme, Inc., for the micro-kjeldahl nitrogen determinations.

Electrophoretic analyses were made by the Tiselius moving boundary method using the Longsworth schlieren scanning technique. Acetate and phosphate buffers, *p*H 4.27, 5.75 and 7.76, ionic strength 0.1, at 2.8°, were used. The three-times crystallized inhibitor used in the electrophoretic studies was neither dialyzed against distilled water nor dried, in order to eliminate any possible denaturation effects; instead the cold crystalline filter cake was dissolved in buffer at 2–5°, dialyzed against several changes of the buffer at 2–5° for two to three days, and then transferred to the electrophoresis cell. Figure 4 illustrates the pattern obtained at *p*H 5.75, where the positively charged component, A, slowly migrated toward the cathode, while the two negatively charged components,

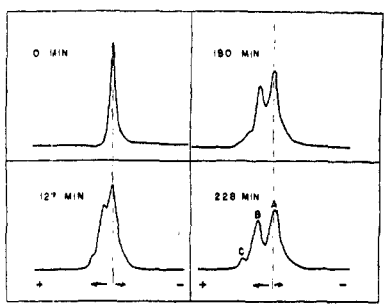

Fig. 4.—Tracing of electrophoretic pattern of the inhibitor after two recrystallizations from trichloroacetic acid solution at *p*H 3.25. The patterns were obtained in an acetate buffer solution of the inhibitor at *p*H 5.75, 0.1 ionic strength, 2.8, potential gradient 4.81 volts/cm., concentration 0.83% inhibitor, after dialysis in the cold for a period of forty-eight hours against two changes of buffer.

B and C, migrated toward the anode. The average composition of the crystalline inhibitor from measurements made in the three buffers was as follows: component A, 48.8 ± 4.3%; component B, 39.8 ± 1.8%; component C, 10.7 ± 2.4%. Figure 5 illustrates the *p*H mobility relationships of the various components, and suggests that an isoelectric separation of the components may be possible.

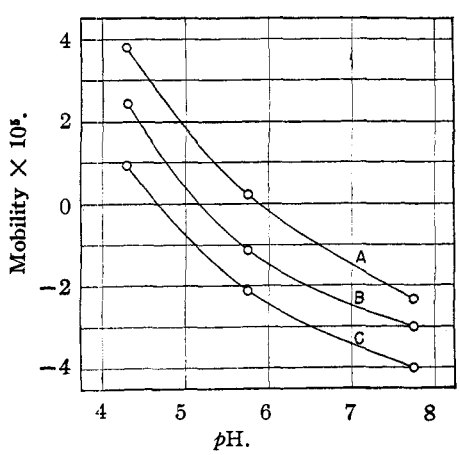

Fig. 5.—The relationship between *p*H and electrophoretic mobility of components A, B and C of the crystalline inhibitor. Mobilities ($\mu \times 10^{-5}$ sq. cm./volt/sec.) were calculated from runs made between 234 and 275 minutes in acetate (*p*H 4.27, 5.75) and phosphate (*p*H 7.76) buffers, 0.1 ionic strength at 2.85° and 4.81 to 5.05 volts per cm. Descending patterns were employed at *p*H 3.27 and 7.76, however, due to poor separation of components B and C at *p*H 5.75 in the descending arm of the cell, it was necessary to calculate the mobilities at this *p*H from the ascending boundaries.

Biochemical Properties.—The crystalline substance obtained from pancreas inhibited the clotting action of thrombin on plasma as well as the digestion of casein by trypsin. Figure 6, curve B, illustrates the results obtained with the anticoagulant assay described above when the concentration of the inhibitor was varied between 0 and 1%. A similar assay (curve A) for the crude inhibitor, Preparation A, is included to illustrate the increased activity of the crystalline over that of a crude preparation.

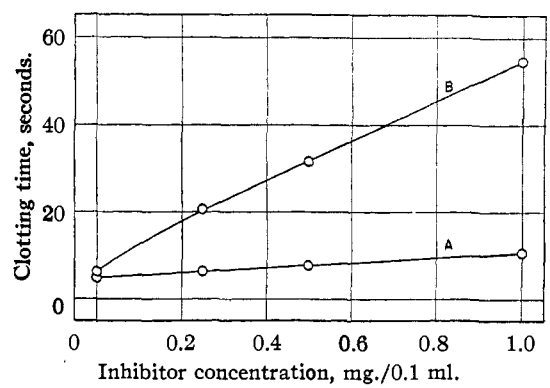

Fig. 6.—Inhibition of the clotting action of thrombin by the inhibitor: Curve A, crude inhibitor, preparation A; curve B, inhibitor recrystallized twice from trichloroacetic acid. 0.1 ml. of inhibitor was incubated with 0.1 ml. of a standardized thrombin for fifteen minutes at 37°, and added to 0.2 ml. of citrated human plasma. Each point is the average of at least three individual determinations.

The trypsin inhibitor activity of the crystalline substance was determined by a modification of the method described by Christensen[15] in which commercial instead of crystalline trypsin was em-

TABLE I

TRYPSIN INHIBITOR ACTIVITY[a]

Inhibitor concentration, micrograms	Tyrosine liberated, micrograms	Inhibition of tryptic digestion, %
0	508	0
0.5	451	11.2
1.0	401	21.0
1.5	353	30.5
2.0	283	44.3

[a] Determined by a modification of the method of Christensen[15] in which the amount of tyrosine liberated from a casein solution by a constant amount of commercial trypsin was determined in the presence of various concentrations of the inhibitor. The results are the average of 2 to 3 determinations. The inhibitor was a preparation that was crystallized from trichloroacetic acid three times, dialyzed and dried from the frozen state.

(15) Christensen, *J. Gen. Physiol.*, **30**, 149 (1946). We are indebted to R. J. Westfall and I. S. Westfall of this department for the trypsin inhibitor assays.

ployed for the digestion of casein, the amounts of each solution were doubled, and the casein solution was dissolved at *p*H 7.6 in the absence of urea. The data are recorded in Table I.

Discussion

A crystalline trypsin inhibitor–anticoagulant substance was obtained from pancreas by a new method, and crystallized from trichloroacetic acid solution at *p*H 3.25. The method was applied successfully in five out of six trials; however, the yields of thrice-crystallized inhibitor were not constant, ranging from 12.7 to 172 micrograms per kg. of frozen pancreas. The method and conditions of crystallization were different from those used by Kunitz and Northrop[1] for the isolation of their inhibitor.

The inhibitor was recrystallized from trichloroacetic acid solution at *p*H 3.25 and 5° in the form of long needles, and from aqueous solution at the same *p*H, in the form of both needles and platelets. The identity of the needles and platelets was not established since the needles but not the platelets could be recovered from the mixture in a crystalline state. However, the remaining amorphous fraction that apparently represented platelets possessed anticoagulant activity, suggesting that the platelets perhaps may represent still another inhibitor.

The chemical and physical properties of the inhibitor were determined on a preparation twice recrystallized from the trichloroacetic acid at *p*H 3.25. The crystalline substance was water-soluble, non-dialyzable, and biuret-positive; it was precipitated by most protein-precipitants, including 5% or higher concentrations of trichloroacetic acid, but not by 2.5% trichloroacetic acid. These properties suggested that the crystalline inhibitor was a protein, probably a proteose or higher polypeptide. The molecular weight was not determined since electrophoretic studies demonstrated the presence of three components in the crystalline inhibitor.

The presence of three well-defined components in the three-times crystallized inhibitor was demonstrated at *p*H 4.27, 5.75 and 7.76. The essentiality of these components for the activity of the crystalline inhibitor has not been established with certainty. Due to lack of an adequate supply of the inhibitor, an electrophoretic separation on a large scale has not been made. However, the results of a preliminary separation[16] at *p*H 5.75, where component A migrated in the opposite direction from that of component B, suggested that components A and B or C possessed anticoagulant activity. Substantiation of these results is necessary before it can be stated with any certainty that there is more than one trypsin inhibitor obtainable from pancreas. In view of these results it would seem better to postpone further chemical and physical characterization of the crystalline inhibitor until such time as its purity[17] or the essentiality of the three components for the crystalline state definitely has been established.

The biochemical activity of the crystalline inhibitor reported in this paper and of the inhibitor of Kunitz and Northrop in general are similar. Both are capable of inhibiting the action of thrombin and of trypsin. However, in the absence of an absolute comparison using the same assay techniques for both inhibitors, it cannot be stated with certainty that the two inhibitors have identical specific activities. Such a comparison could not be made since the inhibitor of Kunitz and Northrop was not available and several attempts to prepare it were unsuccessful. Thus the identity or non-identity of the two crystalline inhibitors was not established by these means. However, the inhibitor crystallized from trichloroacetic acid could not be crystallized from ammonium sulfate solution at *p*H 5.5, conditions employed by Kunitz and Northrop for the crystallization of their inhibitor, nor would it form with crystalline trypsin[18] the crystalline inhibitor-trypsin compound[1] of Kunitz and Northrop, strongly suggesting that the two crystalline inhibitors were not identical. The possibility that the inhibitor of Kunitz and Northrop may be present in combination with some other protein must not be overlooked.

On the basis of chemical properties the two inhibitors likewise do not appear to be identical. The inhibitor of Kunitz and Northrop contained 38.6% carbon, and 11.25% nitrogen compared to values of 43.6% and 16.78%, respectively, for the inhibitor crystallized from trichloroacetic acid. The former was dialyzable through collodion membrane, contained considerable magnesium sulfate as an impurity, and gave a faint biuret test (perhaps due to presence of Mg^{++}), while the latter was non-dialyzable (cellophane), essentially free of inorganic impurities, and gave a strong biuret test. The separated components, A and B + C, of the inhibitor that was crystallized from trichloroacetic acid likewise were non-dialyzable. Since the only properties common to the two inhibitors were specific rotation (0.65° per mg. TN for Kunitz and Northrop, and 0.66° for our inhibitor) and the amino-nitrogen values (6.5 and 7.0%,

(16) At the conclusion of the *p*H 5.75 run (sixteen hours) which was made in the analytical electrophoretic cell, it was possible to separate by compensation techniques pure component A from combined components B and C in sufficient quantity for an assay. After dialysis against distilled water and desiccation, approximately 5 mg. of each fraction was obtained. Both fractions showed considerable anticoagulant activity (A, 34.8 seconds per mg.; B + C, 30.5 seconds per mg.).

(17) The possibility that traces of insulin or of a blood sugar lowering principle may be present in the three-times crystallized inhibitor was suggested by experiments in which the inhibitor was injected intravenously at 10 mg. per kilo levels into two rabbits. The blood sugar was lowered from 99 mg.% to 75 mg.% and from 79 mg.% to 58 mg.%, respectively, in thirty minutes followed by a return to normal in one hour without any convulsions, a response that ordinarily would be elicited by small amounts of insulin. Mr. E. K. Wolf kindly performed this experiment.

(18) We are indebted to Dr. M. Kunitz of the Rockefeller Institute for Medical Research, Princeton, N. J., for his generous sample of crystalline trypsin and for kindly discussing with us the results of our investigation.

respectively) it would appear that the two inhibitors were not identical.

It is obvious that additional studies are required to settle the questions raised by this investigation. An account of many unfinished experiments is included in this report, because an extensive investigation probably will not be possible in the near future.

Summary

A crystalline protein with trypsin inhibitor and anticoagulant properties was obtained from pancreas by a method entirely different from the ammonium sulfate fractionation procedure used by Kunitz and Northrop to isolate their crystalline trypsin inhibitor from pancreas. The inhibitor described in this report was obtained by an acid–alcohol treatment of pancreas; it was precipitated with 31% sodium chloride, separated from insulin by fractionation between 15 and 31% sodium chloride, and then precipitated from aqueous solution between 80 and 90% ethanol. Crystallization from trichloroacetic acid at *p*H 3.25 at 5° produced well-defined needle crystals. The conditions of crystallization and the crystal habit both were different from that of the inhibitor of Kunitz and Northrop.

Some chemical and physical properties of three-times crystallized inhibitor are presented. An extensive study was not made at this time since electrophoretic studies demonstrated the presence of more than one component in the crystallized substance, and since the essentiality of these components for the crystalline state and for biological activity has not yet been established with certainty.

GLENOLDEN, PENNSYLVANIA RECEIVED MARCH 25, 1948

[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Water Absorption of Proteins. III. Contribution of the Peptide Group[2]

BY EDWARD F. MELLON, ALFRED H. KORN AND SAM R. HOOVER

Theoretical considerations by several workers[3,4,5] have established a number of correlations between the number of polar groups in certain proteins and the amount of water absorbed by these proteins. Most workers have based these correlations on the possibility that the polar groups form hydrogen bonds with water, and all of them have based the correlation on the total number of the groups present. Recently we[6] have quantitatively determined the contribution of the amino group to the water-absorbing power of casein. This study indicated that the amino group, which constitutes less than 1% of the weight of the casein molecule, is responsible for about one quarter of the total water absorbed by casein over the entire range between 6 and 93% relative humidity. The amount of water held above 75% relative humidity is consistent with the amount required for complete hydrogen bonding of the water to the amino group. It seemed of interest, therefore, to extend the quantitative studies to other polar groups.

The most numerous polar group in proteins is the peptide group, and its contribution to water absorption has been variously reported. Lloyd and Phillips[3] and Pauling[5] have concluded that the hydration of peptide groups would be slight[7]; but Sponsler, Bath and Ellis[4] indicate a maximum hydrogen-bonding capacity of four moles of water per peptide group. They, however, feel that space restrictions and chain interactions would reduce this figure to less than two. Since it is impractical to modify the peptide groups of a protein to reduce their hydrogen-bonding capacity, we have attempted to study a series of peptides with different numbers of peptide groups per molecule. In order to simplify both the synthesis of the peptides and the interpretation of the data, we have limited this study to the glycine peptides. Except for the terminal carboxyl and amino groups, these peptides have no polar groups except the peptide group and there are no side-chain groups to prevent close packing and high chain interaction between the peptide groups.

Experimental

Preparation of Glycine Peptides.—Glycylglycine was prepared from glycine anhydride.[8] Triglycine,[9] tetraglycine[10] and pentaglycine[10] were prepared by adding chloroacetyl chloride to the next lower peptide and then replacing the halogen by ammonolysis. These four peptides were each recrystallized twice from water by the addition of alcohol. Hexaglycine was prepared by alkaline hydrolysis[11] of its methyl ester, which was prepared by

(1) One of the Laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

(2) This paper is part of a talk presented before the Meeting-in-Miniature of the Philadelphia Section of the American Chemical Society, January 22, 1948.

(3) Lloyd and Phillips, *Trans. Faraday Soc.*, **29**, 132 (1933).

(4) Sponsler, Bath and Ellis, *J. Phys. Chem.*, **44**, 996 (1940).

(5) Pauling, THIS JOURNAL, **67**, 555 (1945).

(6) Mellon, Korn and Hoover, *ibid.*, **69**, 827 (1947).

(7) White and Eyring (*Textile Research J.*, **17**, 523 (1947)) have recently analyzed the whole problem of sorption by swelling high polymers. They accepted Pauling's assumptions regarding the contribution of polar side groups and peptide linkages to sorption by proteins.

(8) Fischer and Fourneau, *Ber.*, **34**, 2868 (1901).

(9) Fischer, *ibid.*, **36**, 2982 (1903).

(10) Fischer, *ibid.*, **37**, 2486 (1904).

(11) Fischer, *ibid.*, **39**, 453 (1906).