ADVANCES IN PROTEIN CHEMISTRY

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PREFACE

The progress of protein chemistry continues, at a pace which appears to accelerate with each passing year. All of the articles presented in this volume record some major landmarks in this progress. The most general in its implications for our knowledge of protein structure is actually the second review in the volume, on X-ray analysis, by Crick and Kendrew. Here the reader will learn, for the first time in any general review, of the great advances that have recently been made in unraveling the structure of protein crystals by the heavy atom technique—with very indication of still greater and more rapid progress in the next few years. Here he will learn, also of new and important developments relating to the structure of silk, of collagen, of synthetic polypeptide fibers, and of many viruses.

The flood of papers on blood clotting continues unabated; in the first article in this volume Scheraga and Laskowski present a detailed and thoughtful discussion of one major aspect of the clotting process—the conversion of fibrinogen to fibrin under the influence of thrombin. The progress made in recent years is such that this subject is now ripe for a prolonged and coherent discussion such as is given here.

The dramatic developments of recent years in the study of the human hemoglobins have provided the most striking set of examples yet available of the genetic control of protein structure, and these studies are also of great clinical and anthropological interest. The field is here reviewed by Itano, who has done so much to advance our knowledge of the human hemoglobins.

In the next article, Li continues his review of the anterior pituitary hormones, dealing with the melanotropins and with prolactin. Here we may comment particularly on the interesting species differences between different melanotropins, and on the fascinating and suggestive relationships between structure and hormone activity in the melanotropins and the corticotropins.

Neurath's article on the activation of zymogens shows the immense progress that has been made in understanding the structural changes involved in forming an active enzyme from its inactive precursor. The results already attained are exciting and illuminating, and they promise much deeper insights, within the next few years, into these fundamental biochemical processes.

Isliker reviews the chemistry of antibodies, showing the major advances made in recent years in their purification and chemical characterization, and correlating these chemical properties with immunological specificity wherever possible.

In the final article, Goodman and Kenner consider the methods available for the synthesis of peptides in the laboratory, taking up the subject from the point at which it had arrived when it was discussed by Fruton in Volume V. Advances in the techniques of peptide synthesis in the intervening years have come rapidly and are numerous. They are none too numerous, however, for the needs of the explorers of protein chemistry and biology, who must constantly extend the scope and power of their synthetic methods if they are to obtain a deeper insight into the structures of these most essential of all natural products.

We are again indebted to Dr. Martha Sinai for her arduous and skillful work in the preparation of the index, and to the staff of the Academic Press for their constant help and cooperation.

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THE FIBRINOGEN-FIBRIN CONVERSION[1]

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CONTENTS

[1] Part of this review was written while one of us (H.A.S.) was at the Carlsberg Laboratorium, Copenhagen, Denmark, as a Fulbright Research Scholar and John Simon Guggenheim Memorial Foundation Fellow.

I. Introduction[2]

The thrombin-induced conversion of fibrinogen to fibrin, which is the primary subject of this review, is essentially the final stage in the complex

[2] The following abbreviations have been used in this chapter: TAMe—p-toluenesulfonyl-L-arginine methyl ester; DFP—diisopropyl fluorophosphate; FDNB—fluorodinitrobenzene; DNP—dinitrophenyl; T—thrombin; F—fibrinogen; f—fibrin monomer; P—fibrinopeptides; f_n—intermediate polymers.

series of processes involved in blood coagulation. Fibrinogen exists in normal blood while thrombin is present as its precursor prothrombin. The coagulation process consists of the conversion of prothrombin to thrombin and of the subsequent action of thrombin on fibrinogen to produce fibrin. Under normal physiological conditions the second reaction commences as soon as a trace of thrombin is produced, i.e. before the first reaction is completed. However, *in vitro* studies of the chemistry of the thrombin-fibrinogen reaction, uncomplicated by the prothrombin-thrombin conversion, can be carried out with isolated and purified thrombin and fibrinogen. We shall, therefore, consider the questions of the purification and characterization of these proteins, and their interaction to form the fibrin clot. In the fibrinogen-fibrin conversion, fibrinogen, already a high polymer of many amino acids, is converted to an even higher polymeric form or coagulum, the fibrin clot.

In delineating the aspects of the fibrinogen-fibrin conversion to be covered in this review it appeared appropriate to omit, except for a brief summary, the work done on the isolation, purification, and characterization of prothrombin, and its conversion to thrombin, since these problems have been treated recently by Seegers (1955). Further, since it is beyond the competence of the reviewers, none of the clinical work on blood clotting has been reviewed. For similar reasons we have limited the subject to work done on relatively pure systems and have, therefore, not discussed the literature on the coagulation of plasma or the role of fibrinolysin. Finally, since the review of Chargaff (1945) covered the subject up to that time, comparatively little attention has been paid here to earlier work.

Brief summaries of work on the fibrinogen-fibrin conversion since 1945 have been presented by Ferry (1948), Astrup (1950), Kowarzyk and Buluk (1950), Janszky (1950b), Laki (1953a), Hughes (1954), Kendrew (1954), Waugh (1954), Bailey and Bettelheim (1955a), and Seegers (1955). A symposium on "Prothrombin and Fibrinogen," published by *Physiological Reviews*, contained articles by Ferry (1954), Laki (1954), Lamy and Waugh (1954), Lorand (1954), Seegers (1954), and Sherry *et al.* (1954). Blood coagulation has frequently been reviewed in several publications such as *Annual Reviews of Biochemistry*, *Annual Reviews of Physiology*, and in several medical and biological journals, e.g. by Quick (1950) and Jaques (1954). Among symposia on blood coagulation dealing with subjects discussed in this review is a series of seminars presented by Alexander (1953), Edsall (1953), Ferguson (1953), Surgenor (1953), and Tullis (1953). Considerable information is also to be found in the "Proceedings of the Conferences on Blood Clotting and Allied Problems," sponsored by the Josiah Macy, Jr., Foundation, and edited by Flynn (1948–1952).

A great number of the advances described in this review were made

during the Second World War and shortly thereafter. However, the breakdown of communications during the war made many investigators unaware of what their colleagues in other countries were accomplishing. An adequate exchange of information was not established in many cases until as late as 1950. Thus, in the years before 1950, there was considerable duplication of research among various workers. In discussing these results, it seemed advisable, in the interest of a more logical presentation, to pay little attention to questions of proper chronology.

II. Thrombin

The enzyme thrombin, which is responsible for initiating the conversion of fibrinogen to fibrin, is not present in normal circulating blood. Instead, normal blood plasma contains the enzyme precursor prothrombin at a concentration of approximately 70 mg. per liter (Hughes, 1954). Since the concentration of the proteins in human blood plasma is approximately 50 g. per liter (Oncley *et al.*, 1947; Cohn *et al.*, 1950) prothrombin represents about 0.14% of the plasma proteins. Hence, a fractionation procedure designed to obtain purified prothrombin must permit the separation of this trace amount of enzyme precursor from large quantities of albumin and globulin impurities.

Prothrombin can be converted to thrombin in a series of highly complex, and as yet not well understood, reactions. More properly, one should speak of a variety of thrombins, since the products obtained from the activation of prothrombin differ according to the procedure used. None of the currently available preparations of thrombin appears to meet even the least stringent criteria for being homogeneous. Thus, our knowledge of the chemical and gross molecular properties of the pure enzyme is practically nonexistent. However, primarily due to the work of Seegers and associates, comparatively pure preparations of *prothrombin* are now available, and have been extensively studied by a number of workers. Some of the problems in the purification of prothrombin and thrombin have been discussed by Ware and Lanchantin (1954). Seegers and Alkjaersig (1956) have also recently summarized the current status of the problem.

1. *Preparation and Properties of Prothrombin*

In Seegers' procedure, prothrombin is first precipitated at pH 5.1 from diluted plasma, then dissolved and adsorbed on magnesium hydroxide, and eluted by decomposing the hydroxide with CO_2. Further refractionation is accomplished with ammonium sulfate, and finally some of the remaining impurities are removed by adsorbing them on barium sulfate (Seegers, 1952; Seegers and Alkjaersig, 1953). The resulting preparation

shows essentially one main peak in electrophoretic experiments (Seegers *et al.*, 1950), migrating similarly to α_2-globulin, together with small and variable amounts of impurities, similar in their electrophoretic behavior to the activation products of prothrombin. The isoelectric point of prothrombin is 4.2, at an ionic strength of 0.1. In the ultracentrifuge, bovine prothrombin sediments as a homogeneous protein. The earlier reports that prothrombin meets the solubility criterion for a single protein were subsequently reevaluated when it was realized that considerable activation to thrombin occurs under the experimental conditions (Seegers, 1955).

Lamy and Waugh (1953) have investigated the hydrodynamic properties of bovine prothrombin. According to their latest report (Lamy and Waugh, 1954) the sedimentation constant is 4.89×10^{-13} sec., the translational diffusion coefficient is 6.25×10^{-7} cm.^{2}/sec., and the intrinsic viscosity (with concentration expressed in grams/100 ml.) is 0.041. As these authors pointed out, the hydrodynamic parameters of bovine prothrombin are almost identical with those of bovine serum albumin. However, the partial specific volume was found to be 0.70 ml./g. a value which is much lower than that of serum albumin (0.734, according to Dayhoff *et al.*, 1952) and unusually low for a globular protein. Using these values, Lamy and Waugh computed a molecular weight of 62,700 for prothrombin. Also from these parameters a value of 2.13×10^6 is obtained for the quantity β, which characterizes the shape of the effective hydrodynamic ellipsoid (Scheraga and Mandelkern, 1953). Since a β-value of 2.13×10^6 corresponds to an essentially spherical hydrodynamic particle, it is likely that the prothrombin molecule is fairly symmetrical. Lamy and Waugh (1954) have also reported the specific optical rotation of prothrombin as $-31.6°$/decimeter for a concentration of 1 g./100 ml.; this value is considerably different from the value of $-60°$ generally obtained for globular proteins.

The amino acid composition of prothrombin has been determined qualitatively by Ray *et al.* (1953) and quantitatively by Laki *et al.* (1954). The quantitative data for prothrombin are listed in Table I. As pointed out by Laki *et al.*, comparison of these data with those in the literature for bovine and human serum albumin shows that there is no resemblance between the amino acid composition of prothrombin and that of serum albumin. Hence, despite the similarity in hydrodynamic parameters, there is no basis for identifying the two proteins with each other.

Although prothrombin contains 18 amino acids, none of these is present in quantities sufficiently small to make the calculation of a minimal molecular weight from amino acid composition meaningful. There is an as yet unexplained discrepancy between the directly determined sulfur

content and that calculated from the combined methionine and cystine content. Prothrombin contains a considerable amount of carbohydrate; the hexose content recently has been shown by Miller and Seegers (1956) to be due to a polymer of glucose. Laki (1954) has pointed out that the carbohydrate components of prothrombin and heparin are different and

TABLE I

Composition of Prothrombin and Citrate Thrombin (in gram moles amino acid/10^5 grams protein)[a]

Amino acid	Prothrombin	Citrate Thrombin	TCA Supernatant
Aspartic acid	75.3	80	66
Threonine	39.2	35	48
Serine	53.8	48	54
Glutamic acid	87.4	81	102
Proline	48.0	39	66
Glycine	59.8	65	51
Alanine	46.0	44	51
Valine	41.6	40	45
Methionine	13.0	9	21
Isoleucine	24.0	24	24
Leucine	58.0	64	48
Tyrosine	24.2	18	36
Phenylalanine	28.5	23	39
Histidine	11.9	10	15
Lysine	39.3	39	39
Arginine	47.0	40	63
Tryptophan	16.3	—	—
Cystine/2	26.3	—	—
Amide NH_3	68.2	(106)	(0)
Acetyl hexoseamine	9.4	—	—
Hexose	36.1	—	—
Pentose	2.5	—	—

[a] Laki, 1954.

that prothrombin does not contain heparin; hence the prothrombin-thrombin conversion cannot be simply a removal of heparin.

Table I also includes data of Laki (1954) for the amino acid composition of the citrate activation product of prothrombin (citrate thrombin) and of the trichloroacetic acid-soluble supernatant solution of the conversion mixture.

If one were to use the molecular weight of 62,700 (Lamy and Waugh, 1954) one could recalculate the prothrombin data of Table I on the basis of the number of residues per mole. However, in view of the heterogeneity of the resulting thrombin, this calculation cannot be carried out with the other data of Table I.

Pure prothrombin may have an activity at least as high as 1400 units per milligram of dry protein (Seegers *et al.*, 1950). The thrombin unit will be defined in Section II, 4a.

2. *Conversion of Prothrombin to Thrombin*

Many of the hypotheses which have been proposed for the mechanism of the conversion of prothrombin to thrombin have been reviewed by Seegers (1955). Most of the recent attention has been paid to four of the processes by which the conversion can be carried out. These are (1) activation by citrate, (2) activation by calcium ions, Ac-globulin and thromboplastin, (3) activation by calcium ions, platelets and thromboplastin, and (4) activation by trypsin. The products resulting from these methods of activation are all thrombins, i.e. they are all capable of clotting fibrinogen. No differences in the enzymatic properties of these molecules have as yet been detected; however their chemical composition and gross molecular properties almost certainly differ. The product of citrate activation is referred to as *citrate thrombin*, those of processes 2 and 3 and related reactions as *biothrombin*, and finally that of trypsin activation as *trypsin thrombin*.

Of the four processes, the most remarkable is the activation by citrate, as it requires no additional protein activators and, unlike the formation of biothrombin, proceeds without the participation of calcium ions. When prothrombin is dissolved in 25% sodium citrate solution there is a rapid initial decrease in prothrombin activity (see Fig. 1); however, the rise in thrombin activity is gradual at first but then increases more rapidly (Lorand *et al.*, 1953). The loss in prothrombin activity is accompanied by the release of carbohydrate and tyrosine-containing fragments that are soluble in trichloroacetic acid. The delay between the disappearance of prothrombin and the appearance of thrombin is thought to be due to the appearance of an intermediate substance as shown in Fig. 1. Evidence for the existence of such an intermediate substance was provided by Seegers *et al.* (1950) who found that prothrombin was converted into a new electrophoretic component before appreciable amounts of thrombin were produced. At this stage of the process, where prothrombin activity has essentially disappeared and appreciable thrombin activity has not yet appeared, the addition of biological activators to the reaction mixture produces no thrombin activity. The citrate activation of prothrombin to thrombin is thought to be autocatalytic, like the activation of trypsinogen, since the kinetics follow a typical autocatalytic sigmoid curve, and since the addition of thrombin at any time may either initiate or accelerate the reaction. However, further work has uncovered a number of complications, many of which are not as yet fully understood (Seegers, 1955).

The liberation of carbohydrate and peptide material is reflected in the

data of Table 1. Aside from the difference in carbohydrate content, the amino acid composition of thrombin is not very different from that of prothrombin. It is worth noting (Laki, 1954) that the TCA-soluble supernatant solution has a high dicarboxylic acid content compared to the basic amino acids, and essentially no amide ammonia. Hence, the solution would contain acidic material, whose liberation in the prothrombin-thrombin conversion conceivably could play a role analogous to the similar release

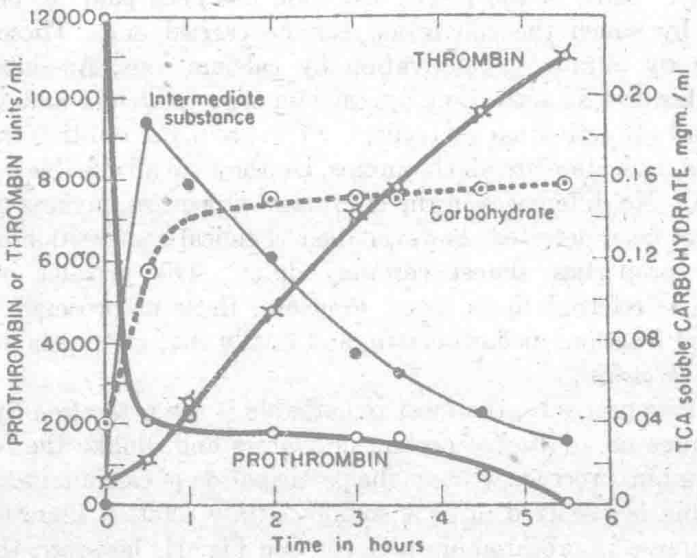

FIG. 1. Rates of various processes in the citrate activation of prothrombin. Purified prothrombin was dissolved in 25% sodium citrate solution. The concentration of added thrombin was 250 units per milliliter. The loss of prothrombin activity coincided with the appearance of TCA-soluble carbohydrate. Later thrombin was formed, and presumably from the intermediate substance. The values for the intermediate substance were obtained by subtracting the sum of the available prothrombin and thrombin activities at any given time from that of prothrombin plus thrombin at the start of the reaction (Lorand *et al.*, 1953).

of acidic peptide material in the fibrinogen-fibrin conversion, as will be discussed in Section VI.

All of these observations give rise to the notion that the activation of prothrombin is a complex series of consecutive reactions, proceeding through the formation of an enzymatically inactive prothrombin derivative, thus accounting for the sigmoid character of the activation curve. The overall reaction, as summarized by Seegers (1955), is:

$$\text{Prothrombin} \rightarrow \text{Prothrombin derivative I} + \text{TCA-soluble material} \qquad (1)$$

$$\text{Prothrombin derivative I} \rightarrow \text{Thrombin} + (?) \qquad (2)$$

The second of these steps is inhibited by 3,4,4′-triaminodiphenyl sulfone and accelerated by 3-chloro-4,4′-diaminodiphenyl sulfone; the difference between accelerated activation of prothrombin and inhibition of the activation is thus determined by the difference between the presence of a chlorine atom or an amino group in a given position of the benzene ring (Seegers *et al.*, 1951). The second step is also inhibited by soybean trypsin inhibitor (Glendening and Page, 1951). The trypsin inhibitor apparently functions by combining with the prothrombin derivative I (Seegers, 1955). The inhibition of the citrate activation by *p*-toluenesulfonyl-L-arginine methyl ester (TAMe) was also tentatively explained by Sherry *et al.* (1954) as being due to a prothrombin-TAMe combination.

The degradative nature of the citrate activation of prothrombin is also indicated by electrophoretic and ultracentrifugal experiments. In electrophoresis experiments, Seegers *et al.* (1950) found that at the end of the activation more than one component had been derived from prothrombin. Ultracentrifugal analyses of Lamy and Waugh (1954) showed four refractive index gradients, the one near the meniscus probably being due to citrate itself. The second, accounting for 15% of the material, had a sedimentation constant of about 2×10^{-13} sec. and a molecular weight of about 15,000; the third represented 32% of the material with a sedimentation constant of 4.1×10^{-13} sec. and a molecular weight of about 45,000; the fourth gradient represented about 50% of the material and was probably caused by aggregates. Separation of the three components showed that thrombin activity resided in the regions of the third and the fourth refractive index gradients.

Little is known about the nature of the bonds broken during the citrate activation. An interesting experiment of Lorand *et al.* (1953) shows that, after the citrate is dialyzed out, the split products (carbohydrate and peptide material) and prothrombin derivative I recombine, and dissociate again on addition of fresh citrate. However, the resulting product is not the original prothrombin. In a more recent paper Seegers and Alkjaersig (1956) have discussed in detail some of the changes occurring in prothrombin reactions, and have described several prothrombin derivatives and their interrelationships.

Carter and Warner (1953) have suggested that S-S bonds are involved in the activation process, since a number of disulfide bond breaking reagents prevent activation.

The role of thrombin in the autocatalytic activation process is uncertain. It is not clear which of the steps thrombin catalyzes and whether any proteolysis is involved.

Activation by biological factors and calcium ions does not seem to require thrombin as a catalyst. During this reaction a small amount of