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Blood coagulation factors II, V, VII, VIII, IX, X and XI

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Methods of Enzymatic Analysis

Third Edition

Volume V

Enzymes 3: Peptidases, Proteinases and Their Inhibitors

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Methods of Enzymatic Analysis

Third Edition

Editor-in-Chief: Hans Ulrich Bergmeyer
Editors: Jürgen Bergmeyer and Marianne Graßl

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Never before has enzymatic analysis been of such importance and had so much potential for further development as today. The number of analytes that can be determined with the aid of enzymes, as well as the number of enzymes that are to be determined in various fields, especially in the clinical laboratory, have grown enormously. The combination of enzymatic reactions with new measuring techniques has opened novel fields of application and will continue to do so.

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3.2 Proenzymes, Enzymes, Inhibitors, Cofactors

3.2.1 Blood Coagulation Factors II, V, VII, VIII, IX, X and XI: Determination by Clotting Assays

Maria C. E. van Dam-Mieras, Annemarie D. Muller and
H. Coenraad Hemker

General

When a blood vessel is damaged the bleeding is stopped by the combined action of the vessel wall, thrombocytes and blood coagulation enzymes. The blood coagulation enzymes occur in plasma as inactive precursors and can become activated in a series of consecutive reactions, some of which take place at lipid/water interfaces. In this reaction sequence the product of the first reaction functions as the enzyme in the second reaction, the product of the second reaction functions as the enzyme in the third reaction, and so on. This so-called coagulation cascade is depicted in Fig. 1.

The classical concept of an intrinsic pathway of coagulation, triggered by the exposure of blood to non-endothelial surfaces, and an extrinsic pathway of coagulation, initiated by the interaction of factor VII with tissue thromboplastin, joining at the factor X activation step (bold lines in Fig. 1) has been shown to be an oversimplification [1, 2]. Recent research has shown that the initiating reactions of both pathways are intimately linked (dotted lines in Fig. 1).

As can be seen in Fig. 1, the reactions in which the vitamin K-dependent coagulation factors (II, VII, IX and X) participate proceed at lipid/water interfaces. The efficiency of the reactions catalyzed by the vitamin K-dependent coagulation factors can be regulated through manipulation of their affinity to lipid/water interfaces. Oral anticoagulation therapy is based on this principle.

The ordered and controlled interplay of the coagulation cascade reactions is accomplished by the high degree of specificity of the coagulation enzymes and by a system of positive and negative feedback mechanisms. The majority of coagulation factors are serine proteases; only the factors V_a and $VIII_a$ do not possess intrinsic enzymatic activity, but they form complexes with the factors X_a and IX_a, respectively, thereby markedly stimulating the activities of the latter enzymes by acting as cofactors or accelerators.

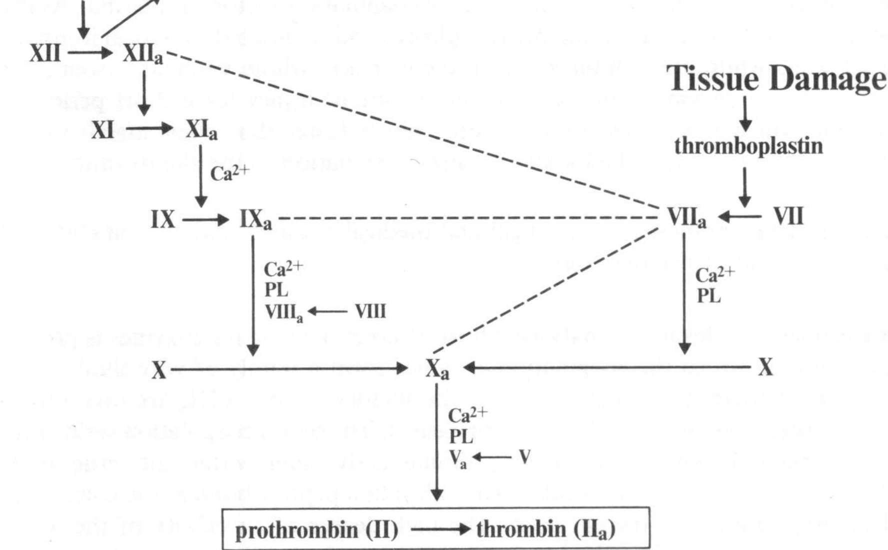

Fig. 1. The blood coagulation cascade, schematic. Possible interrelations between the intrinsic and extrinsic pathways are indicated by dashed lines. PL = phospholipid.

The ultimate visible effect of coagulation is the conversion of soluble fibrinogen into insoluble fibrin by thrombin. However, this is not the only physiological function of this enzyme; other functions of thrombin are:

- the activation of thrombocytes: thrombin causes platelets to aggregate and to make available the phospholipids necessary for the clotting process (the procoagulant lipid/water interface).
- the activation of the factors V and VIII.
- the activation of protein C, a vitamin K-dependent proteinase that inactivates the factors V_a and $VIII_a$.
- partial proteolysis of the prothrombin molecule, thereby converting it into prethrombin 1, a molecule that is activated much more slowly by factor X_a than the original prothrombin molecule; this mechanism seems not to be of relevance under *in vivo* conditions.
- the activation of factor XIII: factor $XIII_a$ stabilizes the polymeric fibrin network by covalent linkage of the polymers.

Present evidence suggests that the coagulation process is autocatalytic and self-limiting and that thrombin plays a central role in the whole haemostasis process.

The circulation of activated coagulation factors in blood would create an extremely dangerous situation [3]; therefore, inhibitors of the activated coagulation factors are also present to prevent the persistence of active coagulation factors in plasma. As the generation of active coagulation factors is explosive and is initiated by a local injury of the vessel wall, whilst the inhibitors are present in the whole vascular system, the active coagulation enzymes can only exist at the site of injury for a short period of time (during which their formation proceeds much faster than their inactivation). Together the processes described above localize coagulation to the site of injury.

Application of the methods: biochemical and medical research and clinical chemical analysis in the field of haematology.

Enzyme properties relevant in analysis: a host of potentially active enzymes is present in plasma among which the coagulation enzymes form a family of very similar proteases. Most of them are serine proteases; the factors V_a and $VIII_a$ are involved as cofactors in reactions catalyzed by serine proteases. The blood coagulation serine proteases have been shown to contain very similar active sites within the structurally homologous regions of their molecules. They all split a peptide bond at the C-terminal site of an Arg residue, as trypsin does. The high degree of specificity of the blood coagulation enzymes is primarily due to the presence of an amino-terminal extension chain, the "pro" part, which is not involved in the catalytic reaction *per se*.

The measurement of a specific clotting factor in plasma is not a simple straightforward procedure: the time course of product formation is very complicated and depends on the concentrations of the coagulation factor to be assayed and that of its potential activator(s) and inhibitor(s). The quantitative determinations of clotting factor activities in plasma are therefore essentially bioassays, in which the results are obtained by comparing the samples to be tested with a series of dilutions of a standard plasma.

Methods of determination: the determination of coagulation enzymes can be based upon at least two different principles: an immunological assay and a biological activity measurement. A general disadvantage of the immunological assay techniques is that they determine an amount of protein and not the biological activity of this protein. Usually the one-dimensional "rocket" immunoassays do not discriminate between the different molecular forms of the coagulation enzymes. Under some circumstances (oral anticoagulation, some types of congenital deficiencies) there may be large discrepancies between the two types of tests.

The quantitative determination of clotting enzymes by biological activity measurement is carried out in a bioassay type of determination by comparing the sample to be tested with various concentrations of the coagulation factor, obtained by dilution of standard plasma. A plasma deficient in the coagulation enzyme to be determined constitutes a medium in which all coagulation factors except the one to be determined

are present in excess. The coagulation enzyme to be determined, present in the suitably diluted sample, will then be rate-limiting.

The amount of enzymatic activity generated can be measured by conventional clotting techniques and by determinations carried out with synthetic substrates.

International reference methods and standards: international standardization of coagulation tests is a very complicated and, so far, an incompletely solved problem. There are at least 4 groups of variables that influence the final outcome of the coagulation test:

1. the collection and treatment of the sample,
2. the composition of the mixture or the type of trigger used to initiate the clotting reaction (e.g. thromboplastin, snake venoms, activated coagulation factor complexes),
3. the preparation of a standard used for the construction of standard curves,
4. the instrumental details of the measurement.

The first attempt to reach international standardization of clotting tests was the introduction of *reference thromboplastins* to which all other thromboplastins could be compared [4–7].

In this thromboplastin calibration, the Prothrombin-Time ratios (quotient of patient PT and mean normal PT) obtained with a series of freshly prepared plasmas from normal subjects and from patients under stable oral anticoagulant treatment, when tested with the thromboplastin preparation to be calibrated, are compared with those obtained when the reference thromboplastin is used. In 1976, the World Health Organisation (WHO) established the human brain-type combined thromboplastin, which contains also fibrinogen and factor V (ICTH reference material 67/40; ICTH = International Committee on Thrombosis and Haemostasis), as the primary WHO reference material. The term International Calibrated Ratio (ICR) was introduced (the ratio obtained by calibration against the primary WHO reference material). In 1979, the bovine combined thromboplastin (ICTH reference material 68/434) and the rabbit brain thromboplastin without added coagulation factors (ICTH reference material 70/178) were accepted as secondary WHO reference materials [8, 9].

In 1979, a report on Prothrombin Time Standardization was published by an Expert Panel of the International Committee on Thrombosis and Haemostasis (ICTH) and the International Committee for Standardization in Haematology (ICSH) [10]. A collaborative study from 199 laboratories was published in this report, in which 9 commercial thromboplastins, 4 thromboplastins held by the National Institute of Biological Standards & Control (NIBSC) and the British Comparative Thromboplastin (BCT) were tested on fresh normal and coumarin plasma, and on three series of freeze-dried plasmas (normal and coumarin plasmas).

Implementation of the WHO recommendations in clinical practice has not been without problems. In the first place the WHO reference material is only available in national reference centres and is not freely available for the calibration of commercial

thromboplastins. Furthermore, the WHO human brain preparation did not fit well into the established British system which is also in use in other countries. Therefore, a thromboplastin calibration study was carried out by the European Community Bureau of Reference (BCR) [11]. The BCR reference materials are available in sufficient amounts for the calibration of commercial thromboplastin preparations. The BCR preparations are a human thromboplastin, a bovine thromboplastin and a rabbit thromboplastin. In this calibration study the traditional approach of calibration of one thromboplastin in terms of another by a straight line through the point 1,1 was found not to be generally valid and a rectilinear relation of the logarithms of the PT-ratios was used [12]. It was proposed to replace the term calibration constant by the term International Sensitivity Index (ISI).

All the standardization studies described above focused upon thromboplastin standardization. International standardization based upon the use of *reference plasmas* has also been proposed [13, 14], but in fact standardization of both thromboplastins and plasmas used in the calibration procedure will be necessary.

Reference plasmas are freeze-dried materials of human origin. Reference plasmas comparable to fresh plasmas of orally anticoagulated patients can be prepared by freeze-drying of plasma samples from coumarin-treated patients, or by selective absorption of the vitamin K-dependent coagulation factors from normal plasma. The first group contains decarboxy coagulation factors (cf. chapter 3.1, pp. 316–351), whereas the second group does not. This discrepancy can possibly explain the different behaviour of both groups of reference plasmas towards thromboplastin preparations [15]. At present, carefully prepared fresh plasma from individual patients under stable oral anticoagulant therapy and from normal subjects still seems to be the best standard for primary calibration of thromboplastins, although evidence is forthcoming that lyophilized plasma prepared from patients under stable oral anticoagulation can also be used for this purpose [16–19].

The problems encountered when working with other activators, such as snake venoms and complexes of activated coagulation factors, will not be much simpler, but as their standardization has not been studied so widely as thromboplastin standardization the subject will not be discussed further here.

For *thrombin* a WHO standard preparation is available (cf. Vol. II, chapter 2.3).

Enzyme effectors: as is common in enzymology, the catalytic efficiency of the coagulation factors is influenced by the pH and ionic strength of the medium and by the type and concentration of ions present. Ca^{2+} is necessary in a variety of ways for most of the interactions of clotting factors. The medium in which the enzymatic reaction is measured has to be specified in these respects.

In the test with synthetic substrates one must always be aware that the relatively small synthetic substrates will never be completely specific. In some instances the specificity of an assay can be improved by adding a compound which specifically inhibits an interfering protease (e.g. hirudin for thrombin, or aprotinin for plasmin).

During the development of a specific test, the presence of unwanted activities must always be checked in control experiments in which all substances but the factor to be determined are present.

Assays

Method Design

Principle: when the biological activity of a clotting factor is determined in a conventional clotting assay, a plasma congenitally deficient in the clotting factor to be determined constitutes a medium in which all coagulation factors except the one to be determined are present in excess. The factor to be determined is added to the sample and, through the experimental design, forms the rate-limiting step in the coagulation cascade. The visible end-point of the process is the conversion of soluble fibrinogen into insoluble fibrin. Quantitative analysis is based upon the measurement of the time interval between the initiation of clotting and the appearance of a visible fibrin clot (the clotting time). The clotting time of the sample is compared to those obtained with a series of dilutions of a standard plasma.

Optimized conditions for measurements: since the activity of the coagulation enzymes varies with pH and ionic strength, the presence of additional metal ions and/or other compounds (e.g. inhibitors of unwanted enzyme activities) and temperature, rigorous standardization of the experimental conditions is necessary. Details are given in the individual assays described below.

The most commonly used buffers are Tris/HCl, Tris/imidazole and *Michaelis* buffer. The desired ionic strength is usually obtained with NaCl. It may further be necessary to add special substances to the reaction medium: 0.1% sodium azide prevents microbial growth; 0.1% – 0.5% Carbowax 6000 (polyethyleneglycol, PEG) prevents absorption of enzymes onto the walls of the reaction vessel. Instead of Carbowax, 1% ovalbumin or serum albumin can be used. In this case a check for the absence of any activity in the albumin preparation should be made. Addition of Carbowax 6000 or albumin is especially important when low concentrations of (purified) enzyme are used. This problem does not arise with plasma samples, and in general at relatively high protein concentrations.

In view of the complicated dependence of the reaction velocity upon the temperature in biological systems, the temperature must carefully be kept constant during the determination. An assay temperature above the ambient temperature is preferable. Coagulation assays are always carried out at 37 °C.

Temperature conversion factors: because phospholipids are an essential reaction component in coagulation systems, and because it has been shown that the temperature dependence of this type of reaction changes abruptly at the melting temperature of the lipid [20], it is strongly recommended to avoid the use of temperature conversion factors.

Equipment

A thermostatted water-bath (37 °C), a *Kolle* hook and stopwatches or a thermostatted coagulometer. Pipettes (plastic), plastic tubes (to prepare the plasma dilutions), glass tubes (for the assay).

Reagents and Solutions

Purity of reagents: all chemicals used to prepare solutions must be of A. R. grade.

Preparation of solutions: all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. *Michaelis* buffer (barbiturate, 0.028 mol/l, acetate, 0.028 mol/l; NaCl, 0.116 mol/l; pH 7.35):

   dissolve 3.86 g acetate-Na · $3H_2O$, 5.86 g sodium barbiturate and 6.8 g NaCl in 600 ml water. After dissolution of the salts the volume is brought to 800 ml with water and finally 200 ml HCl, 0.1 mol/l, is added. The pH is checked and adjusted with HCl, 0.1 mol/l, or NaOH, 0.1 mol/l.

2. $CaCl_2$ solution (0.033 mol/l):

   dissolve 0.49 g $CaCl_2 \cdot 2H_2O$ in water and adjust the volume to 100 ml.

3. Citrate solution (0.109 mol/l):

   dissolve 32.1 g citrate-$Na_3 \cdot 2H_2O$ in water and adjust the volume to 1000 ml.

4. NaCl solution (saline, 0.15 mol/l):

   dissolve 9 g NaCl in 1000 ml water.

5. Platelet-poor plasma:

   obtain plasma from fresh blood collected in plastic tubes containing citrate (9 volumes blood + 1 volume sodium citrate, 0.109 mol/l) by centrifugation (15 min at 13000 *g*).

6. Platelet-free plasma:

   obtain platelet-free plasma by centrifugation of platelet-poor plasma (20 min at 20000 *g* at 4 °C).

7. Standard plasma:

   obtain standard plasma by pooling equal amounts of platelet-free plasma from at least 30 healthy individuals (15 males and 15 females, average age 30 years) and store in small portions at − 20 °C or preferably at − 80 °C until use.

   Plasma samples to be stored should be prepared free from platelets before storage.

8. Thromboplastin:

   prepare from human brain according to a modification of the method of *Owren & Aas* [21 – 23].

Wash human brains under cold running water. After the removal of membranes and blood vessels, repeat washing with NaCl solution (4), cut the brains into small pieces and add 1.5 litre NaCl solution (4), pre-warmed to 37 °C. Homogenize the mixture and keep it at 37 °C for 120 min. Then add 0.5 litre buffered NaCl solution (9 parts NaCl solution (4) + 1 part *Michaelis* buffer (1)) to the suspension. Centrifuge the mixture for 30 min at 1250 *g* and carefully pipette off the supernatant. The supernatant can usually be diluted without significant loss of activity. For this purpose, determine the thromboplastin times of a series of diluted solutions of the supernatant (made in the buffered NaCl solution described above) acting on standard plasma. In practice, the diluted solution that gives a thromboplastin time of 13 seconds is chosen. After dilution store the product in small portions at −20 °C preferably at −80 °C until use. Prior to use pre-warm the thromboplastin solution at 37 °C for at least 20 min and not more than 120 min.

9. Phospholipid (cephalin) suspension (ca. 28 g/l):

   prepare according to a slight modification of the method of *Milstone* [22–24]. Wash human brains under cold, running water. After the removal of membranes and blood vessels, repeat washing with NaCl solution (4). Remove the cortex and homogenize the cortex in 300 ml acetone. Centrifuge the suspension for 15 min at 1250 *g* and remove the supernatant. Repeat the washing about 6 times, using fresh acetone each time. Extract the residue with 1.8 litre ether during about 12 h at room temperature. Remove the ether fraction and evaporate the ether on a water-bath at 35 °C. Wash the residue twice with acetone (washing during 15 min with 900 ml acetone followed by centrifugation for 15 min at 1250 *g*). Dissolve the residue in 200 ml ether. Evaporate the ether as described above and finally evaporate the residue under vacuum for 75 min. Suspend the residue (about 10 g) in 200 ml *Michaelis* buffer (1) with the aid of a blender. Centrifuge the suspension for 20 min at 350 *g* and thereafter for 10 min at 750 *g*. Discard the sediment. Store the supernatant, containing about 2.8 g cephalin per 100 ml, in small portions at −20 °C or −80 °C. Prior to use dilute the phospholipid suspension with *Michaelis* buffer (1); the final dilution depends on the specific test to be carried out.

10. Kaolin/phospholipid suspension (kaolin, 21 g/l; phospholipid, 0.5 g/l):

    suspend 4.17 g koalin in 100 ml NaCl solution (4). Prepare the kaolin/phospholipid suspension immediately before use according to *Horowitz et al.* [25]. After thawing dilute the phospholipid suspension with *Michaelis* buffer (1) to a final concentration of 1 mg/ml. Mix equal volumes of phospholipid suspension (9) and kaolin suspension.

11. Clotting factor-deficient plasma:

    plasma deficient in the factors II, V, VII, VIII, IX, X and XI respectively can be obtained commercially.

    Factor-deficient reagents can also be prepared from plasmas from patients with a congenital deficiency or can be prepared artificially as indicated below.

a) Factor II-deficient reagent: according to a modification of the method described by *Koller et al.* [22, 23, 26].

b) Factor V-deficient reagent: according to *Borchgrevink et al.* [27].

c) Factor VII-deficient reagent: according to *Hemker et al.* [28].

d) Factor X-deficient reagent: according to *Hemker et al.* [28].

e) Factor VIII-deficient reagent: according to *Chantarangkul* [29].

f) Factor IX-deficient reagent: from commercial sources.

g) Factor XI-deficient reagent: according to *Horowitz et al.* [25].

The amount of factor y in a factor y-deficient reagent must be lower than 1%; this can be checked by the method described by *Hemker et al.* [28].

Stability of solutions: store all buffer and salt solutions (1) – (4) at 0 – 4 °C. Buffer and salt solutions will be stable as long as no microbial contamination occurs. Thromboplastin solutions (8), platelet-free plasma (6), factor-deficient reagents (11, 12) and phospholipid suspension (9) have to be stored at – 20 °C or preferably at – 80 °C and will be stable under these conditions for at least 1 year. Once thawed, the solutions must be kept on ice and used within a few hours.

Procedure

Collection and treatment of specimen: collect blood obtained by venepuncture without stasis in plastic tubes containing citrate (9 volumes blood + 1 volume sodium citrate solution, 0.109 mol/l). Discard the first few ml of blood if possible. Some types of vacuum tubes may cause an increased activation of plasma factors.

Prepare platelet-poor plasma by centrifugation (15 min at 13000 *g*). Use only plastic materials during the blood sampling and plasma preparation procedures. Store plasma samples at – 20 °C or – 80 °C prior to testing; the samples must be prepared free from platelets prior to storage (20 min centrifugation at 20000 *g* at 4 °C).

Stability of the enzyme in the sample: if frozen at – 20 °C or – 80 °C the enzymes in plasma will be stable for at least 1 year; once thawed the solutions must be kept on ice and used within a few hours.

The stability in solution of purified factors is at least the same as that described above for plasma; in diluted solutions it may be necessary to prevent adsorption of the enzymes to the vessel wall by the addition of 0.1% – 0.5% Carbowax 6000 or 1% ovalbumin or serum albumin.

Assay conditions: clotting assays are carried out in glass tubes in a thermostatted water-bath or in a thermostatted coagulometer at 37 °C; dilutions of sample and

standard plasma are made in plastic tubes. Pre-warm thromboplastin solution (8) (for at least 20 and not more than 120 min) and $CaCl_2$ solution (2) to 37 °C.

Prior to measurement the sample to be determined is diluted 1:10 or 1:20 in *Michaelis* buffer (1). All determinations are carried out in duplicate. Assay volume 0.4 ml; volume fraction of sample 0.25.

Measurement: factors II, V, VII, and X

Pipette successively into a glass tube:		
congenital or artificial factor II, V, VII or X-deficient reagent	(11 a/b/c/d)	0.1 ml
diluted sample or diluted standard plasma	(7)	0.1 ml
thromboplastin solution	(8)	0.1 ml
incubate 30 s		
$CaCl_2$ solution*	(2)	0.1 ml
record the clotting time (time interval between $CaCl_2$ addition and the appearance of the fibrin clot).		

* final concentration 8.25 mmol/l

The clotting time measured with the unknown sample must lie within the range obtained with the diluted solutions of the standard plasma. The drawbacks of this method are numerous [28].

Standard curve: the following dilutions of standard plasma in *Michaelis* buffer (1) are used for the construction of the reference curve: 10%, 5%, 2.5%, 1.67%, 1.25%, 1%, 0.5 %.

The clotting times obtained with the dilutions of the standard plasma are traditionally plotted against the factor y concentration (in % of standard plasma; y = II, V, VII or X) on double logarithmic paper and this graph is used to read the factor concentration in an unknown sample, the coagulation time of which is known.

Calculation: the amount of factor II, V, VII or X, respectively, in the inknown sample has to be determined by comparison of the clotting time obtained with the diluted sample to the clotting times obtained with a series of diluted solutions of standard plasma.

For the factors of the extrinsic system (II, V, VII, X) the relationship found by *Hemker et al.* can be used:

$$t_\rho = t_{\min} + \rho_{2t_{\min}} \times t_\rho \times \frac{1}{\rho_r + \rho_s}$$

in which

t_ρ coagulation time, min,
t_{min} minimum coagulation time at infinite concentration of the rate limiting factor, min,
$\rho_{2t_{min}}$ clotting factor concentration that will cause a $t_\rho = 2 \times t_{min}$; % of standard plasma,
ρ_r clotting factor concentration in the reagent; % of standard plasma,
ρ_s clotting factor concentration in the sample; % of standard plasma.

In a given assay system t_{min}, $\rho_{2t_{min}}$ and ρ_r are constants which may be deduced from the data obtained with known sample concentrations, by calculation or by a computer program [28]. The constants t_{min}, $\rho_{2t_{min}}$ and ρ_r must be determined each time a new batch of thromboplastin or reagent solution is used. The level of clotting factor in the unknown sample can be read from the double logarithmic plot or from a computer-constructed reference table [28].

Measurement: factors VIII, IX and XI

Pipette successively into a glass tube:			concentration in assay mixture	
congenital or artificial factor VIII, IX or XI-deficient reagent	(11 e/f/g)	0.1 ml		
diluted sample or diluted standard plasma	(7)	0.1 ml		
kaolin/phospholipid suspension	(10)	0.1 ml	kaolin phospholipid	0.12 g/l 5.21 g/l
incubate 20 min at 37°C				
$CaCl_2$ solution	(2)	0.1 ml	$CaCl_2$	8.25 mmol/l
record the clotting time (time interval between the addition of $CaCl_2$ and the appearance of the fibrin clot).				

The clotting time measured with the diluted unknown sample must lie within the range obtained with the dilutions of the standard plasma.

Standard curve: the following dilutions of standard plasma (in buffer 1) are used for the construction of the standard curve: 2.5%, 1.67%, 1.25%, 1.0%, 0.5%. The

clotting times obtained with the dilutions of the standard plasma are plotted against the factor y concentration (in % of standard plasma; y = VIII, IX or XI) on double logarithmic paper, as here the relationship $t_\rho = f(\rho)$ is unknown.

Calculation: the amount of factor VIII, IX or XI, respectively, in the unknown sample has to be determined by comparing the clotting time obtained with the sample to the clotting times obtained with a series of diluted solutions of standard plasma. The amount of the corresponding factor in the unknown sample can be read from the double logarithmic plot and expressed in % of standard plasma.

Validation of Methods

Precision, accuracy, detection limit and sensitivity: in the concentration range of 10 – 100% of standard plasma, one-stage estimations of the extrinsic coagulation factors (II, V, VII, X) show a standard deviation in our hands of 7 – 10%. The standard deviations of one-stage estimations of the intrinsic system factors (VIII, IX, XI) are from 10 – 16%.

The detection limit is about 1% of the activity present in standard plasma. The lower detection limit is dependent upon the residual activity of the respective coagulation factor in the reagent used.

A very precise method to calculate concentrations from 0.05 – 2% of standard plasma is offered by the use of the computer program available from the authors.

Sources of error: in a clotting assay, the amount of a specific clotting factor in the sample has to be determined from its influence upon the overall coagulation process. Therefore, the activity measured is determined not only by the total amount of the clotting factor but also by its actual molecular structure (slight differences in the molecular form of a clotting factor may have very important consequences for its biological activity), and by the presence of potential activators(s) and inhibitor(s) in the test system. It is evident that in bioassays of this type a rigorous standardization of the experimental conditions is necessary.

Specificity: the experimental design of the clotting assay is such that the clotting factor to be determined forms the rate-limiting step in the coagulation cascade. However, when a specific test is carried out in a complicated biological system, the presence of unwanted activities must always be checked in control experiments in which all substances but the factor to be determined are present.

Reference ranges: given as approximate concentrations in plasma in nmol/l corresponding to the 100% values of the respective standard plasma:

Factor	II	V	VII	VIII	IX	X	XI
Ref. range	1500	30	10	0.2	70	180	30

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3.2.2 Blood Coagulation Factors II, V, VII, VIII, IX, X and XI: Determination with Synthetic Substrates

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General

For information on the coagulation cascade, enzyme properties, application of methods, methods of determination and standardization cf. chapter 3.2.1, pp. 352 – 356.

Assays

Method Design

Principle: with the use of synthetic substrates it is no longer necessary to measure a coagulation enzyme through its influence upon the complete coagulation cascade; synthetic substrates make possible the measurement of isolated steps of the coagulation process. Synthetic substrates contain a peptide part, designed to be relatively

specific towards a given coagulation factor, and an additional group which produces a physically measurable effect when it is split off from the substrate (there are, e.g., chromogenic, fluorigenic, luminogenic and electrogenic leaving groups [1]).

The principle of activity determinations with synthetic substrates is depicted in Fig. 1. Because the first step in the reaction sequence is rate-limiting, the liberation of the leaving group is a measure of the velocity of substrate hydrolysis.

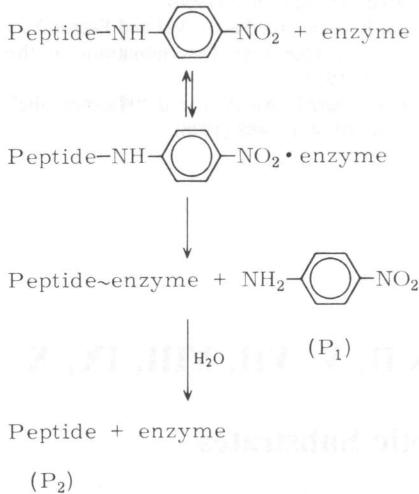
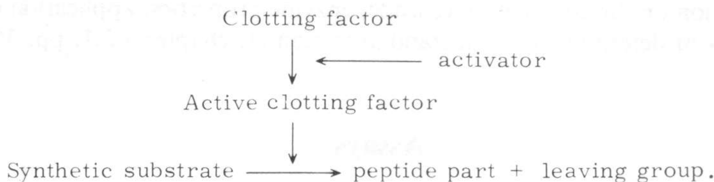


Fig. 1. Principle of the clotting factor activity determination with a chromogenic substrate. For details, see text.

A problem encountered when setting up an assay of coagulation parameters with a synthetic substrate is that clotting factors occur in blood as inactive zymogens, which must be activated before the activity can be determined in a synthetic substrate assay. Therefore, the procedure must be as follows:



Test procedures with synthetic substrates are relatively simple and can be automated. However, the small synthetic substrates will never be completely specific and differences between the enzymatic activities towards natural and synthetic substrates will occur. Therefore, the determination of coagulation enzymes in biological material with synthetic substrates can only be carried out properly in a "bioassay type" determination. This means that the change in the physical parameter produced by the

enzyme to be determined must be compared with the changes produced in that parameter by a series of dilutions of standard plasma. Only in model systems with purified coagulation factors can real "chemical determinations" be carried out with synthetic substrates.

Examples of chromogenic substrates*:

S 2222	Bz-Ile-Glu-(-OR)-Gly-Arg-4-NA
S 2337	Bz-Ile-Glu-(γ-Piperidyl)-Gly-Arg-4-NA
S 2238	H-D-Phe-Pipq-Arg-4-NA
S 2160	Bz-Phe-Val-Arg-4-NA
Chromozym® TH	Tos or CBz-Gly-Pro-Arg-4-NA

* The substrates of the "S" series are made by *Kabi Vitrum AB,* Stockholm, Sweden and the "Chromozyme" series by *Pentapharm,* Basel, Switzerland.

When synthetic substrates are used for the detection of enzymatic activity they should preferably be added at a concentration at least two times K_m. Also, the change in the physical parameter to be measured should be large enough to give reliable data. The implications of the latter requirement for the substrate concentration to be used depend on the type of leaving group. These conditions will be specified in the individual assays to be described.

Equipment

A thermostatted water-bath (for pre-warming the bulk medium), a spectrophotometer or spectral-line photometer capable of exact measurement at a wavelength in the range of 380 – 410 nm with a thermostatted cuvette holder, a fluorimeter with a thermostatted cuvette holder, a recorder or stopwatch, pipettes, cuvettes.

3.2.2.1 Determination of Factor II_a (Thrombin) and Other Assays Based on Factor II_a Determination

Thrombin can be measured with the chromogenic substrates S 2238, S 2160 and Chromozym® TH*. These substrates are not only split by α-thrombin, but also by β-thrombin, γ-thrombin, meizothrombin and coagulase-thrombin.

The specific activities of the various forms of thrombin towards different chromogenic substrates may vary [2, 3, 4]. Much less variation is encountered, however, with synthetic substrates than with natural substrates. Therefore, there is not necessarily a fixed relation between biological and amidolytic activity in different thrombin preparations.

* Registered trademark of *Pentapharm AG,* Basel, Switzerland, supplied by *Boehringer Mannheim.*

Factor II_a estimation is the basis of various assays for *factor II, antithrombin III* (heparin cofactor), *heparin* and antiheparins such as *platelet factor 4*. In addition, the factors constituting the *prothrombinase complex* can be measured by assessing the velocity of factor II activation; this leads to *factor V* and *platelet factor 3* determinations.

Equipment

Spectrophotometer capable of exact measurement at 405 nm; thermostatted cuvette holder.

Reagents and Solutions

Purity of reagents: all chemicals used must be of analytical grade.

Preparation of solutions: all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. Tris buffer (Tris, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8):

   dissolve 6.05 g tris(hydroxymethyl)aminomethane and 5.84 g NaCl in 900 ml water. Bring the pH to 7.8 with 37% HCl and afterwards bring the volume to 1000 ml with water. Measure the pH and adjust it to 7.8 with HCl, 0.1 mol/l or NaOH, 0.1 mol/l.

2. Tris buffer (Tris, 50 mmol/l; NaCl, 0.1 mol/l; human serum albumin, 0.5 mg/ml; pH 7.8):

   supplement Tris buffer (1) with 0.5 g human serum albumin per 1000 ml.

3. Tris buffer (Tris, 50 mmol/l; NaCl, 0.1 mol/l; human serum albumin, 0.5 mg/ml; EDTA, 20 mmol/l; pH 7.8):

   supplement Tris buffer (1) with 0.5 g human serum albumin and 7.44 g EDTA-$Na_2 \cdot 2\,H_2O$ (Titriplex III, *Merck*, Darmstadt) per 1000 ml.

4. $CaCl_2$ solution (0.1 mol/l):

   dissolve 1.47 g $CaCl_2 \cdot 2\,H_2O$ in water and adjust the volume to 100 ml.

5. Citrate solution (0.109 mol/l):

   dissolve 32.1 g citrate-$Na_3 \cdot 2\,H_2O$ in water and adjust the volume to 1000 ml.

6. Platelet-poor plasma:

   cf. solution (5), p. 358.

7. Platelet-free plasma:

   cf. solution (6), p. 358.

8. Standard plasma:

   cf. solution (7), p. 358.

9. *Russell*'s viper venom (RVV*) solution (0.1 mg/ml):

   dissolve 1 mg RVV in water and adjust the volume to 10 ml.

10. Soybean trypsin inhibitor (SBTI) solution (inhibitor, 4 mg/ml; Tris, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8):

    dissolve 40 mg soybean trypsin inhibitor in Tris buffer (1) and adjust the volume to 10 ml.

11. Phospholipid suspension (0.75 mg/ml; 25% of which is brain phosphatidylserine (PS) and 75% egg phosphatidylcholine (PC); Tris, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8):

    purify brain PS from brain extract (*Folch* III extract, *Sigma*) according to *Comfurius & Zwaal* [5]. Dissolve 500 mg brain extract in 10 ml chloroform and apply to a CM-cellulose column (CM-52, *Whatman*) equilibrated with chloroform. Use 1 ml CM-cellulose per 5 mg total lipid. Subsequently elute the column with 3 (column) volumes chloroform, 3 volumes chloroform/methanol 9:1, and 20 volumes chloroform/methanol 8:2. Elute the PS from the column with 5 volumes chloroform/methanol 1:1. Discard the first 0.5 volume of the 1:1 eluate. Dry the remaining 4.5 volumes by rotary evaporation and dissolve the PS in chloroform/methanol 1:1 in a concentration of 10 mg/ml.

    Obtain a 10 mg/ml solution of egg PC in chloroform/methanol 1:1 by diluting a solution containing 100 mg egg PC per 100 ml chloroform/methanol (1:1) (*Sigma*) 1:10 with chloroform/methanol 1:1.

    Prepare the phospholipid suspension by mixing 0.37 ml PS solution (10 mg/ml in chloroform/methanol 1:1) with 1.13 ml PC solution (10 mg/ml in chloroform/methanol 1:1). Remove the organic solvent by a stream of nitrogen. To the dry lipids add 2 ml Tris buffer (1) and suspend the lipids by agitating on a vortex mixer for 5 minutes. Prepare single bilayer (unilamellar) vesicles by sonicating the

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* RVV *Russell*'s viper venom
SBTI soybean trypsin inhibitor
PS phosphatidylserine
PC phosphatidylcholine

lipid suspension for 10 min at 0 °C, using a *MSE* Mark II 150 watt ultrasonic desintegrator. Place the end of the tip of the sonication probe in the middle of the lipid suspension. Energy output is at 8 micron peak-to-peak amplitude. Dilute the stock solution of 7.5 mg/ml 10-fold in Tris buffer (1) and store the solutions (0.75 mg/ml) at −70 °C. After thawing, re-sonicate the phospholipid suspension as described above.

12. S 2238 solution (2.35 mmol/l):

    dissolve the content of one vial S 2238 (25 mg) in 17 ml water to obtain a stock solution of 2.35 mmol/l.

13. Factor II solution (10 µmol/l):

    in Tris buffer (2); cf. Appendix, p. 390, or obtain commercially.

14. Factor X_a solution (150 nmol/l and 0.05 nmol/l, respectively):

    in Tris buffer (2); cf. Appendix, p. 392, or obtain commercially.

Stability of solutions: store all buffer and salt solutions (1) – (5) at 0 – 4 °C; they are stable as long as no microbial contamination occurs.

Plasma (6) – (8), factor II (13) and factor X (14) solutions and phospholipid suspension (11) have to be stored at −20 °C or −80 °C and are stable under these circumstances for at least one year. Once thawed, the plasma, factor II and factor X solutions must be kept on ice and used within a few hours. The phospholipid suspension must be re-sonicated after thawing (2 min at 0 °C, 8 micron peak-to-peak amplitude) and is stable for 2 weeks at 0 °C. SBTI and RVV solutions (10), (9) are stable at 0 °C for several days.

Chromogenic substrates are best dissolved in sterile distilled water not above a temperature of 50 °C. This will give a pH of 4 – 6. Maximal stability is obtained around pH 4; lower pH values are to be avoided as they can cause acid hydrolysis of the substrate. Synthetic substrate solutions are stable for at least 1 month at 5 °C or below. No destruction by light has been observed. Micro-organisms may break down the substrate rather quickly: nitroaniline formation is the first sign of breakdown. The addition of a drop of thymol will protect against microbial growth.

Procedure

Collection and treatment of sample: cf. p. 360.

Stability of the enzyme in the sample: to prevent adsorption of the enzyme to the vessel wall, all dilutions of enzymes and plasma are made in buffer containing 0.5 mg/ml human serum albumin or 0.1 – 0.5% polyethylene glycol (Carbowax 6000).

Assay conditions: wavelength 405 nm; plastic cuvettes, 10 mm light path; 37 °C; assay volume 1000 µl. Pre-warm buffer to 37 °C.

Measurement: factor II_a (thrombin) [1]

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(3)	800 µl	Tris NaCl albumin EDTA	40 mmol/l 80 mmol/l 0.4 mg/ml 16 mmol/l
S 2238 solution	(12)	100 µl	S 2238	0.24 mmol/l
sample, factor II_a (thrombin) solution		100 µl	volume fraction	0.1
mix, monitor the absorbance increase due to 4-nitroaniline production continuously or at fixed time intervals (every 15 s) during 3 min.				

If $\Delta A/\Delta t$ is greater than 1 min^{-1}, the samples must be diluted appropriately with buffer (3).

If interference by factor X_a in a thrombin-containing sample is suspected, add 10 µl of soybean trypsin inhibitor solution (10); the final concentration will be 40 µg/ml.

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed as % of standard plasma.

Calculation: the reaction velocity is best compared to that obtained with dilutions of a thrombin standard. For determinations in plasma, standard plasma can serve this purpose; for measurements in systems containing purified factor II_a an active-site titrated factor II_a preparation is the best standard.

Measurement: factor II (prothrombin) [1, 6]

Factor II (prothrombin) in plasma can be determined after quantitative conversion into factor II_a (thrombin) by a suitable activator. A satisfactory arrangement for measuring prothrombin is the following: factor X_a, phospholipid and Ca^{2+} are added to plasma to convert prothrombin into thrombin; the amount of thrombin formed is measured with a chromogenic substrate. Under the experimental conditions described below, the quantitative conversion of prothrombin into thrombin is achieved within 120 s, provided that the plasma sample contains at least 30% of factor V. Soybean trypsin inhibitor must be added thereafter to inhibit factor X_a, which also hydrolyzes the chromogenic substrate.

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
sample, undiluted plasma or another factor II (thrombin)-containing solution		1 – 4 μl	volume fraction	0.001 – 0.004
phospholipid suspension	(11)	10 μl	PS PC	1.88 mg/l 5.62 mg/l
factor X_a solution (150 nmol/l)	(14)	10 μl	F X_a	1.5 nmol/l
$CaCl_2$ solution	(4)	40 μl	$CaCl_2$	4 mmol/l
Tris buffer	(2)	330 μl		
incubate 120 s at 37 °C				
SBTI solution	(10)	10 μl	SBTI	0.04 mg/ml
Tris buffer	(3)	500 μl	Tris NaCl albumin EDTA	43 mmol/l 0.086 mol/l 0.42 g/l 10 mmol/l
S 2238	(12)	100 μl	S 2238	0.24 mmol/l
mix, monitor the absorbance increase due to 4-nitroaniline production continuously or at fixed time intervals (every 15 s) during 3 min.				

If the $\Delta A/\Delta t$ is greater than 1 min^{-1} the samples must be appropriately diluted with buffer (2).

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed as % of standard plasma.

Calculation: the results are expressed as percentage factor II (prothrombin) relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Measurement: factor V [1]

Factor V in plasma can be determined after quantitative conversion into factor V_a by *Russell*'s viper venom (RVV). Factor V_a is an essential cofactor in the activation of prothrombin by the prothrombinase complex (thrombin activating complex consisting of factor X_a, factor V_a, phospholipids and Ca^{2+}). By a suitable experimental design, factor V_a can become the rate-determining reaction constituent. The principle of the factor V determination presented below is the following: factor V in plasma is rapidly

activated into factor V_a by RVV. Factor X_a, phospholipid and Ca^{2+} are added and the mixture is incubated for 5 minutes to allow assembly of factor X_a and factor V_a at the lipid surface. Thrombin formation is started by addition of prothrombin. The initial rate of thrombin formation is determined from the amount of thrombin present after 2 minutes. Under the experimental conditions described here, the rate of thrombin formation is proportional to the amount of factor V_a present in the assay mixture.

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
sample (normal plasma should be diluted about 1 : 10000 in this assay)		50 µl	volume fraction	0.05*
Russell's viper venom solution	(9)	5 µl	RVV	0.5 mg/l
incubate 1 min at room temperature				
Tris buffer	(2)	245 µl		
phospholipid suspension	(11)	5 µl	PS	0.93 mg/l
			PC	2.80 mg/l
factor X_a solution	(14)	100 µl	FX_a	5 µmol/l
$CaCl_2$ solution	(4)	50 µl	$CaCl_2$	4.98 mmol/l
incubate 5 min at 37 °C				
prothrombin solution	(13)	50 µl	factor II	0.5 µmol/l
wait exactly 2 min				
soybean trypsin inhibitor solution	(10)**	400 µl	SBTI	0.04 g/l
			Tris	42 mmol/l
			NaCl	84 mmol/l
			albumin	0.42 g/l
			EDTA	7.78 mmol/l
mix				
S 2238 solution	(12)	100 µl	S 2238	0.23 mmol/l
monitor the absorbance increase due to the 4-nitroaniline production continuously or at fixed time intervals (every 15 s) during 3 min.				

* 0.05×10^{-4} calculated on undiluted plasma.

** 10 µl solution (10) diluted with Tris buffer (3) to 400 µl.

If $\Delta A/\Delta t$ is greater than 1 min^{-1} the samples must be appropriately diluted in buffer (2).

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed in % of standard plasma.

Calculation: the results are expressed as percentage factor V relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Validation of Methods

Precision, accuracy, detection limit and sensitivity: in a concentration range of 10 – 100% of standard plasma, chromogenic substrate assays of the factors II and V show a standard deviation of 3 – 4% in our hands.

Spectrophotometric determinations are most accurate in the range between A = 0.080 and A = 0.800; when the chemical system allows, it is best to adapt it so that this range is covered by the assay. Because of optical limitations, some spectrophotometers may give non-linear responses at $A > 1.0$. The absorbance of 4-nitroaniline is linear with concentration from about 1 – 200 µmol/l (roughly from A = 0.010 to A = 2.0) in water and in Tris buffer, 0.05 mol/l.

The lowest velocity that can be measured with accuracy in a typical spectrophotometer is about $\Delta A/\Delta t = 0.010\ min^{-1}$.

The detection limit of chromogenic substrate assays in biological systems is not only determined by the optical system but also by the presence of other (unwanted) activities in the sample.

The concentration of S 2238 in the cuvette is 0.235 mmol/l. This is about 8 times the K_m value of thrombin for S 2238. Thus, extensive substrate conversion by thrombin will not lead to non-linearity of 4-nitroaniline production due to substrate depletion. Chromozym® TH and S 2238 have similar kinetic parameters for thrombin, and both are suitable for thrombin measurements. However, Chromozym® TH is more easily converted by bovine factor X_a than S 2238. When Chromozym® TH and bovine factor X_a are used, addition of soybean trypsin inhibitor is recommended.

Sources of error: as the chromogenic substrate assay remains a bioassay in principle, rigorous standardization of the reaction conditions is necessary. Furthermore, chromogenic substrates are never completely specific and other enzymes may be present in biological samples which are also active towards the chromogenic substrate used, and which will therefore interfere with the determination.

In all tests in which thrombin is formed, fibrin clots interfering with the measurement can be formed. This source of error can be eliminated by the use of defibrinated plasma or by working with diluted plasma samples. Opalescence in the assay solutions (e.g. caused by heparin, thromboplastin, protamine sulphate, lipaemic plasma, etc.)

prevents precise optical determinations. This source of error can also be ameliorated by the use of diluted plasma samples.

The choice of a factor II activator in the factor II assay is crucial. Non-physiological activators such as staphylocoagulase and snake venoms will not discriminate between normal prothrombin and decarboxyprothrombin (i.e. the abnormal prothrombin present during oral anticoagulant therapy, also called PIVKA). Hence, these activators are unsuitable for the estimation of the level of functional prothrombin in vitamin K deficiency (and also in various forms of liver disease) or during oral anticoagulation. The only known activator that differentiates between the two species is the physiological prothrombinase complex consisting of factor X_a, factor V_a and a suitable phospholipid [6]. The factor II test described above is specific for normal prothrombin and is independent of factor V and factor X levels in plasma. It can be made independent of heparin by the addition of polybrene.

It has been observed that the generation of factor II_a by factor X_a (plus phospholipid and factor V_a) is markedly inhibited in the presence of chromogenic substrates for thrombin [7]. This hampers the direct monitoring of factor II_a generation by following chromogenic substrate hydrolysis. Moreover, production of 4-nitroaniline will not be linear in an assay system containing both factor X_a and prothrombin.

In assays for factor II, therefore, the activation by factor X_a has to take place in the absence of the chromogenic substrate, i.e. a pre-incubation is always necessary. For the same reasons the presence of chromogenic substrate in the activation mixture of the factor V assay is better avoided.

The determination of factor V depends on the activation of factor II by a prothrombinase complex in which the factor V concentration is rate-limiting. In this respect it must be stressed that the V and K_m values of the factor II activation reaction are dependent upon the amount and composition of the lipid present [8], which makes standardization of crucial importance.

Reference ranges: factor II_a (thrombin) is absent from normal plasma. In the test system described above a thrombin concentration in the cuvette of 10^{-9} mol/l (= 1 pmol/ml = 0.1 NIH unit/ml) will cause a change in absorbance at 405 nm of 0.095 absorbance units/min.

The concentration of factor II in normal plasma is 1.5×10^{-6} mol/l. 1 µl of normal plasma will cause an increase in absorbance of about $\Delta A/\Delta t = 0.150 \text{ min}^{-1}$ in the test system described above.

The concentration of factor V in normal plasma is 3×10^{-8} mol/l. 1 µl of normal plasma diluted 1:10000 with buffer will cause an increase in absorbance of about $\Delta A/\Delta t = 0.020 \text{ min}^{-1}$ in the assay system described above.

3.2.2.2 Determination of Factor X_a and Other Assays Based on Factor X_a Determination

Factor X_a can be readily measured with the chromogenic substrates S 2222 and S 2337. Bovine plasma contains two distinct forms of factor X, called factor X_1 and

factor X_2 [9]; only one form is present in human plasma. From each form of factor X two forms of factor X_a can be produced, called factor X_a α and factor X_a β [10]. There are no indications that the kinetic properties of the α and β forms towards synthetic substrates are different [11]. The abnormal factor X_a that is formed by activation of decarboxy-factor X with *Russell*'s viper venom (RVV) has the same kinetic properties towards S 2222 as the normal factor X_a. However, decarboxy-factor X is activated at a much slower rate by RVV than normal factor X. Therefore, an RVV test for factor X can be used to monitor oral anticoagulant therapy.

The estimation of factor X_a can serve as the basis for the assessment of *factor X, antithrombin III* (and thus *heparin* and *platelet factor 4*) and the constituents of the factor X activating complexes, i.e. *factor VII, factor VIII, factor IX, platelet factor 3* and *tissue thromboplastin*.

Equipment

Spectrophotometer capable of exact measurement at 405 nm; thermostatted cuvette holder.

Reagents and Solutions

Purity of reagents: all chemicals used must be of analytical grade.

Preparation of solutions: all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

Solutions (1) – (8): cf. solutions (1) – (8), pp. 368, 369.

9. *Michaelis* buffer:

   cf. solution (1), p. 358.

10. NaCl solution:

    cf. solution (4), p. 358.

11. Thromboplastin:

    cf. solution (8), p. 358.

12. *Russell*'s viper venom (RVV) solution (0.12 mg/ml):

    dissolve 1.2 mg RVV in water and adjust the volume to 10 ml.

13. Phospholipid suspension (0.75 mg/ml; 40% phosphatidylserine, PS, 60% egg phosphatidylcholine, PC; Tris, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8):

    purify PS from brain extract as described for suspension (11), p. 369.
    Obtain a 10 mg/ml solution of egg PC in chloroform/methanol 1:1 by diluting a 100 mg/ml solution of egg PC in chloroform/methanol 1:1 (*Sigma*) 1:10 with chloroform/methanol 1:1.
    Prepare the phospholipid suspension by mixing 0.6 ml PS solution (10 mg/ml in chloroform/methanol 1:1) with 0.9 ml PC solution (10 mg/ml in chloroform/methanol 1:1). Continue the procedure as described under suspension (11), p. 369.

14. S 2337 solution (1.92 mmol/l):

    dissolve the content of 1 vial S 2337 (24 mg) in 15 ml water and dilute further with water until a 1:100 dilution of the stock solution gives an absorbance at 316 nm of $A = 0.250$.

15. Factor II_a solution (90 nmol/l):

    in Tris buffer (2); cf. Appendix, p. 390, or obtain commercially.

16. Factor IX_a solution (2.5 µmol/l):

    in Tris buffer (2); cf. Appendix, p. 392, or obtain commercially.

17. Factor X_a solution (1 nmol/l):

    in Tris buffer (2); cf. Appendix, p. 392.

18. Factor X solutions (1 µmol/l (a) and 10 µmol/l (b), respectively):

    in Tris buffer (2); cf. Appendix, p. 390.

19. Factor XI_a solution (0.225 mg/ml):

    in Tris buffer (2); cf. Appendix, p. 391.

Stability of solutions: as given on p. 370; this includes factor IX, factor XI and thromboplastin solutions as well.
For the stability of chromogenic substrates cf. p. 370.

Procedure

Collection and treatment of sample: cf. p. 360.

Stability of the enzyme in the sample: cf. p. 360 and p. 370.

Assay conditions: wavelength 405 nm; plastic cuvettes, light-path 10 mm; 37 °C; assay volume 1000 µl. Pre-warm buffer to 37 °C.

Measurement: factor X_a [1]

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(3)	800 µl	Tris NaCl albumin EDTA	40 mmol/l 80 mmol/l 0.4 g/l 16 mmol/l
S 2337 solution	(14)	100 µl	S 2337	0.19 mmol/l
sample (containing factor X_a)		100 µl	volume fraction	0.1
mix, monitor the absorbance increase due to 4-nitroaniline production continuously or at fixed time intervals (every 15 s) during 3 min.				

If the $\Delta A/\Delta t$ is greater than 0.4 min^{-1}, the samples must be appropriately diluted with buffer (3).

If factor X_a has to be measured in intact plasma, the plasma (blood) must be mixed with an excess of antithrombin III-directed immunoglobulins immediately upon venepuncture. This test has been described by *Vinazzer & Heimburger* [12] who used 6 µl blood in the assay described above.

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed in % of standard plasma.

Calculation: the reaction velocity is best compared to velocities obtained with dilutions of a factor X_a standard. For determinations in plasma, standard plasma can serve this purpose; for measurements in systems containing purified factor X_a an active-site titrated factor X_a preparation is the best standard.

Measurement: factor X [1, 13]

Measuring factor X means measuring factor X_a after suitable activation. For the determination of factor X in plasma, the factor X activating protease from *Russell's* viper venom (RVV-X) seems to be the activator of choice. No lipid is necessary in this reaction; in fact, lipid inhibits activation because RVV-X acts only on free, non-adsorbed factor X [14]. In the absence of lipids negligible amounts of thrombin are formed. Thus a possible source of error is excluded.

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(2)	385 µl		
citrated plasma		15 µl	volume fraction	0.015
RVV-X solution	(12)	50 µl	RVV-X	6 mg/l
$CaCl_2$ solution	(4)	50 µl	$CaCl_2$	5 mmol/l
incubate 60 s at 37 °C				
diluted S 2337 solution	(14)*	500 µl	S 2337	0.19 mmol/l
			Tris	39 mmol/l
			NaCl	78.5 mol/l
			albumin	0.39 mg/l
			EDTA	8 mmol/l
monitor the increase in absorbance due to 4-nitroaniline production at 405 nm continuously or at fixed time intervals (every 15 s) during 3 min.				

* 100 µl solution (14) diluted with Tris buffer (3) to 500 µl; pre-warmed.

If the $\Delta A/\Delta t$ is greater than 0.4 min^{-1}, the samples must be appropriately diluted with buffer (3).

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations expressed as % of standard plasma.

Calculation: the results are expressed as percentage factor X relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Measurement: factor VII [1, 15, 16]

In principle, both factor VII and tissue thromboplastins can be determined through their activating action on factor X; the experimental design must be such that the factor of interest becomes rate-limiting. The principle of the factor VII determination described below is as follows: factor VII in plasma is converted into factor VII_a by trace amounts of factor X_a in the presence of thromboplastin and Ca^{2+}. The activity of factor VII_a is measured by determination of the initital rate of factor X activation, in the presence of thromboplastin and Ca^{2+}.

Pipette into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(2)	110 µl		
plasma (diluted 1 : 100 with Tris buffer (2))		40 µl	volume fraction	0.04*
tissue thromboplastin		10 µl		
factor X_a solution	(17)	20 µl	F X_a	0.02 nmol/l
$CaCl_2$ solution	(4)	20 µl	$CaCl_2$	2 mmol/l
incubate 5 min at 37 °C				
factor X solution	(18 b)	10 µl	F X	0.1 µmol/l
incubate exactly 4 min at 37 °C				
Tris buffer	(3)	700 µl	Tris	44 mmol/l
			NaCl	87 mol/l
			albumin	0.44 g/l
			EDTA	14 mmol/l
S 2337 solution	(14)	100 µl	S 2337	0.19 mmol/l
monitor the increase in absorbance due to 4-nitroaniline production at 405 nm continuously or at fixed time intervals (every 15 s) during 3 min.				

* 0.04×10^{-2} calculated on undiluted plasma.

If $\Delta A/\Delta t$ is greater than 0.4 min^{-1}, the samples must be appropriately diluted with buffer (2).

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations expressed as % of standard plasma.

Calculation: the results are expressed as percentage of factor VII relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Measurement: factor VIII

A substrate specific for factor X can be used to monitor the velocity of factor X_a formation from a known amount of factor X and hence to assess the activity of the tenase complex (= factor X activating complex consisting of factor $VIII_a$, factor IX_a,

phospholipids and Ca^{2+}). If factor IX_a is present in a fixed and relatively high concentration, then, in the presence of a fixed amount of phospholipid and Ca^{2+}, factor $VIII_a$ (produced by the activation of factor VIII by thrombin) becomes the rate-determining reaction constituent.

Factor $VIII_a$ generation

Pipette into a plastic tube:			concentration in incubation mixture	
sample (plasma)		40 µl	volume fraction	$\varphi_1 = 0.04$
Tris buffer	(2)	910 µl	Tris	48 mmol/l
			NaCl	96 mmol/l
			albumin	0.48 g/l
factor II_a solution	(15)	50 µl	F II_a	4.5 nmol/l
mix, incubate exactly 1 min at 37 °C, use a 500 µl aliquot for further treatment of the activated sample.				

Generation of factor X activating complex, factor X_a (factor $VIII_a$ rate-limiting)

Pipette successively into a plastic tube:			concentration in incubation mixture	
Tris buffer	(2)	350 µl	Tris	46 mmol/l
			NaCl	92 mmol/l
			albumin	0.46 g/l
factor IX_a solution	(16)	20 µl	F IX_a	0.05 µmol/l
phospholipid suspension	(13)	20 µl	PS	60 mg/l
			PC	9 mg/l
$CaCl_2$ solution	(4)	60 µl	$CaCl_2$	6 mmol/l
mix, equilibrate temperature at 37 °C				
factor X solution	(18b)	50 µl	F X	0.5 µmol/l
activated sample		500 µl	volume fraction	$\varphi_2 = 0.5$*
mix, incubate exactly 1 and 2 min, use a 100 µl aliquot for colour reaction of factor X_a.				

* 0.02 calculated on the original sample.

Photometry

Pipette successively into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(3)	800 µl	Tris NaCl albumin EDTA	45 mmol/l 89.2 mmol/l 0.45 g/l 16 mmol/l
S 2337 solution	(14)	100 µl	S 2337	0.19 mmol/l
mix				
factor X activation complex		100 µl	volume fraction F IX_a PS PC $CaCl_2$ F X	$\varphi_3 = 0.1$* 5 nmol/l 0.6 mg/l 0.9 mg/l 0.6 mmol/l 50 nmol/l
mix, monitor the increase in absorbance due to 4-nitroaniline production at 405 nm continuously or at fixed time intervals (every 15 s) during 3 min.				

* 0.002 calculated on the original sample.

If $\Delta A/\Delta t$ is greater than 0.4 min^{-1}, the samples must be appropriately diluted with buffer (2).

Correct results by subtracting the blank value determined with Tris buffer (2) instead of plasma.

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed as % of standard plasma.

Calculation: the results are expressed as percentage of factor VIII relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Measurement: factor IX [17]

A substrate specific for factor X_a can be used to monitor the velocity of factor X_a formation from a known amount of factor X by a tenase complex (= factor X activating complex consisting of factor IX_a, phospholipids and Ca^{2+}) in which, because of the experimental design, factor IX_a is the rate-determining reaction constituent. The factor IX assay described below involves the following steps:

a) conversion of plasma factor IX to factor IX_a by purified factor XI_a,
b) incubation of factor IX_a with factor X, phospholipids and Ca^{2+},
c) addition of EDTA to stop factor X_a formation by IX_a,
d) determination of the amount of factor X_a present from the rate of hydrolysis of S 2337.

Factor IX_a generation

Pipette into a plastic tube:			concentration in incubation mixture	
Tris buffer	(2)	90 µl	Tris	45 mmol/l
			NaCl	90 mmol/l
			albumin	0.45 mg/l
$CaCl_2$ solution	(4)	15 µl	$CaCl_2$	10 mmol/ml
factor XI_a solution	(19)	15 µl	F XI_a	23 mg/l
plasma (diluted 1 : 10 in Tris buffer (2))		30 µl	volume fraction	$\varphi_1 = 0.2$*
mix, incubate 25 min at 37 °C, use a 100 µl aliquot of this activation mixture for further treatment of activated sample.				

* 0.02 calculated on undiluted plasma.

Generation of factor X activating complex, factor X_a (factor IX_a rate limiting)

Pipette successively into a plastic tube:			concentration in incubation mixture	
Tris buffer	(2)	200 µl	Tris	44 mmol/l
			NaCl	88 mol/l
			albumin	0.44 g/l
factor X solution	(18a)	100 µl	F X	0.2 µmol/l
phospholipid suspension	(13)		PS	30 mg/l
		50 µl	PC	45 mg/l
$CaCl_2$ solution	(4)	50 µl	$CaCl_2$	10 mmol/l
mix, equilibrate at 37 °C				
activated sample		100 µl	volume fraction	$\varphi_2 = 0.2$*
mix, incubate exactly 10 min at 37 °C, use a 400 µl aliquot of this incubation mixture for colour reaction of factor X_a.				

* 0.04 calculated on original sample; 0.004 calculated on undiluted plasma.

Photometry

Pipette successively into a plastic semi-micro cuvette:			concentration in assay mixture	
Tris buffer	(3)	400 µl	Tris	42 mmol/l
			NaCl	84 mol/l
			albumin	0.42 mg/l
			EDTA	8.9 mmol/l
factor X activation complex		400 µl	volume fraction	$\varphi_3 = 0.44$*
S 2337 solution	(14)	100 µl	S 2337	0.21 mmol/l
			F X	0.02 µmol/l
			PS	3 mg/l
			PC	5 mg/l
			$CaCl_2$	1.1 mmol/l
mix, monitor the increase in absorbance due to 4-nitroaniline production at 405 nm continuously or at fixed time intervals (every 15 s) during 3 min.				

* 0.018 calculated on original sample; 0.0018 calculated on undiluted plasma.

If $\Delta A/\Delta t$ is greater than 0.4 min^{-1}, the samples must be appropriately diluted in buffer.

Standard curve: construct a standard curve by plotting $\Delta A/\Delta t$ in min^{-1} obtained with dilutions of the standard plasma against their concentrations, expressed as % of standard plasma.

Calculation: the results are expressed as percentage of factor IX relative to a standard plasma. Therefore, the reaction velocity obtained with the sample must be compared to velocities obtained with a series of dilutions of standard plasma.

Validation of Methods

Precision, accuracy, detection limit and sensitivity: given on p. 374 for factors II and V; the same for factors VII and X.

The relative standard deviations (RSD) of the chromogenic substrate assays based on factor X_a measurement are (in %) [17]:

Factor	RSD_{assay}	$RSD_{tot.}$	$RSD_{biol.}$
VII	5.1	21.8	21.1
VIII	6.9	29.7	–
IX	5.1	11.0	9.7
X	3.0	15.0	14.8

RSD_{assay} relative standard deviation (standard deviation expressed as percentage of the mean)
$RSD_{tot.}$ inter-individual relative standard deviation determined in 33 healthy normal individuals
$RSD_{biol.}$ biological variation determined from $(RSD_{tot.})^2 = (RSD_{assay})^2 + (RSD_{biol.})^2$.

Sources of error: in principle, the same as given on pp. 374, 375. The presence of unspecific activities must be checked in control experiments in which all substances occurring in the assay system are present, except the enzyme to be determined. Furthermore, the enzyme reaction can be inhibited by the chromogenic substrate. Therefore, assay standardization is of utmost importance.

If factor X_a has to be measured in intact plasma, the plasma (blood) must be mixed with an excess of anti-antithrombin III-directed immunoglobulins [12] immediately upon venepuncture. This lengthens the survival time of factor X_a without abolishing factor X_a inactivation altogether, possibly because of the presence of factor X_a inhibitors other than antithrombin III or α_2-macroglobulin (whose action will hardly be observed because the α_2-macroglobulin-factor X_a complex is still able to split small synthetic substrates).

If instead of *Russell*'s viper venom (RVV-X) thromboplastin and factor VII are used as an activator, less factor X_a seems to be formed than with RVV-X. With thromboplastin as an activator, the amount of factor X_a formed is linearly related to the amount of plasma from standard samples only over a narrow concentration range, and the coefficient of variation is larger than with the RVV-X method [18].

In the factor VII assays, total factor VII is determined on the basis of a complete activation of this factor. However, in this respect several difficulties have not yet been overcome. The problems arise primarily from the fact that factor VII is present in plasma in two molecular forms, as single-chain and two-chain molecules. The single-chain form seems to have a distinct species specificity with respect to thromboplastin, whereas the two-chain form has not [19]. The two-chain form arises in certain plasmas upon storage at 4 °C. It has also been suggested that the two-chain form is indicative of different forms of disease, such as metastases from carcinoma of the prostate and generalized atherosclerosis. As yet there are no methods available to differentiate between the two forms in a chromogenic substrate assay. On the other hand, if tissue thromboplastins of different species have to be compared, the result of the test will depend upon the relative amount of the two-chain form of factor VII present in the reaction mixture.

Inter-laboratory differences in thromboplastin preparations also greatly hamper standardization of the factor VII determination. A further complication may result from the fact that factor X_a can activate factor VII. The effect of a chromogenic substrate occupying the active site of factor X_a on the interaction with factor VII remains to be investigated.

The determination of the factors VIII and IX is based on the activation of factor X by the tenase complex. Through manipulation of the experimental conditions, factor VIII or factor IX can become the rate-limiting reaction component in this complex. In this respect it must be stressed that the V and K_m values of the tenase complex are

dependent upon the amount and composition of the lipids present, which makes standardization in this respect of crucial importance.

Reference ranges

Factor X_a is absent from normal plasma. A factor X_a concentration in the cuvette of 1 nmol/l will cause a $\Delta A/\Delta t = 0.070 \text{ min}^{-1}$ at 405 nm.

The concentration of *factor X* in normal plasma is 180 nmol/l; 15 µl of normal plasma will cause an increase in absorbance at 405 nm of about $\Delta A/\Delta t = 0.200 \text{ min}^{-1}$.

Factor VII concentration in normal plasma is 10 nmol/l; 40 µl of normal plasma, diluted 1 : 100, will cause an increase in absorbance at 405 nm of about $\Delta A/\Delta t = 0.065 \text{ min}^{-1}$ in the test system described above.

Factor VIII concentration in normal plasma is 0.2 nmol/l; 40 µl of normal plasma will cause an increase in absorbance of about $\Delta A/\Delta t = 0.050 \text{ min}^{-1}$ after 2 min of factor X activation.

Factor IX concentration in normal plasma is 70 nmol/l; 30 µl of normal plasma, diluted 1 : 10, will cause an increase in absorbance at 405 nm of about $\Delta A/\Delta t = 0.028 \text{ min}^{-1}$.

3.2.2.3 Determination of Factors XI_a and XI [1, 20]

It has been reported recently by *Iwanaga et al.* [1] and *Kato et al.* [20] that factor XI_a can be determined with the fluorigenic substrate BOC-Phe-Ser-Arg-MCA (MCA = 7-amino-4-methyl-coumarine), which is relatively specific for factor XI_a. This assay can be used also to measure factor XI after activation with factor XII_a. However, the authors only described a test for the determination of factor XI in model systems containing purified coagulation factors; no adaptation for its use in the determination of factor XI in plasma has been reported. As we have no experience with this assay, we will only give a schematic description of the method but no further experimental details.

Preparation of solutions

1. Tris buffer (Tris, 50 mmol/l; NaCl, 0.15 mol/l; bovine serum albumin, 0.1 mg/ml; pH 8.0):

   dissolve 6.05 g tris (hydroxymethyl) aminomethane and 8.76 g NaCl in 900 ml water; proceed further as described for solution (1) on page 368 but adjust the pH to 8.0. Supplement this Tris buffer with 0.1 g bovine serum albumin per 1000 ml.

2. Tris buffer (Tris, 20 mmol/l; NaCl, 0.15 mol/l; bovine serum albumin, 0.1 mg/ml; pH 8.0):

   dissolve 2.42 g tris (hydroxymethyl) aminomethane and 8.76 g NaCl in 900 ml water; proceed further as described for solution (1) on page 368 but adjust the pH to 8.0. Supplement this Tris buffer with 0.1 g bovine serum albumin per 1000 ml.

3. BOC-Phe-Ser-Arg-MCA (10 mmol/l in dimethylformamide):

   dissolve 67 mg substrate in 10 ml dimethylformamide.

4. Factor XII_a solution (78 µg/ml in Tris buffer (1)):

   cf. Appendix or obtain commercially.

Assay conditions: fluorigenic substrate assays are carried out in glass cuvettes in a fluorimeter with a thermostatted cuvette holder at 37 °C. Pre-warm buffer to 37 °C. Excitation wavelength 380 nm; emission wavelength 440 nm.

Measurement: factor XI_a [1, 20]

Pipette into a glass cuvette:			concentration in assay mixture	
Tris buffer	(1)	500 µl	Tris NaCl albumin	48.5 mmol/l 145.6 mmol/l 0.1 g/l
sample (containing approx. 10 µg factor XI_a)		10 µl	F XI_a	19.4 mg/l
mix, incubate 5 min at 37 °C				
BOC-Phe-Ser-Arg-MCA solution	(3)	5 µl	BOC-Phe-Ser-Arg-MCA	0.097 mmol/l
mix, monitor the initial rate of the increase of the relative fluorescence continuously or at fixed time intervals during 10 min (excitation at 380 nm, emission at 440 nm).				

Standard curve: construct a standard curve by plotting the fluorescence increase per min of dilutions of the factor XI_a standard against their concentrations, analogously to the photometric determinations.

Calculation: read the concentration of the sample from the standard curve as for the photometric determination, p. 384.

Measurement: factor XI [1, 20]

Factor XI_a generation

Pipette into a glass tube:			concentration in incubation mixture
sample (containing approx. 500 μg factor XI/ml)		10 μl	volume fraction $\varphi_1 = 0.016$
Tris buffer	(2)	600 μl	
factor XII_a solution	(4)	20 μl	F XII_a 2.48 mg/l Tris 19.7 mmol/l NaCl 147.6 mmol/l albumin 0.1 g/l
mix, incubate 30 min at 37 °C; use a 10 μl aliquot for fluorimetry.			

Fluorimetry

Pipette successively into a glass cuvette:			concentration in assay mixture
Tris buffer	(1)	500 μl	Tris 48.9 mmol/l NaCl 148.5 mmol/l albumin 0.1 g/l
activated sample		10 μl	volume fraction $\varphi_2 = 0.019$*
mix, incubate 5 min at 37 °C			
BOC-Phe-Ser-Arg-MCA solution	(3)	5 μl	BOC-Phe-Ser-Arg-MCA 0.097 mmol/l
mix, monitor the initial rate of the increase of the relative fluorescence continuously or at fixed time intervals during 10 min (excitation at 380 nm, emission at 440 nm).			

* 0.0003 calculated on the original sample.

Standard curve: construct a standard curve by plotting the fluorescence increase per min of dilutions of the factor XI standard against their concentrations, analogously to the photometric determinations.

Calculation: read the concentration of the sample from the standard curve as for the photometric determination, p. 384.

Validation of Methods

The authors stated that the substrate BOC-Phe-Ser-Arg-MCA is very susceptible to cleavage by trypsin: therefore, soybean trypsin inhibitor (SBTI) should be added to the reaction mixture if trypsin can be expected to be present in the sample. They further reported that the hydrolysis rate is constant over a period up to 10 minutes and that proportionality between the amount of enzyme used and the activity measured is observed over a 10-fold range. Factor XI_a activity is quantitatively measured up to a concentration of 0.5 µg/ml. Moreover, the sensitivity of the measurement of the fluorescence increase due to AMC release can be amplified 10 times, depending on the instrument used; therefore, the limit of detection of factor XI_a is 50 nmol/l.

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Appendix

Purification of coagulation factors and preparation of reagents for chromogenic substrate assays.

Gerbrand van Dieijen and Truus Janssen-Claessen

The purification procedures described here are currently used in the Department of Biochemistry in Maastricht and are adapted from several publications, mainly from the laboratories of *E. Davie,* Seattle WA; *C. M. Jackson,* St. Louis, MO and *Y. Nemerson,* New York, NY [1, 2].

Preparation of bovine prothrombin, factor X and factor IX [3 – 5]

At the slaughterhouse collect 50 l ox blood using a mixture of citrate and inhibitors as anticoagulant. Mix 9 l blood with 1 l of a solution containing: 36 g sodium citrate; 7.2 g benzamidine; 10 ml trasylol (100000 KIU); 0.6 g heparin (100000 U/l). Centrifuge the blood for 30 min at 2500 rpm in the *MSE* Mistral centrifuge with the 6 × 1 litre rotor. Aspirate the plasma from the red cells and add 80 ml $BaCl_2$, 1 mol/l, to the plasma solution. Stir the suspension for 60 min at 4 °C. Collect the barium-citrate pellet by centrifugation (10 min; 2500 rpm) and wash the sediment twice (150 ml buffer per litre plasma) with buffer containing per litre: 9 g NaCl; benzamidine, 10 mmol; $BaCl_2$, 5 mmol; 10 mg soybean trypsin inhibitor (SBTI). Elute proteins from Ba-citrate with 2 l of a buffer containing: Na-citrate, 0.2 mol/l; benzamidine, 10 mmol/l; soybean trypsin inhibitor, 40 mg/l; 2 ampoules trasylol (200000 KIU); pH 6.9 adjusted with citric acid, 1 mol/l. Add solid $(NH_4)_2SO_4$ to the eluate to achieve 35% saturation. The precipitate is discarded and the supernatant is brought to 65% $(NH_4)_2SO_4$ saturation. Collect precipitates by centrifugation (30 min, 12000 rpm,

JA-14 Rotor, *Beckman*). Dissolve the final 65% $(NH_4)_2SO_4$ precipitate in 50 – 75 ml of a buffer consisting of morpholino ethanesulphonic acid (MES)/NaOH, 20 mmol/l, and benzamidine, 1 mmol/l; pH 6.0. Dialyze the protein solution against two 1 l changes of the same buffer and adsorb the protein onto a DEAE-Sephadex column (2.5 × 35 cm), equilibrated with MES/NaOH (pH 6.0) buffer. Wash the column with 300 ml MES/NaOH buffer containing NaCl, 0.15 mol/l, and elute with a NaCl gradient (2 × 500 ml), ranging from 0.15 to 0.6 mol/l in MES/NaOH, 20 mmol/l, benzamidine, 1 mmol/l (pH 6.0).

The first strongly adsorbing fraction of the eluate from the DEAE-column contains prothrombin, factor IX and factor VII, and the weakly adsorbing fraction at the end of the gradient contains factors X_1 and X_2. Make two protein pools, the first containing prothrombin and factors VII and IX, the second pool containing factors X_1 and X_2.

Separate factor X_1 and X_2 by the method of *Fujikawa et al.* [4]. Adsorb the proteins to a DEAE-Sephadex column (2.5 × 30 cm) and use a gradient (2 × 1 litre) of sodium citrate from 0.1 mol/l to 0.2 mol/l, pH 6.9, containing benzamidine, 10 mmol/l, to elute the column. Pool fractions containing factor X_1 and fractions containing factor X_2 separately, concentrate by ultrafiltration (*Amicon* PM 10) and dialyze extensively against Tris/HCl buffer (50 mmol/l; NaCl, 0.1 mol/l; pH 7.8).

Dialyze the pooled fractions from the DEAE-column containing prothrombin, factor IX and factor VII and adsorb to a heparin-agarose column (2.2 × 20 cm) using a buffer containing: imidazol, 50 mmol/l; benzamidine, 1 mmol/l; $CaCl_2$, 2.5 mmol/l; pH 6.0. Elute prothrombin and factor VII stepwise using imidazol buffer (50 mol/l) containing NaCl, 0.15 mol/l respectively 0.25 mol/l.

Elute factor IX with a salt gradient (NaCl, 2 × 300 ml) in imidazol buffer ranging from 0.25 to 0.7 mol/l [5]. Pool the prothrombin-containing fractions, concentrate and fractionate using a Sephadex-G100 column (2.5 × 90 cm). Fractionate factor IX under the same conditions.

The prothrombin, factor IX and factor X_1 and X_2 preparations were apparently pure as judged by SDS-gel electrophoresis. The specific activities determined in a clotting assay were 4 units/mg for prothrombin, 100 units/mg for factors X_1 and X_2, and 145 units/mg for factor IX, assuming the presence of 1 unit of each of the factors in normal bovine plasma.

Factor XI_a preparation

Isolate the factor XI_a preparation from "bovine contact product" prepared according to *Nossel* [6] and further purify this preparation by heparin/agarose affinity chromatography as suggested by *Østerud et al.* [7]. To obtain "bovine contact product" stir the supernatant obtained after Ba-citrate adsorption of bovine plasma (see above) with Celite (*535 Johns Manville*), 50 g/l plasma, during 45 min at room temperature. Wash the Celite 4 – 5 times with a buffer containing: Tris/HCl, 20 mmol/l; 0.9% NaCl; pH 7.0 (1 litre/ 100 g Celite). Elute contact product from Celite with buffer containing: Tris/HCl, 20 mmol/l; NaCl, 2 mol/l; pH 7.0.

Concentrate the eluate and dialyze against sodium acetate buffer, 50 mmol/l, pH 5.5. Adsorb crude contact product to a heparin-agarose column (1.5 × 20 cm) [7], wash the column with 250 ml of a buffer containing sodium acetate, 50 mmol/l; NaCl, 0.3 mol/l; pH 5.5 and elute subsequently with a salt gradient (NaCl, 2 × 250 ml) from 0.3 mol/l to 1.0 mmol/l in the same buffer. Pool fractions containing factor XI_a, identified by a clotting assay using factor XI-deficient plasma, concentrate in an *Amicon* cell and dialyze against a buffer containing: Tris/HCl, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8. Determine the concentration from absorbance, assuming that one A_{280} unit/ml is equivalent to 1 mg protein per ml.

Thrombin preparation [8]

Incubate 10 mg prothrombin and 25 μg factor X_a overnight at 37 °C in a reaction mixture of 5 ml containing: Tris/HCl, 50 mmol/l; NaCl, 0.1 mol/l; $CaCl_2$, 20 mmol/l; pH 7.8. After incubation remove the precipitate and dialyze the protein against a sodium phosphate buffer, 50 mmol/l, pH 8.5. Adsorb the protein to a SP-Sephadex column (1.5 × 15 cm) [8]. Wash the column with 150 ml phosphate buffer containing NaCl, 0.15 mol/l, and develop with a NaCl gradient (2 × 200 ml) from 0.15 to 0.45 mol/l in sodium phosphate buffer, 50 mmol/l, pH 6.5. Pool the thrombin containing fractions, concentrate and dialyze against Tris/HCl buffer, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8.

Factor X_a preparation [9]

Prepare bovine factor X_a by activation of factor X by *Russell*'s viper venom [9]. Incubate 2 mg factor X and 0.1 mg RVV in 2 ml Tris/HCl buffer, 50 mmol/l; NaCl, 0.1 mol/l and $CaCl_2$, 10 mmol/l; pH 7.8. Follow the activation by transferring aliquots from the reaction mixture to a cuvette containing S 2337. When a plateau level of activation is reached, add EDTA to a final concentration of 20 mmol/l, and dialyze the reaction mixture against a buffer containing: Tris/HCl, 50 mmol/l; citrate, 60 mmol/l; pH 7.1. After dialysis adsorb the proteins of the sample onto a DEAE-Sephadex column (1.5 × 15 cm) equilibrated with a buffer containing Tris, 50 mmol/l, and citrate, 0.1 mol/l; pH 8.0.

Elute the column with a NaCl gradient (2 × 150 ml) from 0 – 0.6 mol/l in a buffer containing: Tris, 50 mmol/l; citrate, 0.1 mol/l; pH 8.0. Pool fractions containing factor X_a (identified with S 2337 as substrate) and dialyze against Tris/HCl buffer, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8.

Factor IX_a preparation [10]

Incubate 10 mg factor IX and 0.2 mg factor XI_a in a reaction volume of 5 ml for 90 min at 37 °C in Tris/HCl buffer, 50 mmol/l; NaCl, 50 mmol/l; $CaCl_2$, 10 mmol/l; pH 8.5. After 90 min the activation is complete. Add EDTA (pH 7.5) to a final con-

centration of 30 mmol/l. Adsorb the proteins of the mixture onto a DEAE-Sephadex column (1 × 13 cm) equilibrated with Tris/HCl buffer, 0.15 mol/l; NaCl, 50 mmol/l; benzamidine, 20 mmol/l; pH 7.9.

Wash the column with 200 ml of the same buffer containing NaCl, 0.1 mol/l and elute with a NaCl gradient (2 × 150 ml) from 0.1 to 0.4 mol/l in the same buffer. Pool factor IX_a fractions, identified by a clotting assay in absence of kaolin using a IX_a-deficient reagent, concentrate and dialyze against Tris/HCl buffer, 50 mmol/l; NaCl, 0.1 mol/l; pH 8.

Dialyze the protein preparations against a Tris/HCl buffer, 50 mmol/l; NaCl, 0.1 mol/l; pH 7.8.

Estimation of protein concentration of prothrombin, factor X_1 and X_2 and factor IX

Estimate the protein concentrations from absorbance at 280 nm using A_{280} values of 15.5 for prothrombin [3], 12.4 for factor X_1 and X_2 [4] and 14.9 for factor IX [5]. The molecular weights used to calculate molar concentrations are: 72000 for prothrombin, 55000 for factor X_1 and X_2 and factor IX.

Determine the concentrations of thrombin, factor X_a and factor IX_a by active-site titration or calculate from absorbance using A_{280} values of 20.0 for thrombin, 12.4 for factor X_a and 14.3 for factor IX_a [3, 8, 9]. The molecular weights used are 36000 for thrombin and 44000 for factors X_a and IX_a.

Active-site titration of thrombin, factor X_a, and factor IX_a [11 – 13]

All coagulation factors are diluted to the desired concentration in a buffer containing Tris, 50 mmol/l; NaCl, 100 mmol/l; pH 7.9, and albumin, 0.5 mg/ml. The factors are frozen and stored in small aliquots at −70 °C.

Dialyze the proteins against sodium barbital buffer, 50 mmol/l, pH 8.3. Place thermostatted cuvettes in a double-beam spectrophotometer, set wavelength at 405 nm. Typically, the sample cuvette contains 40 – 200 µg of protein in 800 µl barbital buffer, 50 mmol/l; $CaCl_2$, 20 mmol/l. The reference cuvette contains calcium barbital but no protein. After 5 min equilibration add 5 µl 4-nitrophenyl 4′-guanidino-benzoate solution, 20 mol/l. A rapid increase in A_{405} absorbance ("burst") is observed. After 2 – 3 min (for factor IX_a) no further or a small constant increase is observed. Determine the burst by extrapolation to time zero. Calculate the molar protein concentration from the burst using a millimolar absorption coefficient for 4-nitrophenol at 405 nm of $1.841 \times \text{mmol}^{-1} \times \text{mm}^{-1}$, and assuming the liberation of 1 mol of 4-nitrophenol per mol enzyme protein.

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3.2.3 Blood Coagulation Factor XII

Hageman Factor

Cornelis Kluft, Petronella Los and Lars Svendsen

General

The proteolytic enzyme factor XIIa is present in mammalian plasma in an inactive form, factor XII or *Hageman* factor. The factor was identified by *Ratnoff & Colopy* in 1955 because of its congenital absence from Mr. *Hageman*'s blood [1]. Later, it was isolated from blood and characterized [2].

The activation of factor XII is triggered by negatively-charged surfaces such as glass, kaolin or silica, or negatively-charged soluble polymers such as dextran sulphate. In addition it requires the participation of the plasma factors prekallikrein and high molecular weight kininogen [3]. Factor XII participates in the first step of the intrinsic pathway of coagulation and in various other processes such as fibrinolysis,

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