Analysis of Coagulation

New Methods for the Analysis of Coagulation Using Chromogenic Substrates

Neue Methoden der Gerinnungsanalyse mit chromogenen Substraten

Proceedings of the Symposium of the
Deutsche Gesellschaft für Klinische Chemie
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Foreword

The conference in Titisee on new methods of coagulation analysis using chromogenic substrates was the first large meeting of scientists working in this field.

The development and application of these new methods is only just beginning, so the discussion of methodological problems and further possibilities and goals was an essential part of the conference.

Since the results were to be made available to all interested parties as quickly as possible, it was necessary to overlook a certain diversity in the external form of the manuscripts as well as a number of superficial errors.

Even so, it was only possible to publish the results within 8 months of the conference thanks to the willingness of the authors to provide manuscripts which were ready to print and the cooperation of the publisher, de Gruyter. For this, I appreciate the help of all those involved.

Special thanks go to Dr. Lars Svendsen for his help with difficult passages of the discussion, and to Mrs. G. Wahl, Miss A. Kleffmann, Miss H. Sachs, Dipl. chem. R. Tesch and Mr. M. Schmid for their help in organizing the conference.

I. Witt
Freiburg i. Br. November 1976

Vorwort

Die Konferenz in Titisee über neue Methoden der Gerinnungsanalyse mit chromogenen Substraten war das erste größere Treffen von Wissenschaftlern, die auf diesem Gebiet arbeiten.

Die Entwicklung und Anwendung dieser neuen Methoden steht noch ganz am Anfang. Daher war die Diskussion der methodischen Probleme sowie der weiteren Möglichkeiten und Ziele ein wesentlicher Teil der Konferenz.

Da die Ergebnisse der Tagung möglichst schnell allen Interessierten zugänglich gemacht werden sollten, mußten gewisse Uneinheitlichkeiten der äußeren Form der Manuskripte sowie einige Schönheitsfehler in Kauf genommen werden.

Die Veröffentlichung innerhalb von 8 Monaten nach der Konferenz war aber nur möglich durch die Kooperation der Autoren bei der Herstellung druckfertiger Manuskripte und die gute Zusammenarbeit mit dem de Gruyter-Verlag. Dafür danke ich allen Beteiligten.

Mein besonderer Dank gilt Dr. Lars Svendsen für seine Hilfe bei der Interpretation von schwierigen Diskussionsabschnitten sowie meinen Mitarbeitern Frau G. Wahl, Fräulein A. Kleffmann, Fräulein H. Sachs, Herrn Dipl. Chem. R. Tesch und Herrn M. Schmid für ihre Hilfe bei der Organisation der Konferenz.

I. Witt
Freiburg i. Br., November 1976

Begrüßung

Meine Damen und Herren,

ich möchte Sie herzlich willkommen heißen zum Symposium der Deutschen Gesellschaft für Klinische Chemie über „Neue Methoden der Gerinnungsanalyse mit chromogenen Substraten".

Ich freue mich, daß auch der Präsident unserer Gesellschaft, Herr Delbrück, Zeit gefunden hat, an unserem Treffen teilzunehmen.

Ich glaube, alle Teilnehmer sind davon überzeugt, daß die Gerinnungsanalytik mit chromogenen Substraten ganz neue Wege öffnet, sowohl für die Routineanalytik als auch für die Grundlagenforschung.

Die Entwicklungen stehen noch ganz am Anfang. Daß dieses Thema dennoch für eine Workshop-Konferenz gewählt wurde, geschah aus folgenden Überlegungen:
Gerade weil wir erst am Anfang neuer Entwicklungen stehen, sollten möglichst frühzeitig alle daran arbeitenden Gruppen die Möglichkeit haben, Kontakt miteinander aufzunehmen und durch diesen ersten Erfahrungsaustausch zu einer Kooperation zu kommen.

Da bisher nur wenige Veröffentlichungen vorliegen, war es nicht einfach, die „Chromogenen-Substrat-Spezialisten" aufzuspüren und ich bin sicher, daß ich nicht alle gefunden habe. Erfreulicherweise haben wir aber einen der Urväter der chromogenen Substrate, Herrn Svendsen, für unser Treffen gewinnen können.

Es wäre zu begrüßen, wenn die Kooperation der verschiedenen Arbeitsgruppen zur Entwicklung optimaler und standardisierter Methoden führen würde, die dann in der Routine-Analytik eingesetzt werden könnten.

Ich meine, daß wir es im Augenblick noch in der Hand haben, das zu vermeiden, was früher bei vielen klinisch-chemischen Parametern üblich war und zum Teil auch noch heute üblich ist:
In vielen Laboratorien wurde zwar der gleiche Parameter bestimmt, aber mit ganz unterschiedlicher Methodik. Ich möchte nur an die Bestimmung der alkalischen Phosphatase erinnern. die nach Wroblewski, King-Armstrong, Bessi-Lowry oder Schulze oder Meyer bestimmt werden konnte. Für einen angenäherten Vergleich brauchte man ständig Umrechnungsfaktoren.

Ich brauche nicht näher auszuführen, daß diese Praxis für die Patienten-Versorgung in keiner Weise optimal war. Jahrelange Bemühungen waren und werden noch nötig sein, um einheitliche, standardisierte Methoden für die wichtigsten klinisch-chemischen Parameter einzuführen.

Die Chancen, diesen Weg für die neuen Methoden der Gerinnungsanalytik zu verhindern, sind noch offen. Im Hinblick auf eine optimale Versorgung der Patienten und einen effektiven Einsatz der heute überall beschränkten finanziellen Mittel sollten wir diese Chance nicht ungenutzt lassen.

In diesem Sinne sollten wir unsere ersten Erfahrungen mit den chromogenen Substraten eingehend und kritisch diskutieren. Ob etwas von den hier geäußerten Hoffnungen zu realisieren ist, wird uns vielleicht unsere Abschluß-Diskussion zeigen.

I. Witt

Begrüßung

Meine Damen und Herren,

ich möchte allen, die hier versammelt sind, die Grüße des Vorstandes der Deutschen Gesellschaft für Klinische Chemie überbringen und hoffe, daß dieses Symposium in dem von Frau Witt angedeuteten Sinne ein Erfolg sein wird.

Die Deutsche Gesellschaft für Klinische Chemie veranstaltet nun schon seit mehreren Jahren Kleinkonferenzen, die Experten Gelegenheit geben sollen, in einem geschlossenen und für eine fruchtbare Diskussion limitierten Kreis über neue Entwicklungen oder auch Erfahrungen zu diskutieren. Wir wollen die Ergebnisse, die hier erzielt werden, nicht in diesem kleinen Kreis einzementieren. Das Ziel einer solchen Konferenz ist auf der einen Seite, die Diskussion im kleinen Kreis zu führen, auf der anderen Seite, die so erarbeiteten Informationen an alle auf diesem Gebiet Tätigen zu vermitteln. Die Ergebnisse dieser Konferenzen werden in Form eines Abschlußberichtes in der Zeitschrift für Klinische Chemie und Klinische Biochemie publiziert und durch Sonderdrucke allen Mitgliedern unserer Gesellschaft zur Kenntnis gegeben, so daß jeder mit den Entwicklungen vertraut bleibt, die sich in den einzelnen Fachrichtungen der Klinischen Chemie abspielen.

Ich möchte allen danken, die es auf sich genommen haben, bei dem nicht gerade sehr angenehmen Reisewetter hierherzukommen und ich hoffe, daß sie für ihre Reisemühen entschädigt werden, einmal durch den Ertrag der hier zu leistenden Arbeit und zum anderen auch durch die Atmosphäre, in der wir hier dank der Vorbereitungen von Frau Witt und ihrem Helferkreis diese Konferenz halten können.

Ich wünsche gutes Gelingen.

A. Delbrück

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1 Theory of Coagulation Analysis with Chromogenic Substrates
Theoretische Grundlagen der Gerinnungsanalyse mit chromogenen Substraten

Moderator: L. Róka

1.1 Molecular Aspects of Fibrinogen-Fibrin-Transition

E.A. Beck, M. Furlan, T. Seelich, W. Villiger

Introduction

Human fibrinogen (MW 340'000) is composed of three pairs of polypeptide (Aα-, Bβ- and γ-) chains. Although the primary structure of fibrinogen has been elucidated in part, much less is known about its spatial arrangement (1). Thrombin attacks the N-terminal portion of the α- and β-chains and thereby removes at least four small peptides (2A and 2B, respectively). By convention, fibrinogen-fibrin transition can be summarized as follows:

$$(A\alpha\ B\beta\ \gamma)_2 \xrightarrow{\text{Thrombin}} (\alpha\ \beta\ \gamma)_2 + 2A + 2B$$

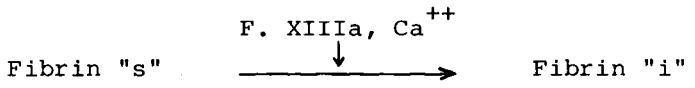
The reaction product is often called "fibrin monomer", although, in chemical terms, both fibrinogen and fibrin have a symmetrical dimeric structure. Under physiological conditions, fibrin molecules aggregate and form a visible gel or

clot. Visible fibrin formation has been the "universal indicator" for indirect measurement of thrombin during decades. This Symposium largely serves to discuss coagulation assays which have become independent of fibrin formation. However, it will be difficult to convince clinical chemists and "coagulationists" to abandon one of the most convenient test systems in existence, i.e. manual or automated measurements of "coagulation times". It is the purpose of this review to show that measurement of thrombin in conventional coagulation assays depends on a complex series of molecular events which, as discussed subsequently, are still poorly understood.

What is fibrin?

Morphologically, fibrin is a gel composed of a fibrous network which is stable enough to enclose the whole content of blood in a semi-solid state, at least until clot retraction starts to extrude serum or fibrinolysis begins to liquefy the clot. Mechanical properties of the fibrin clot, however, depend on a further, rather slow reaction which does not usually influence the "clotting time", the socalled "fibrin stabilization" produced by covalent cross-linking (transpeptidation) in the presence of activated factor XIII (factor XIIIa, Fibrinoligase, Plasma-Transpeptidase). Non-stabilized fibrin is characterized by its solubility at high ionic strength or in dissociating agents, such as urea, weak acid or alkali, and is, therefore, often referred to as "fibrin s" (s for soluble). "Insoluble" fibrin, or fibrin "i", corresponds to a partially or completely cross-linked fibrin network. Factor XIII (precursor) is activated by thrombin. The cross-linking

reaction involves formation of intermolecular γ-glutamyl-ε-lysine bridges between adjacent γ- or α-chains, respectively, and depends on the presence of Ca^{++}-ions. This second reaction can, therefore, be summarized as follows:



The fundamental work leading to these now well established concepts has been summarized in several reviews (e.g. 1, 2).

Recent studies of Kanaide and Shainoff (3) suggest that factor XIIIa is also capable of cross-linking native fibrinogen, although fibrinogen is much less susceptible to the action of factor XIIIa than fibrin. This difference of reactivity could indicate that the fibrinogen-fibrin transition is accompanied by a conformational change. As already mentioned, non-cross-linked fibrin can be kept in solution at high ionic strength (e.g. 1M NaCl). At this salt concentration, the activity of factor XIIIa is not significantly impaired. This system, therefore, seemed of interest to us for studies using various means to simulate the hypothetical conformational change characterized by increased reactivity of fibrinogen with factor XIIIa. The activity of factor XIIIa can be estimated by measuring the amount of cross-linked subunit γ- and α-chains by electrophoresis of reduced fibrin(ogen) on 5 % polyacrylamide gels (subsequently used abbreviation: PAGE). A more convenient means of showing this effect is based on the observation that radioactive or fluorescent labelled amines, such as cadaverine, competitively inhibit fibrin cross-linking by their incorporation and covalent binding to acceptor groups in the presence of factor XIIIa (4). By

measuring the fluorescence of reduced γ- and α-chains on PAGE it is, therefore, possible to study conditions which increase the reactivity of fibrinogen towards factor XIIIa.

Hudry-Clergeon recently proposed a model for fibrinogen-fibrin transition which implies a profound conformational change (5). In contrast to previous studies suggesting an elongated shape of fibrinogen, the French workers assumed, on the basis of electron-microscopic and neutron diffraction studies, that the shape of native fibrinogen in aqueous solutions might rather be spheroid. In this arrangement, the C-terminal parts could be in proximity with the strongly negative N-terminal "fibrinopeptides". By removal (thrombin action) or electrostatic neutralization of some N-terminal negative groups the molecule might open up and expose functional sites responsible for polymerization and interaction with factor XIIIa, i.e. come into an arrangement with neighboring molecules suitable for cross-linking. The cross-linking sites of the γ-chains have been localized in the C-terminal part (6). Since there is only one donor and one acceptor group available for transpeptidation per gamma chain, an antiparallel arrangement of γ-chains has been proposed. In fact, following reduction of cross-linked fibrin, the only species visible on PAGE is a dimer (7). Since this pattern has now been confirmed in many laboratories, every model of fibrin formation has to take account of the γ-chain arrangement as a solid basis. The polymerization and cross-linking sites of the α-chains are less well documented. However, cross-linked and reduced α-chains form large polymers suggesting interactions with more than two adjacent α-chains in fibrin strands (7).

Recent studies performed in our laboratory (8) suggest that neither high ionic strength (1M NaCl) nor 1M urea are capable of dissociating the presumed non-covalent bonds between the N-terminal and C-terminal regions of fibrinogen, since these agents do not increase the susceptibility of fibrinogen towards factor XIIIa. In contrast, protamine, a positively charged protein, indeed increases incorporation of dansyl cadaverine in a very similar way as thrombin itself. It is well known that protamine causes "paracoagulation" of fibrinogen without cleavage of the N-terminal fibrinopeptides. The fibrinogen, precipitated by protamine, presents the characteristic cross-striation of fibrin when studied by electron microscopy (9). In conclusion, fibrin or fibrinogen may, under certain conditions, attain similar reactivity. Thus, the differentiation between "fibrinogen" and "fibrin" depends on the point of view - either macroscopic - physical, or chemical. In order to avoid further confusion by the nomenclature which has been used for the various stages of fibrin formation, we suggest to reserve the term "fibrin" for fibrinogen devoid of fibrinopeptides, and describe gelation as a physical process (aggregation of fibrinogen and/or fibrin), primarily without formation of covalent bonds. The misleading term "fibrin monomer" should be altogether abandoned.

The issue is further complicated by the observation that even "fibrin" is not a functionally homogenous species. Laurent and Blombäck suggested many years ago that cleavage of fibrinopeptides A precedes that of the fibrinopeptides B. A clotting enzyme extracted from Bothrops venom preferentially removes the A peptides and thereby induces clot formation (10). Herzig et al.subsequently showed,by using the venom of another snake,Contortrix, which preferentially removes the B peptides, that visible gelation depends on the cleavage of additional A

peptides (11). Therefore, gelation provoked by proteolytic alteration of the N-terminal part of fibrinogen clearly depends on the removal of fibrinopeptide(s) A. The appearance of the resulting clot resembles that of fibrin clots produced by thrombin at high ionic strength or alkaline pH (10, 12), i.e. the gels are transparent ("fine clot"). This observation led to the general belief that the sole removal of fibrinopeptides A results in "end-to-end" aggregation of fibrin $(\alpha\ B\beta\ \gamma)_2$ with resulting thin fibres. In contrast, the additional removal of fibrinopeptide(s) B by thrombin would produce, at neutral pH and physiologic ionic strength, a turbid or "coarse" clot corresponding to lateral aggregation of fibrils.

These conclusions were challenged by recent experiments performed in our laboratory. First, we found that chemical cross-linking by glutaraldehyde or tetranitromethane of both fibrinogen and fibrin, even at high ionic strength or in 1M urea, is identical (13). This observation indicates that a major conformational change or difference in the pattern of aggregation, consequent to fibrinopeptide cleavage, is rather improbable, but it does not exclude possible minor changes responsible for reactivity with factor XIIIa. Nor do these results exclude secondary shape changes when fibrin molecules are arranged within a fibrin network and exposed to external mechanical forces.

More direct evidence for the type of fibrin aggregation under various conditions was obtained by preferential removal of fibrinopeptides A or B using again the Bothrops enzyme or bat roxobin (14). If peptide cleavage was sufficiently slow it was possible to cross-link resulting intermediate aggregates

using preactivated, thrombin-free factor XIIIa. Further aggregation was prevented by addition of SDS and urea, and the molecular size of intermediate aggregates was measured by PAGE (3 % gels). Preferential removal of fibrinopeptide(s) A resulted in gelation at a time when still significant amounts of fibrin(ogen) "monomers", besides large-polymer aggregates, were present. In contrast, primordial removal of fibrinopeptide(s) B resulted in the appearance of several species of intermediate size aggregates before, as a result of additional removal of fibrinopeptide(s) A, visible clotting occured. It would have been tempting to assume that early elongation of fibrin chains might be associated with γ-chain aggregation and cross-linking. However, no such clear-cut evidence emerged from this experimental approach. We conclude that, to-date, evidence is lacking to ascribe a definite shape to fibrinogen and fibrin. The preferential removal of an N-terminal portion of the β-chain seems to favor complex formation between fibrin and/or fibrinogen molecules which is not necessarily accompanied by visible clotting.

Finally, results and interpretations of studies using electron microscopy should be briefly mentioned with respect to fibrin formation. According to Hall and Slayter (15), fibrinogen can be visualized as an elongated trinodular structure, the linear alignment of which might explain the typical cross-striation of fibrin strands. The three nodules might also correspond to the major "disulphide knots" or the three plasmin-resistant core fragments (two D and one E) of fibrinogen. However, most recent studies failed to show a trinodular structure of fibrinogen molecules (5, 16). By freeze-etching techniques a sausage-like appearance of hydrated fibrinogen molecules was suggested (17), whereas spraying of fibrinogen in volatile buffers onto conventional membranes mostly revealed spheroid

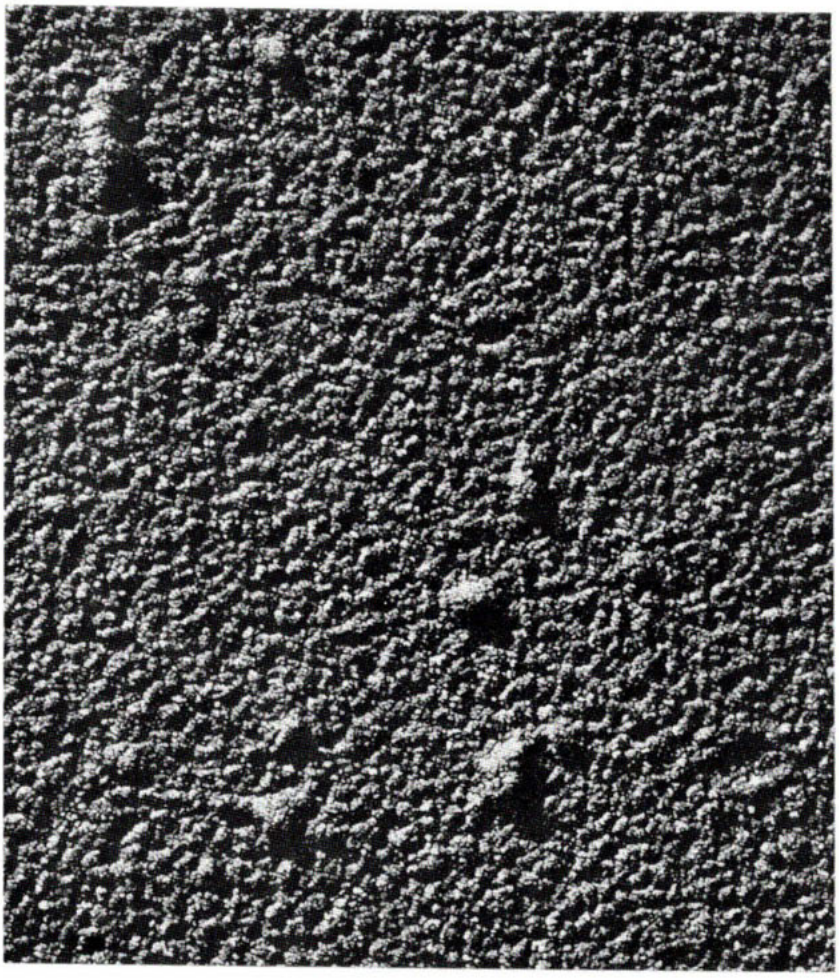


Fig. 1: Single molecules and small aggregates of fibrinogen. A carbon-coated formvar membrane was flushed with fibrinogen (Kabi, grade L, 10 mg/ml). Shadow-cast picture. Original magnification ∿ x 240'000. The smaller molecular species probably corresponds to albumin which was added to improve an even distribution of fibrinogen (0.1 mg albumin per ml fibrinogen solution).

structures. Fig. 1 illustrates the typical appearance of fibrinogen spread on carbon-coated formvar membranes. Our unpublished experiments suggest that a spheroid structure is maintained even at an early stage of fibrin aggregation (fig. 2). The appearance of such aggregates is not strictly linear. It is also of interest to note that smaller aggregates often contact larger fibres at a more or less right angle (fig. 3). More evidence is, however, needed to support or discard the old concept of "end-to-end" and "side-to-side" aggregation of fibrin under various conditions.

Thus, we are still unable to draw definite conclusions concerning the molecular events responsible for fibrinogen-fibrin transition from our own and other published results. A minor conformational change, comparable e.g. with intramolecular shifts of oxygenated versus deoxygenated hemoglobin, might have profound effects on the reactivity of fibrin and/or fibrinogen (for the latter cf. effects of protamine). It is indeed disappointing to note that a plasma protein which has been isolated in reasonably pure form a century ago still holds so many secrets.

Disorders of fibrinogen-fibrin transition and their detection by laboratory tests

As long as fibrin formation serves as "indicator" for studies of thrombin formation, results depend both on a sufficient fibrinogen concentration, an adequate aggregation of fibrin and absence of substances interfering with this latter reaction,