The Coagulability of Ammonium Sulfate Precipitated Canine Fibrinogen[1] (35660)

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The purity of fibrinogen which has been isolated and purified by fractional salt precipitation with ammonium sulfate as well as the usefulness of this preparation in turnover studies has been questioned recently (1). Fibrinogen purified by this method has been used in turnover studies in the rabbit (2), man (3), and the dog (4, 5). In our studies on fibrinogen turnover in the beagle dog (4, 5) the purity or coagulability of the fibrinogen used was determined to establish the mass loss and isotope loss in the split-off peptides (6, 7) following thrombin addition. A quantitative knowledge of these losses is necessary for the accurate determination of canine plasma fibrinogen concentration by an isotope dilution method (8). The present communication reports these results on the coagulability of canine fibrinogen isolated and purified by the ammonium sulfate fractionation method by: (a) UV absorption at 280 mμ; (b) fibrinogen and fibrin nitrogen determination; and (c) by the weighing of dried fibrin.

Methods. Fibrinogen preparation. A femoral artery of a previously heparinized (5000 units) dog under sodium pentobarbital anesthesia, 3 mg/kg (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa) was cannulated with a short polyethylene catheter, and the blood was allowed to flow freely into a beaker. Additional heparin was added to the swirled blood being collected in the beaker to insure anticoagulation. The plasma was separated and stored at approximately 4° for several days before the fibrinogen was isolated. During the period of storage in the cold, cold-insoluble material formed which was removed by centrifugation on the day of the isolation. The fibrinogen was isolated following the method of Atencio *et al.* (8), the details of which have been described elsewhere (4).

The fibrinogen concentration in the purified and dialyzed (0.01 *M* sodium citrate) sample was determined by measurement of the optical density at 280 mμ (Beckman DU spectrophotometer, Beckman Instrument Co., Fullerton, Calif.) against the dialysis diffusate and the use of the extinction coefficient $E^{0.1\%}_{1\text{ cm}} = 1.583$ (9) determined for the dog. A solution containing approximately 4 mg/ml was then prepared by dilution of a determined volume of the dialyzed fibrinogen solution with the dialysis diffusate.

Coagulability determinations. A. 280 mμ ultraviolet absorption. The coagulability by UV absorption was determined by the method of Atencio *et al.* (8) as has been previously described (6).

B. Gravimetric analysis of dried fibrin. One-ml samples of the fibrinogen solution, containing 0.5–1.5 mg of protein nitrogen, were pipetted into Kjeldahl flasks containing concentrated H_2SO_4 and approximately 500 mg of a mixture of K_2SO_4 and mercuric sulfate. The samples were digested for 3 hr, and distilled with an NaOH solution containing thiosulfate, into a boric acid solution containing methyl red and bromocresol green as indicator. Powdered zinc was added before distillation to free any nitrogen which may have

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formed into mercury complexes during digestion. Blank samples and protein standard solution containing a known amount of protein nitrogen were run simultaneously (Protein Standard Solution, Lot No. C4508, Armour Pharmaceutical Co., Kankakee, Ill.). The samples were tritated with 0.01 *N* HCl, using the protein standard as end point. The determination of the protein standard solutions allows certain corrections to be made which result from day to day variations in end point determination. These nitrogen values were converted to milligrams of fibrinogen by use of the conversion factor 5.92 [16.9% nitrogen in fibrinogen (10, 11)] and thus establish the amount of fibrinogen in the original sample.

The fibrin removed on siliconized glass rods following thrombin addition in the determination of UV coagulability was washed thoroughly in distilled water. The clots were then removed from the rods and placed into the bottom of weighing bottles which had been previously dried and tared to constant weight. The fibrin was dehydrated by adding a mixture of ethanol and ether (v/v, 3 parts to 2 parts) in 5 successive small volumes, approximately 5 ml each. Following dehydration, the weighing bottles were dried at 110°, again to constant weight. This measured fibrin weight can be compared with the fibrinogen weight calculated from total nitrogen to establish coagulability on a weight basis.

C. Fibrinogen and fibrin nitrogen determination. The nitrogen of the original fibrinogen sample was determined as described above in the gravimetric procedure.

The fibrin, which was dried to constant weight (W_1) during the gravimetric procedure, was freed from the bottom of the weighing bottle and transferred to a Kjeldahl flask, where the above-described procedure was repeated to establish the nitrogen present in the fibrin transferred (N_t). The residual fibrin remaining in the weighing bottle following the transfer was once again dried to constant weight (W_2) in order to determine the transferred dry weight of the fibrin ($W_t = W_1 - W_2$). The total nitrogen content in the dried fibrin sample before transfer was calculated as $N_t W_1/W_t$. The ratio of this value to the nitrogen content in the original fibrinogen sample times 100 is thus the coagulability of the sample based on nitrogen recovery. The percentage nitrogen in dried canine fibrin is calculated as $100\ N_t/W_t$.

Results. The coagulability of purified and dialyzed, ammonium sulfate precipitated dog fibrinogen as determined by three different methods is shown in Table I. The average value determined by UV absorption at 280 mμ is 93.9 ± 1.2%. Coagulability determined by a comparison of the weight of dried fibrin collected from a fibrinogen aliquot to the fibrinogen weight in the aliquot determined by nitrogen analysis has an average value of 94.7 ± 3.1%. The coagulability, expressed on the basis of the nitrogen content of an aliquot of fibrinogen and of the fibrin collected from a similar aliquot following thrombin addition has an average value of 96.5 ± 4.3%. The average value for the coagulability of canine fibrinogen based on three different

TABLE I. The Coagulability of Ammonium Sulfate Precipitated and Purified Canine Fibrinogen as Determined by Three Different Methods and the Percentage Nitrogen in the Fibrin Collected from This Fibrinogen.

Method	*n*	(%; mean ± SD)	Range
		Coagulability (%)	
1. 280 mμ UV analysis	18	93.9 ± 1.2	92.1– 95.4
2. Dried fibrin wt	18	94.7 ± 3.1	89.5–102.3
3. Fibrinogen and fibrin N determination	18	96.5 ± 4.3	88.2–101.5
Av	54	95.0	
		Nitrogen (%)	
1. Fibrin	18	17.3 ± 0.8	15.2– 18.5

methods is 95%.

The determination of the nitrogen content of dried fibrin samples allows one to calculate the nitrogen percentage of fibrin which is listed in Table I and has an average value of 17.3 ± 0.8%.

Discussion. The present results on the coagulability of purified and dialyzed dog fibrinogen measured by the three methods used, show that this molecule is 95% coagulable by the addition of thrombin. The value of 95% has been repeatedly obtained in this laboratory by UV analysis at 280 mμ on canine fibrinogen (4, 6, 9, 12) purified by fractional salt precipitation according to the method of Atencio *et al.* (8). To the knowledge of the authors, coagulability of dog fibrinogen has not been previously studied or reported; however, it has been reported from the use of the same precipitation techniques that both rabbit fibrinogen (2) and human fibrinogen (3) are 95% coagulable. It would thus appear that canine fibrinogen has a similar coagulability. The lack of 100% coagulability by the UV absorption method is to be expected from the findings that the split-off peptides during the canine fibrinogen–fibrin transformation do contain tyrosine (7, 13, 14). Other possible explanations are the presence of some nonclottable protein contaminant or the presence of denatured fibrinogen.

The coagulability obtained by comparing the weight of fibrinogen (calculated from the determined nitrogen and the conversion factor of 5.92) with the milligram dry weight of fibrin collected from an identical amount of fibrinogen shows an average value of 94.7% (Table I). This result of approximately 5% weight loss can also be obtained in the following manner. If the milligrams of protein are calculated from the nitrogen amounts, using the conversion factor of 5.92 for fibrinogen [(16.9% nitrogen (10, 11,15)] and 5.78 for fibrin (17.3% nitrogen found in this study) the coagulability on a weight basis is calculated to be 95.2%. Thus it would appear that a 3% loss in nitrogen corresponds approximately to a 5% loss in weight, a finding which is a corollary to the difference in the percentage nitrogen in fibrinogen and fibrin.

The coagulability calculated from nitrogen determinations in fibrinogen and fibrin was found to have an average value of 96.5%. This value shows that in dog fibrinogen, as in many other species, there is an approximate 3–4% loss of the fibrinogen nitrogen in the formation of fibrin. Bovine fibrinogen of the highest purity has been stated to be no more than 97% coagulable based on the determination of clottable nitrogen (16) and the results of this study utilizing canine fibrinogen are in close agreement.

If it is assumed that the 3% loss in nitrogen and the 5% loss in weight during the fibrinogen–fibrin transformation are accounted for by the nitrogen content and weight of the split-off peptides, then the canine fibrinogen prepared in this study by ammonium sulfate precipitation is close to being 100% coagulable. The agreement between the three methods further suggests that the isolation and purification procedure can produce a fibrinogen molecule of sufficient purity to be successfully used in turnover studies. The inability of this fractionation technique to produce a fibrinogen of sufficient purity for metabolic studies, which has been advanced by Zetterquist (1), would thus seem to be unfounded. With the use of fibrinogen purified by this method, a complete study of the metabolism and distribution of both homologous and autologous ^{131}I-fibrinogen in the dog has been performed (4) and the half-time values determined are in agreement with those reported by others (17–20) for the dog.

The fibrin collected from the purified and dialyzed fibrinogen of this study, has been found to have 17.3 ± 0.8% nitrogen. This value compares closely with the value of 17.0% reported for bovine fibrin (15) and 17.1% for human fibrin (21). The percentage nitrogen in bovine fibrinogen has been reported to be 1.5% lower than in the fibrin of this same species (15) and if this percentage difference exists for canine fibrinogen, its nitrogen percentage would be 17.0, calculated from 17.3% nitrogen in canine fibrin. This calculated value of 17.0% is in agreement with the value of 16.9% reported for many species (10, 11, 15).

Summary. Fibrinogen, isolated from the plasma of mongrel dogs by fractional ammonium sulfate precipitation, extensively purified and finally dialyzed into 0.01 *M* sodium citrate was used in a study of coagulability. Coagulability determined by UV absorption at 280 mμ has a value of 93.9 ± 1.2%. Clottability, determined by a comparison of the dry weight of fibrin collected from a fibrinogen solution in which the fibrinogen amount was determined by nitrogen analysis and the conversion factor of 5.92 (16.9% nitrogen in fibrinogen) has a value of 94.7 ± 3.1%. On the basis of total nitrogen from a fibrinogen aliquot and the nitrogen present in the dried fibrin from a similar aliquot the nitrogen coagulability was found to be 96.5 ± 4.3%. The average of the three methods is 95%, showing that approximately 5% of the fibrinogen molecule is lost in the conversion to fibrin. The close agreement between the results of all three methods and the average coagulability of 95% show that the ammonium sulfate precipitation and purification procedures can produce a protein of high purity which is suitable for metabolic studies. The percentage nitrogen of purified fibrin has been found to be 17.3 ± 0.8%.

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