New Perspectives in Coagulation Testing

Jawed Fareed, Harry L. Messmore, and Edward W. Berme

The field of coagulation testing has undergone some major technological and conceptual developments, which are briefly reviewed here. The assessment of coagulation parameters is no longer restricted to the study of clot formation and its dissolution. The understanding of the biochemical nature of coagulation processes, coupled with the development of new therapeutic agents in the treatment of hemostatic disorders, has brought about the development of fast, reliable, and clearly defined laboratory test procedures to evaluate the components of this system. The introduction of methods involving synthetic substrates has been very significant because many of the coagulation parameters can now be measured with a spectrophotometer or fluorometer, by methods that lend themselves to the automation found in most large clinical chemistry laboratories. In our laboratory, we use automated synthetic-substrate methods for antithrombin-III, plasminogen, and prothrombin, and are developing the synthetic-substrate assay equivalent of clot-based prothrombin time and partial thromboplastin. Immunological methods such as laser/rate nephelometry, enzyme-linked immunosassays, electroimmunodiffusion, and radioimmunoassays have been utilized to evaluate coagulation proteins. The relation of functional and immunological properties of these proteins to their physiological function is being studied. In coming years the testing of coagulation function will undergo some major changes and will require input from clinical chemists and other laboratory scientists to facilitate the technology transfer and proper standardization of new methods.

Additional Keyphrases: hemostasis · prothrombin · coagulation proteins · synthetic coagulation substrates · enzymic methods · immunochemistry · platelets · prostaglandins · fibrinogen

Blood coagulation is now understood to be a well-defined molecular process regulated by a delicate interaction of the following three systems: coagulation and fibrinolytic proteins, platelets, and endothelium.

Hemostasis is the containment of circulating blood. Blood consists of a fluid phase (plasma) and a particulate phase (cellular elements); blood vessels are lined with the vascular endothelial cells, which form the blood-contacted surface of the normal vessel lumen. The three systems—coagulation and fibrinolytic proteins, blood cells, and vascular endothelium—exist in concert and function in hemostasis. The extent to which they participate in thrombotic and hemorrhagic processes depends on the severity of the damage, circulatory complications, and influences of other physiological systems.

A pathophysiological process is capable of altering the composition of each of the components of the total hemostatic system. Because the molecular nature of each of these processes is now understood and many alterations are detectable by newer techniques, a new era in the testing of hemostatic function has begun (1–10). Major breakthroughs have occurred in the understanding of coagulation enzymology, immunology, and drug modulation of the components of hemostatic pathways. The understanding of hemostatic processes and alterations produced by pathophysiological changes and drug modulation of this system is facilitated by input from various disciplines. The introduction of synthetic peptide substrates and immunological methods represent new dimensions in coagulation testing. We will review some of the recent trends in this area, emphasizing the role of clinical chemistry in this developing field.

Concept of Serine Proteases and Their Inhibitors

In the past few years coagulationists have studied intensely the inhibitors of the coagulation, fibrinolytic, kallikrein, and other serine protease enzymes. The coagulation cascade (Figure 1) of fibrinolytic and prekallikrein/kallikrein pathways plays a key role in the regulation of hemostasis. Most of the enzymes participating in these processes mimic trypsin and are known as serine proteases; this group includes the following enzymes (and their precursors): Factor XIIa (XII), Factor XIa (XI), Factor IXa (IX), Factor Xa (X), Factor VIIa (VII), kallikrein (prekallikrein or Fletcher’s factor), thrombin (prothrombin or Factor II), and plasmin (plasminogen). Both the intrinsic and extrinsic pathways of coagulation result in the eventual activation of Factor X, which in turn converts prothrombin into thrombin. The thrombin formed transforms the soluble fibrinogen into an insoluble fibrin clot, which is eventually digested by plasmin into the soluble fibrin-split products. It is generally believed that, in the coagulation cascade, fibrinolytic pathways are simultaneously activated; that is, once the coagulation process has been activated, these clotting enzymes are capable of triggering the fibrinolytic pathways through a feedback mechanism. If the activities of these enzymes proceed without any regulatory mechanism, the net result is fibrinolysis. The activities of coagulation and fibrinolytic enzymes are, however, modulated by numerous serine protease inhibitors, including C1’ inhibitor; α2-macroglobulin, α1-antitrypsin, α2-antiplasmin, and most important, antithrombin-III (AT-III). Although the other inhibitors are also involved in the regulation of hemostatic function, AT-III plays a central role in the modulation of coagulation processes. Some of the properties of the major inhibitors of clotting and of the fibrinolytic enzymes in human plasma are given in Table 1. The response to therapeutic anticoagulants, thrombolytic agents, and immunosuppressive

1 Nonstandard abbreviations used: AT-III, antithrombin-III; pNA, p-nitroanilin; P/SK, plasminogen/streptokinase complex; VIII R:Ag, Factor VIII-related antigen.
drugs is also dependent on the functional concentrations of these inhibitors. Laboratory evaluation of these proteins is an extremely important area, requiring sophisticated methodologies and an in-depth understanding of the physiology of hemostasis. Newer methodologies offer a unique opportunity to utilize an enzyme-specific substrate for the study of the regulators of hemostasis without significant interference by the other proteins.

Antithrombin-III (Heparin Cofactor)

AT-III, also known as heparin cofactor and Factor Xa inhibitor, is a plasma protein that migrates during electrophoresis in the region of α2-globulin; its relative molecular mass is 65,000. It circulates in blood in a nonactivated state, which is the form responsible for the progressive inhibition of numerous serine proteases such as thrombin, Factors XIIa, IXa, Xa, and XIa. Although AT-III is a key inhibitor capable of inhibiting various serine proteases at different sites of the coagulation cascade, its main action is against thrombin and Factor Xa. In its natural state AT-III is a relatively weak inhibitor of these enzymes; in the presence of heparin, however, AT-III is activated and becomes a very potent inhibitor of Factors Xa and thrombin (Figure 2). The therapeutic efficacy of heparin depends almost totally on the presence of AT-III, so that individuals lacking this inhibitor fail to respond to heparin therapy. AT-III concentrations are decreased in the following conditions: liver diseases, disseminated intravascular coagulation, carcinoma, acute leukemia, advanced stages of sickle-cell anemia, major post-surgical trauma, deep venous thrombosis, thrombophlebitis (hereditary deficiency of AT-III), Gram-negative septicemia, endotoxemia, and oral contraceptive therapy.

**Table 1. Fibrinolytic Enzymes and Major Inhibitors of Clotting in Human Plasma**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>M₀</th>
<th>Plasma conc. a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/L</td>
<td>µmol/L</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>54,000</td>
<td>2,900 ± 450</td>
</tr>
<tr>
<td>α₁-Antichymotrypsin</td>
<td>69,000</td>
<td>487 ± 65</td>
</tr>
<tr>
<td>Inter-α-trypsin-inhibitor</td>
<td>160,000</td>
<td>500 ± 71</td>
</tr>
<tr>
<td>Antithrombin-III</td>
<td>65,000</td>
<td>290 ± 29</td>
</tr>
<tr>
<td>C₁-Inactivator</td>
<td>104,000</td>
<td>235 ± 30</td>
</tr>
<tr>
<td>α₂-Macroglobulin</td>
<td>820,000</td>
<td>2,600 ± 700</td>
</tr>
<tr>
<td>α₂-Antiplasmin</td>
<td>65,000</td>
<td>116 ± 19</td>
</tr>
</tbody>
</table>

Table 2. Synthetic Chromogenic Peptide Substrates and Their Applications

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>CHEMICAL STRUCTURE</th>
<th>APPLICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromozym-TH</td>
<td>Tos or Cbz-Gly-Pro-Arg-pNA</td>
<td>Prothrombin, Antithrombin-III, Antithrombin, Monitoring of Oral Anticoagulants, Heparin Platelet Factor III and Platelet Factor IV</td>
</tr>
<tr>
<td>S-2160</td>
<td>Bz-Phe-Val-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2236</td>
<td>H-D-Phe-Pip-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2366</td>
<td>Pyro-Glu-Pro-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>Abbott &quot;I&quot;</td>
<td>Chy-Gly-Pro-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2222</td>
<td>Bz-Ile-Glu-(y-Or)-Gly-Arg-pNA</td>
<td>A, Xa, Anti-Xa and Platelet Factor IV</td>
</tr>
<tr>
<td>S-2337</td>
<td>Bz-Ile-Glu-(y-D-Piperydyl)-Gly-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2251</td>
<td>H-D-Val-Leu-Pip-Arg-pNA</td>
<td>Plasmin, Plasminogen, Antiplasmin, and Plasminogen Activators</td>
</tr>
<tr>
<td>Chromozym-PL</td>
<td>Tos-Gly-Pro-Lys-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2322</td>
<td>H-D-Val-Gly-Arg-pNA</td>
<td>Tissue Activator of Plasminogen</td>
</tr>
<tr>
<td>S-2302</td>
<td>H-D-Pro-Phe-Arg-pNA</td>
<td>Plasma Kallikrein, Antikallikrein and Kallikrein Activators</td>
</tr>
<tr>
<td>Chromozym-PK</td>
<td>Bz-Pro-Phe-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>Chromozym-UK</td>
<td>Bz-Val-Gly-Arg-pNA</td>
<td>Urokinase</td>
</tr>
<tr>
<td>S-2444</td>
<td>Pyro-Glu-Gly-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>Chromozym-Try</td>
<td>H-D-Val-Gly-Arg-pNA</td>
<td>Trypsin and Antitrypsin</td>
</tr>
<tr>
<td>S-2422</td>
<td>Cbo-Val-Gly-Arg-pNA</td>
<td></td>
</tr>
<tr>
<td>S-2433</td>
<td>Acct-Ile-Gly-Gly-Arg-pNA</td>
<td>Endotoxin, Limulus Procoagulant enzyme</td>
</tr>
</tbody>
</table>

The substrates of the Chromozym series are manufactured by Pentapharm (Basel, Switzerland) and the substrates of the "S" series are manufactured by Kabi A B (Stockholm, Sweden).

Synthetic Peptide Substrate Methods for Coagulation Testing

Since the introduction of enzyme-specific synthetic substrates for thrombin, the whole field of coagulation enzymology has undergone a dramatic change. Although it has only been a few years since the first chromogenic tripeptide, Bz-Phe-Val-Arg-pNA (S-2160), was introduced by Svendsen et al. (11) as a specific substrate for thrombin, more than 30 synthetic chromogenic and fluorogenic peptides have been introduced for the specific evaluation of thrombin, Factor Xa, plasmin, plasma and glandular kallikrein, plasminogen activator, urokinase, trypsin, limulus-coagulant enzyme, and many other serine protease enzymes (11–53). Methodologies for the active enzymes, their zymogens, and their inhibitors are continually being developed. In addition, coagulation and related pathways have been investigated with these substrates and with specific reagents. Because the peptide substrate-based methods follow the principles of enzymology, a whole new concept of coagulation analysis has evolved.

Some of the commercially available synthetic chromogenic peptide substrates are listed in Table 2. All of these substrates are coupled with p-nitroaniline (pNA) as a chromophore at the terminal carboxyl group and contain arginine or lysine. Table 3 lists commercially available fluorogenic substrates currently available for the determination of serine protease enzymes and their inhibitors. The action of serine proteases on these fluorogenic substrates results in the release of various fluorophores, which can be measured with a suitable instrument.

Currently, reliable methods for the determination of AT-III, plasminogen, α2-antiplasmin, and absolute concentrations of heparin are available. Recently, chromogenic assays for Factors X and II have also become available (46–50). Aminolytic assays equivalent to prothrombin time and partial thromboplastin time are being developed (51–53). Assay methods based on synthetic substrates are expected to contribute significantly to the laboratory testing of coagulation function.

Development of Synthetic Substrates

Fibrinopeptide A is a known inhibitor of the action of thrombin on fibrinogen. Svendsen et al. (11) and Blomback (12) have reported on the amino acid sequence of fibrinopeptide A in various animal species. They found that certain amino acids in the carboxyl terminal of fibrinopeptide A were identical in all species, which suggested that the affinity to thrombin may be determined by these amino acids. At the positions 9, 2, and 1 of fibrinopeptide A are phenylalanine, valine, and arginine, respectively. This peptide fragment was shown to possess an anticoagulant activity and an affinity for thrombin.

Most synthetic substrates are developed after the peptide sequence of the site of action on the natural substrate has been established. The tripeptide or tetrapeptide sequence is isolated and chemically synthesized, and a chromophore or
Fluorophore is attached at the carboxyl terminal. Because this attachment is via an amide bond (CONH), the enzyme can cleave this bond and release a chromophore or fluorophore whose optical properties are significantly different from those of the native substrate.

The chemical structure of the first peptide substrate, S-2160, is shown in Figure 3. The amino acid sequence of this substrate is identical to the peptide sequence in fibrinogen at which the thrombin acts during the clotting process. The pNA group attached to the carboxyl end is released during the enzymatic process, the release being proportional to the activity of the enzyme. Most of the other peptide substrates are designed on the same concept (17). The representative absorption spectra of equal amounts of S-2160 and pNA are shown in Figure 4. Obviously, these two compounds have quite distinct spectral characteristics. Note that when pNA is bound to the synthetic tripeptide Bz-Phe-Val-Arg, it does not absorb significantly in the 400-410 nm range, whereas in the free form it absorbs strongly there. This is the basic principle of the application of chromogenic substrates, where both kinetic and endpoint methods are in use.

Application of Synthetic Peptides in Clinically Important Coagulation Parameters

A list of various synthetic peptide substrate methods for the coagulation parameters is given in Table 4. At present, the following commercially available methods have been developed (11-43): AT-III, plasminogen, heparin, and α2-antiplasmin.

Monitoring AT-III: AT-III activity is measured with specific substrates, S-2238 and chromozym-TH. A known amount of thrombin is incubated with a sample of diluted plasma in buffer containing heparin. The residual amidolytic activity of thrombin is measured and compared with standard AT-III or a normal human plasma preparation.

Heparin + AT-III → activated AT-III

Thrombin + activated AT-III

→ thrombin/activated AT-III complex

+ residual thrombin

Table 3. Synthetic Fluorogenic Substrates and Their Applications

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>CHEMICAL STRUCTURE</th>
<th>APPLICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>Boc-Val-Pro-Arg-MCA*</td>
<td>Prothrombin, Antithrombin-III, Monitoring of Oral Anticoagulants, Heparin, Platelet Factor III and IV</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>Boc-Ile-Glu-Gly-Arg-MCA**</td>
<td>X, Xa, Anti-Xa, Platelet Factor IV, Heparin and Endotoxin</td>
</tr>
<tr>
<td>Urokinase</td>
<td>Glut-Gly-Arg-MCA**</td>
<td>Urokinase, Urokinase Activators and Inhibitors</td>
</tr>
<tr>
<td>Plasmin</td>
<td>H-D-Val-Leu-Lys-AIE*</td>
<td>Plasminogen, Antiplasmin, Plasmin, and Plasminogen Activators</td>
</tr>
<tr>
<td>Urinary Kallikrein</td>
<td>Pro-Phe-Arg-MCA**</td>
<td>Kallikrein and Anti-Kallikrein</td>
</tr>
<tr>
<td>Pancreatic Kallikrein</td>
<td>Pro-Phe-Arg-MCA**</td>
<td>Glandular Kallikrein and Prekallikrein</td>
</tr>
<tr>
<td>Plasma Kallikrein</td>
<td>Z-Phe-Arg-MCA**</td>
<td>Plasma Kallikrein and Prekallikrein</td>
</tr>
<tr>
<td>Limulus Clotting Enzyme</td>
<td>Boc-Leu-Gly-Arg-MCA**</td>
<td>Endotoxin, Bacterial Activators</td>
</tr>
<tr>
<td>Ila, Xla</td>
<td>Boc-Phe-Ser-Arg-MCA**</td>
<td>Contact Factors</td>
</tr>
<tr>
<td></td>
<td>Boc-Leu-Thr-Arg-MCA**</td>
<td></td>
</tr>
</tbody>
</table>

*Substrate available in the Dade Protopath Kits.
**Substrate available from Peptide Institute, Protein Research Foundation, Osaka, Japan.
Table 4. Status of Synthetic Substrate Methodologies for the Evaluation of Coagulation Function

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-III</td>
<td>Satisfactory, all reagents used are readily available. Many commercially available kits. Standardization needed. Diagnostic value established.</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Satisfactory. Reagents are available. Standardization?</td>
</tr>
<tr>
<td>α2-Antiplasmin</td>
<td>Reagents are not available readily. Availability of plasmin is limited. One kit available.</td>
</tr>
<tr>
<td>Heparin</td>
<td>Only measures absolute concentrations of heparin. Of questionable benefit for the monitoring of therapeutic heparinization.</td>
</tr>
<tr>
<td>Platelet factor 4</td>
<td>Methodologies are standardized. Measures antiheparin activity and only reliable in samples not containing heparin.</td>
</tr>
<tr>
<td>Platelet factor 3</td>
<td>Complex method; ultra-pure non-activated prothrombin complex is needed.</td>
</tr>
<tr>
<td>Prothrombin (Factor II)</td>
<td>Still in developmental stages. A reliable assay is possible.</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>Satisfactory. All reagents available. Diagnostic significance is to be established.</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>Satisfactory. Helpful in the diagnosis of hypercoagulable state.</td>
</tr>
<tr>
<td>Factor X</td>
<td>Reliable method available. Useful in the management of oral anticoagulants.</td>
</tr>
<tr>
<td>Partial thromboplastin time (amidolytic equivalent)</td>
<td>Numerous methods available. Clinical efficacy not proven.</td>
</tr>
<tr>
<td>Prothrombin time (amidolytic equivalent)</td>
<td>Numerous methods available.</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Under development.</td>
</tr>
<tr>
<td>Factor VII</td>
<td>Under development.</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Under development.</td>
</tr>
<tr>
<td>Tissue activator of plasminogen</td>
<td>Under development.</td>
</tr>
<tr>
<td>Total serine protease activity</td>
<td>Under development.</td>
</tr>
</tbody>
</table>

Residual thrombin + H-D-Phe-Pip-Arg-pNA (S-2238)

\[ \rightarrow \text{H-D-Bz-Phe-Pip-ArgCOOH + pNA} \]

or

Residual thrombin

\[ \rightarrow \text{Tos-Gly-Pro-Arg-pNA(\text{chromozym-TH})} \]

\[ \rightarrow \text{Tos-Gly-Pro-ArgCOOH + pNA} \]

In the presence of heparin, suppression of the amidolytic activity of thrombin is inversely proportional to AT-III activity. An exactly identical principle is applicable to the assays involving fluorogenic substrates. Bovine Factor Xa and thrombin can be used instead of human α-thrombin.

**Plasminogen assay:** Plasminogen has always been a very difficult assay for coagulation laboratories, because of potent antiplasmin substances in human plasma. With the introduction of S-2251 and chromozym-PL, it has become easier to perform amidolytic assays on samples having true plasmin activity and devoid of antiplasmin substances (which is rarely the case). Methods for plasminogen determination have not been studied as extensively as those for AT-III. However, it is possible to measure plasminogen as a plasminogen/streptokinase complex (P/SK), whose amidolytic activity is directly proportional to the plasminogen content of the sample:

Plasminogen (P) + streptokinase (SK) \(\rightarrow\) P/SK

H-D-Val-Leu-Lys-pNA (S-2251)

\[ \text{P/SK} \rightarrow \text{H-D-Val-Leu-Lys-COOH + pNA} \]

Tos-Gly-Pro-Lys-pNA (chromozym-PL)

\[ \text{P/SK} \rightarrow \text{Tos-Gly-Pro-Lys-COOH + pNA} \]

All plasminogen assays are based on two reactions. First, the plasminogen in plasma reacts with a known amount of streptokinase to form a complex (P/SK). Second, the amidolytic activity of the complex is determined by using S-2251 or chromozym-PL as a substrate; the \(\Delta A_{405/\text{min}}\) is proportional to the amount of plasminogen. In the fluorogenic substrates the fluorophore released after the action of P/ SK action is measured to quantitate the plasminogen.

**Heparin methods:** An assay based on the heparin-accelerated inhibition of the activated Factor X was available long before the emergence of synthetic chromogenic substrates. In standard procedures for the synthetic-substrate methods, heparin is analyzed as a heparin/AT-III complex. The presence of AT-III in the test plasma is crucial; either normal plasma or purified AT-III can be used to assure proper complexation. These assays can be adapted for both endpoint and kinetic methods. Recently, a kit based on the chromogenic methodologies has been introduced by Kabi AB and Ortho Diagnostics: it contains the substrate, Factor Xa, AT-III, buffer, and normal human plasma in lyophilized form. The procedure is as follows:

Heparin + AT-III \(\rightarrow\) heparin/AT-III

Heparin/AT-III + factor X

\[ \rightarrow \text{heparin/AT-III/Xa + residual Xa} \]

Residual Xa + R-pNA

\[ \rightarrow \text{R-COOH + pNA (S-2222 or S-2337)} \]

Because the purified Xa preparations are difficult to obtain, Larsen et al. developed a heparin assay involving S-2238 and thrombin (39):

Heparin + AT-III \(\rightarrow\) heparin/AT-III

Heparin/AT-III + thrombin \(\rightarrow\) heparin/AT-III/thrombin + residual thrombin

Residual thrombin + R-pNA \(\rightarrow\) R-COOH + pNA (S-2238 or chromozym-TH)

Fluorogenic substrates for the assay of thrombin and factor Xa can also be used for the quantitation of absolute amounts of heparin.

**Antiplasmin methods:** α2-Antiplasmin is now known to be the primary antiplasmin in blood because of its rapid and relatively specific inhibition of plasmin activity. Numerous
studies have shown that the amount of $\alpha_2$-antiplasmin fluctuates in certain disorders (54, 55). Various chromogenic and fluorogenic methods have been proposed for the quantitation of this inhibitor. A known amount of plasmin is incubated with the test material for 30 to 60 s. The amount of residual plasmin is quantitated with plasmin-specific substrates, such as S-2251 or chromozym-PL:

$$\text{Plasmin + plasma (} \alpha_2 \text{-antiplasmin)}$$
$$\rightarrow \text{Plasmin/} \alpha_2 \text{-antiplasmin + residual plasmin}$$

Residual plasmin + R-pNA (S-2251 or chromozym-PL)
$$\rightarrow R-\text{COOH} + \text{pNA}$$

**Automation of Synthetic-Substrate Methods**

Because synthetic-substrate methods are based on the release or the suppression of release of a chromophore or fluorophore, most of the enzyme analyzers used in clinical chemistry laboratories can be used for these analyses. Scully and Kakkar have used an LKB analyzer (LKB Instruments, Rockville, MD 20852) to measure thrombin, AT-III, and antithrombin, with S-2238 as substrate (21). Using the LKB analyzer, Bergstrom and coworkers automated a method for determining plasma prothrombin (56, 57). The application of various centrifugal analyzers for the determination of AT-III have been reported (58, 59). Recently, automated determination of antiplasmin and AT-III with the ABA-100 system (Abbott Diagnostics, N. Chicago, IL 60064) has been described (60). Yamada and Meguro (61) have recently described a novel method of activated partial thromboplastin time, using the chromogenic substrate for thrombin and an AutoAnalyzer (Technicon Instruments, Tarrytown, NY 10591). A fully automated chromogenic-substrate method for measuring plasma Factor Xa to monitor patients receiving coumarin therapy has also been reported (62).

**Standardization of Synthetic-Substrate Methodologies**

Because most of the synthetic substrates are used in activator, zymogen, enzyme, and inhibitor assays and follow the principles of enzymology, eventually their use will have to follow the recommendations of the International Commission of Enzymology.

Standardization of reagents available for the synthetic-substrate methodologies is a growing concern. Although the whole field of synthetic-substrate methodologies has grown very rapidly, the availability of purified reagents is still a serious limitation. Commercially available reagents such as thrombin, Factor Xa, plasmin, AT-III, and kallikrein vary greatly from source to source and from batch to batch. A classic example is the available human thrombin preparation: despite the potency values assigned to a known thrombin lot, wide variations have been detected. Moreover, most of the coagulation enzymes are standardized in terms of their relative amidolytic or lytic properties, which are not the same as their amidolytic activity. Availability of a complete kit for AT-III, antiplasmin, prekallikrein, heparin, and their coagulation parameters undoubtedly puts the responsibility on the manufacturer's shoulders, and we have seen good quality control measures taken by these manufacturers.

**Future Trends in Measurement of AT-III and Heparin**

The introduction of synthetic-substrate assays will play a key role in the development of diagnostic methodologies to test hemostatic function. AT-III determination has already become a routine diagnostic test in major medical centers throughout Europe and the United States. The amounts of heparin/AT-III complex may possibly be used in the study of kinetics of heparin and the evaluation of heparin products by clinical pharmacologists. One area where the application of synthetic-substrate methodologies for heparin may have a value is the study of the release of endogenous heparin or heparin-like substances in certain pathophysiologic conditions. The clinical significance of endogenous heparin has yet to be established. The ratio of the functional AT-III and heparin concentrations may also have some diagnostic value. Although methods based on the clotting principle for the measurement of heparin are available, these tests are time consuming, expensive, and difficult to perform. In addition, reproducibility is often a problem, and the biological reagents, once reconstituted, cannot be used repeatedly.

Mass screening of AT-III is possible where automated methodologies are available; at the present, automated and semiautomated methods are used with the LKB analyzer, the Abbott Bichromatic analyzer, the Beckman Model 35 kinetic analyzer (Beckman Instruments, Fullerton, CA 92634), the Gilford Model 3500 analyzer (Gilford Instrument Labs., Oberlin, OH 44074), and Technicon analyzers. The application of centrifugal analysis is quite attractive, as is the use of a discrete analyzer for the determination of antithrombin and most of the other coagulation parameters.

Testing of hemostatic function is at present considered to be a highly specialized test battery, performed only in the coagulation laboratories. With synthetic substrates the determination of AT-III may also be within the scope of the routine clinical chemistry laboratory. In the near future coagulation panels based on clinical chemistry principles will be available. The role of industry in the development of such panels is a crucial one. Preliminary studies in many laboratories indicate that AT-III, antiplasmins, plasminogen, anti-Xa, antikallikrein, and progressive antithrombin activity can run as one panel.

Methods based on synthetic peptide substrates are currently more expensive than the existing methods, with reagents and substrates being the main contributors to the cost. Improving the methods for synthesizing these substrates may also lower costs. There is no serious problem with the specificity with the existing substrates; however, it may be profitable to provide more sensitive substrates. The development of agents such as the tetrazolium in dyes is desirable. Coupling substrates with a fluorophore may also help reduce costs and increase the sensitivity of the assay system.

Education of laboratory personnel is important. At this time the biochemical concepts involved in the coagulation processes are generally poorly understood by laboratory personnel. Extensive educational material and notes on application should be provided for review of the biochemical concepts and their pertinence to the pathology of hemostatic defects.

The role of various government agencies in the development of chromogenic substrates can be summarized as follows:

1. Promotion of basic research in the developmental areas.
2. Standardization of methodologies by the National Bureau of Standards and the Bureau of Biologic Standards.
3. Coordination of clinical trials in conjunction with professional organizations such as the American Society of Clinical Pathologists and the American Association for Clinical Chemistry.
4. Implementation of well-defined regulations regarding use of these new concepts.

Because they involve a new concept, the synthetic-substrate methodologies need a great deal of work to be accepted in the routine laboratory. An important consideration is the availability of reliable reagents, especially the Factor Xa and thrombin preparations. These enzymes must be consistent, and a known standard should always be available for comparison.

Previously stated problems with the chromogenic-substrate
methodologies such as nonspecificity, formation of fibrin in the reaction mixture, possible inhibition of thrombin by substrate or products, appearance of turbidity, instability of thrombin, substrate instability, and precipitation of the pNA have already been worked out, and the present assays do not suffer from these problems. Any diagnostic laboratory test that correlates well with the clinical status of the patient and that will accurately and reproducibly detect biologic events should be available to the physician. The synthetic substrates lend themselves extremely well to the assay of the enzymes of coagulation and related systems, and are equally useful in the assay of the inhibitors in these systems.

The demonstration of the clinical usefulness of these tests is the first step in their adoption as routine laboratory methods. More clinical studies comparing the standard methods with these newer methods are needed, but we feel that all indications at present are that they are well suited for routine use.

Commercially Available Kits

We have examined commercially available AT-III methods and have found them reliable. The assay methods for prekallikrein and antiplasmin need further clinical work to be utilized in the diagnosis of a given coagulopathy. A comparison of results obtained with various commercially available kits for AT-III is given in Table 5; obviously, most of these methods give comparable results for AT-III. The results for the radial immunodiffusion methods in a normal population and with a standard preparation of AT-III are comparable. In abnormal plasma samples higher results were obtained with radial immunodiffusion; however, both the clotting and the chromogenic methods gave comparable results (unpublished data).

Fluorogenic Substrate Methods

Recently, Dade Division (American Hospital Supply Corp., Miami, FL 33152) introduced a complete system, including a dedicated instrument ("Protopath"), for the fluorometric quantitation of AT-III, plasminogen, and heparin in plasma (65-65). The principles of the fluorometric method are identical to the ones used in the chromogenic substrate method except that instead of pNA a fluorogenic material, 5-amidoisophthalic acid dimethyl ester, is released and kinetically measured with the Protopath fluorometer.

In addition, the heparin methodologies in this system make use of thrombin wherever Factor Xa was used in the chromogenic methods. The assay methods for the quantitation of AT-III and plasminogen determination are exactly the same as those applied in the chromogenic analysis, except that the fluorophore released is designed to be measured only with the Protopath enzyme analyzer. These kits cannot be conveniently used with other spectrophotofluorometers, which limits the user to only one instrument. Table 6 compares the concentrations of AT-III, plasminogen, and heparin as determined by the chromogenic and fluorogenic methods; both methods gave comparable results.

Although we believe that AT-III and plasminogen analysis based on the synthetic peptide methods provide reliable results, in our hands, we still feel that the therapeutic anticoagulant action of heparin is best monitored with the activated partial thromboplastin time and other coagulant assays. The determination of absolute amount of heparin in a patient’s plasma in meaningless, in that many endogenous factors such as AT-III, platelet factor 4 (antithrombin factor), and some of their proteins affect the anticoagulant action of heparin. These studies are limited, and we need to gather more data.

Immunological Method in Coagulation Testing

The specificity and sensitivity of the antigen/antibody reaction has provided a unique tool to determine minute quantities of antigenic proteins in serum and other biologic fluids. Immunologic methods have been widely utilized for the absolute quantitation of coagulation proteins (2-9). Newer methods such as radioimmunoassays, laser and rate nephelometry, enzyme-linked and other fluorimunoassays have been introduced and are being used in most clinical and research laboratories. For a general account of immunological principles and techniques, see the reviews by Clausen (66) and by Rose et al. (67).

Radial Immunodiffusion

Since the introduction of radial immunodiffusion methods (68-70), many commercially prepared radial immunodiffusion plates for coagulation proteins have become available. For a critical review on this methodology see Ritzman (71). Al-

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Table 5. AT-III Determination by Commercially Available Methods and by a Method Developed at Our Laboratory

<table>
<thead>
<tr>
<th>Method</th>
<th>Human plasma</th>
<th>AT-III concn.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Coagulopathy</td>
</tr>
<tr>
<td>Kabi</td>
<td>107 ± 16</td>
<td>67 ± 31</td>
</tr>
<tr>
<td>Stago</td>
<td>113 ± 16</td>
<td>17 ± 26</td>
</tr>
<tr>
<td>Boehringer-Mannheim</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Abbott</td>
<td>118 ± 13</td>
<td>79 ± 24</td>
</tr>
<tr>
<td>Pacific Hemostasis</td>
<td>107 ± 21</td>
<td>64 ± 30</td>
</tr>
<tr>
<td>Loyola University</td>
<td>94 ± 13</td>
<td>58 ± 11</td>
</tr>
<tr>
<td>Radial immunodiffusion</td>
<td>109 ± 31</td>
<td>89 ± 11</td>
</tr>
</tbody>
</table>

(Celbiochem)

Kabi, Stockholm, Sweden; Stago, Asnières, France; Boehringer-Mannheim, Garmisch, F.R.G.; Abbott, North Chicago, IL 60064; Pacific Hemostasis, Bakersfield, CA 93301; Celbiochem, San Diego, CA 92112.

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Table 6. Comparison of AT-III, Plasminogen, and Heparin, As Determined with Chromogenic and Fluorogenic Substrates

<table>
<thead>
<tr>
<th></th>
<th>Chromogenic methods</th>
<th>Fluorogenic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-III (n = 100)</td>
<td>78.1 ± 11.8</td>
<td>82.0 ± 13.6</td>
</tr>
<tr>
<td>Plasminogen (n = 100)</td>
<td>81.9 ± 12.6</td>
<td>84.6 ± 12.9</td>
</tr>
<tr>
<td>Heparin (n = 50)</td>
<td>0.48 ± 0.08</td>
<td>0.51 ± 0.01</td>
</tr>
</tbody>
</table>

The Dade "Protopath" System was used for all three analyses. Kinetic methods developed at Loyola were used for the AT-III and plasminogen determinations, whereas a commercially available kit (Ortho Diagnostic) was used to quantitate heparin.
though these procedures are reliable, their turnaround time often exceeds 48 h, making them somewhat undesirable in a clinical situation where results are required immediately.

Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis is extensively used in immunology and clinical chemistry (72, 73). The technology has been used extensively in the study of Factor-VIII-related antigen, AT-III, and α2-antiplasmin.

The rocket method offers several advantages over conventional immunodiffusion methods. Quantitation is achieved in only a few hours instead of days, and antigenically related coagulation proteins can be identified and quantitated. Recently, an automated electro-immuno quantitation system ("EIQ"; ICL Scientific, Fountain Valley, CA) has become available for quantitating α1-antitrypsin and other serum and plasma proteins.

Radioimmunoassay

Ever since radioimmunoassay was introduced by Yalow and Berson (74) to measure endogenous human plasma insulin, efforts have been made to apply this technique in practically all areas of laboratory medicine. An extensive review on this technology as it pertains to clinical chemistry is available (75). Fibrinopeptide A, platelet-release-specific proteins, AT-III, and many of the other components of coagulation system have been measured by radioimmunoassay (7).

Nephelometry

Several laboratories are now utilizing commercially available laser and rate nephelometers to quantitate coagulation proteins. In 1977 Sternberg (76) introduced a rate nephelometer to monitor the rate of light-scatter change. This particular feature eliminated the necessity of blank corrections, which is necessary in equilibrium nephelometry (i.e., light scatter is measured at the equilibrium of antigen/antibody reactions). At present, laser and rate nephelometers are satisfactorily utilized for estimating the quantities of several proteins. Coagulation proteins that can be quantitated by nephelometric methods include: fibrinogen, AT-III, plasminogen, α2-macroglobulin, α2-antiplasmin, fibrinopeptides, fibrin-split products, and prothrombin. The methods are reportedly reproducible and highly sensitive, and correlate well with radial immunodiffusion methods (77).

Fluorimmunoassay

A solid-phase fluorimmunoassay has been developed recently to quantitate Factor-VIII-related antigen (VIII R:Ag) (78). In this assay a fluorescein-labeled anti-VIII R:Ag is allowed to react with the test plasma, and the unbound antibody is then removed by using a specially designed sampler ("Stiq") precoated with a purified VIII R:Ag. The fluorescence on the polymeric surface of the sampler is measured with a special fluorometer system (FIAX, International Diagnostics Technology, Santa Clara, CA 95050). The precision of the assay reportedly compares favorably with electro-immunoassay. We have adopted this system in our laboratory to quantitate VIII R:Ag, and the results so far indicate a correlation coefficient (r) of 0.96 between the FIAX and the electro-immuno detection methods. Almost all of the coagulation protein/serine protease-inhibitor complex can be detected with the FIAX and electro-immuno diffusion methods.

Enzyme-Linked Immunoassay

Various competitive and noncompetitive enzyme-linked immunosassays have been developed to measure several coagulation proteins (79, 80). The principle of the assay is similar to fluorimmunoassay, except that an enzyme-conjugate is utilized instead of the fluorescein-labeled antibody. Substrate hydrolysis by the enzyme-conjugate is inversely proportional to the amount of test protein in plasma. Ness and Perkins (81) have quantitated VIII R:Ag by this method and claim to have increased sensitivity over the Laurell method. However, enzyme immunoassays do not seem to offer serious advantages over the Laurell method or fluorimmunoassays. The technique requires additional steps of: (a) purifying VIII R:Ag on a polysacrylamide agarose column, (b) isolating goat anti-rabbit IgG by immunoadsorption, (c) conjugating the enzyme to the antibody, (d) preparing substrate solution and buffers, and (e) performing several incubation steps. Aside from the increased sensitivity with this method, no particular advantage is seen in the diagnosis of Factor VIII-related disorders.

Immunologic Studies of Serine Protease Inhibitor Complexes

The role of various serine protease inhibitors in regulating the function of coagulation and fibrinolytic enzymes such as thrombin, Factor Xa, and plasmin is quite clear. The existence of an AT-III-serine protease complex, which has been reported previously (9, 10, 88), indicates that the process of activation has taken place and that the active enzyme has formed a complex with a given inhibitor. The detection of an enzyme/inhibitor complex will provide a useful mean to evaluate the activation of a given system.

Clinical Considerations

During the last two decades, rapid advances have been made in immunological concepts and immunologic methods. Clinical investigators must incorporate these newer concepts and methods to interpret results of laboratory tests performed to aid in diagnosis, therapeutic monitoring, and prognosis of a hemostatic and thrombotic disorder; therefore, specificity and sensitivity of a given method are important considerations. Although immunodiagnosis depends to a large extent on antibody specificity, there are no widely available international standards for most coagulation proteins. Commericially available antisera to most coagulation proteins and related proteins show inappropriate specificities when assayed with a panel of antigens (82). To overcome this problem, several recommendations on the characterization and clinical use of immunologic reagents have been made (83). At present, each immunologic method should be critically evaluated for the significance of its results and its suitability in a coagulation laboratory. Immunologic methods provide important information on the qualitative nature of a coagulation protein. In rocket immunoelectrophoresis the genetically variant and complex forms of coagulation protein are distinguishable.

Immunologic methods, however, quantitate coagulation protein in terms of absolute amounts, which does not truly reflect the function of a given coagulation protein. It is, therefore, important to consider the limitations of these methods before making any firm diagnosis.

Study of Fibrinogen and Its Derivatives

Molecular elucidation of fibrinogen has played a key role in the understanding of coagulation process and has provided useful diagnostic information in certain coagulation disorders. The study of fibrinogen can be divided into the following categories: the study of the native fibrinogen and its variants, cleaved fibrinopeptide A and B, proteolytic degradation products of fibrinogen and fibrin, soluble fibrin monomer/polymer complexes, and fibrinolytic enzymes and inhibitors.

Fibrinopeptide A and B can be analyzed by thin-layer chromatography and thin-layer high-voltage electrophoresis. Reliable radioimmunoassays are also available for the quantitation of fibrinopeptide A (84). The quantities of fibrino-
peptide A are increased in hypercoagulable states and related disorders (65). Gel electrophoresis has been extensively used to study fibrinogen and its derivatives (86, 87). From a diagnostic standpoint increased amounts of fibrinopeptide A in plasma are indicative of the production of fibrin. Nossel et al. have developed a radioimmunoassay for fibrinopeptide A that is applicable in clinical situations (88). A commercial kit (IMCO Corp., Stockholm, Sweden) is also available. As discussed previously, the serine protease inhibitor complex can be quantitated by radioimmunoassay methods. Pre-existing plasmin/antiplasmin complexes that circulate in blood for some time after the induction of pathologic or therapeutic fibrinolysis may reflect the degree of activation (10). Colleen and de Cock (10) have used the appearance of a neoantigen in such complexes to produce an agglutination test readily applicable in clinical situations. They reported a high titre of such complexes in plasma samples from patients with such fibrinolytic conditions as disseminated intravascular coagulation.

**Platelet Functions and Activities**

Our understanding of the platelet physiology and biochemistry has recently been enhanced by the availability of new methodologies; only some aspects, related to clinical chemistry, are considered here. Several recent reviews cover newer tests for evaluating platelet function (89-97), for which, from a diagnostic standpoint, the following areas are of interest: platelet-release-specific proteins, prostaglandins and their derivatives, and biochemical aspects of platelet aggregation and adhesion.

Most existing platelet-function tests such as bleeding time, platelet retention, aggregation, release reaction, platelet factor 3 availability, circulating aggregates, and survival studies suffer from certain limitations. The problem arises from attempting to evaluate cellular function in an in vitro situation, where an essential constituent (vascular endothelium) is absent and where coagulation is controlled by use of exogenously added anticoagulants. Except for imaging and other sophisticated tests, a reliable test to evaluate the endothelial function is not available. However, the study of prostaglandin derivatives in circulating plasma indirectly gives an indication of the role endothelium plays in the regulation of prostaglandins and their derivatives, thereby modulating platelet function (98-100). Obviously, in vivo tests are highly desirable, but except for bleeding time tests, which can be influenced by factors other than platelets, no such easily performed test is available.

**Release of Platelet-Specific Proteins In Vivo**

The biochemical and ultrastructural considerations of platelet-release reactions have been elegantly reviewed by MacIntyre (101) and White (102). Isolation and characterization of platelet-specific proteins have made it possible to monitor the release reaction in vivo by radioimmunoassay methods. ADP and serotonin (5-hydroxytryptamine) can be measured during a release reaction, but because of their relatively short half-lives and insensitive methods for their determination, the quantities of these two release substances provide only limited information.

The Scottish group isolated two platelet-specific antigens, platelet factor 4 and β-thromboglobulin, and radioimmunoassays for both have been developed (103-106). Preliminary applications of these tests to clinical situations indicated that the tests were a reliable measurement of the platelet-release reaction in vivo. Ellis et al. have reported that platelet factor 4 is increased in patients with coronary diseases (107). Although numerous publications on these two proteins have appeared, our current knowledge of the basic physiology of platelet-release reactions in normal and disease states is still incomplete; hence, the diagnostic efficacy of these two proteins needs additional clinical support data.

Localization of various substances in platelet granules has been investigated indirectly by their susceptibility to different concentrations of aggregating agents. This technique indicates that there are apparently three types of granules (101): α-, or light, granules with platelet-specific proteins; β-, or dense, granules with amines and nucleotides; and other α-granules with lysosomal hydrolases. Another interesting release protein is the cell-growth-stimulating (mitogenic) factor, which has been implicated in the proliferation of smooth muscle cells within the vascular intima; it is located in the same α-granules as β-thromboglobulin and platelet factor 4. The diagnostic role of this protein is yet to be evaluated.

**Prostaglandins and Platelets**

Many of the substances formed by platelets during the release reaction arise through the liberation of arachidonic acid from platelet or arterial membrane phospholipids, either through the action of phospholipase A2 (108, 109) or through the sequential actions of phospholipase C and diglyceride lipase (110). When arachidonic acid is acted upon by cyclooxygenase, the short-lived aggregating agents PGD2, PGE2, and thromboxane A2 are formed (111-115). Although all investigators agree that thromboxane A2 is a potent aggregating agent, there is some controversy concerning whether PGG2 and PGH2, which give rise to thromboxane A2, are themselves aggregating agents (98, 116). Thromboxane A2 also causes vasoconstriction (117). The stable end-product, thromboxane B2, is chemotactic (able to stimulate mobility) for leukocytes. A small proportion of the PGH2 may give rise to PGE2 and PGD2 instead of thromboxane A2 (118). PGE2 affects vessel permeability and causes vessel contraction (119, 120). In some animal species and in humans PGD2 inhibits platelet aggregation (118). Arachidionate molecules that are acted upon by platelet lipoxygenase are converted to several products, including 1,2-hydroxy-5,8,10,14-eicosatetraenoic acid, which is also chemotactic (121). Other substances that appear in the medium when platelets interact with release-inducing agents are collagenase elastase and a factor that interacts with the fifth component of complement (C5) to form a chemotactic substance (122-124).

As far as is known, all the agents that cause the release of platelet granule contents also activate phospholipase A2. The link between the release reaction and activation of this enzyme has not been established, although both probably involve the movement of internal platelet calcium (98, 116, 117).

Malondialdehyde, a metabolite of labile endoperoxide PGG2, is formed during platelet aggregation (125). Because it can easily be measured by incubation with thiobarbituric acid to form a pink pigment, it is a useful indicator of platelet prostaglandin synthesis. Stuart et al. first estimated platelet survival by measuring the rate of return of such synthesis after its inactivation by aspirin (126).

Serial determinations of malondialdehyde are performed on platelet samples aggregated with thrombin, epinephrine, or N-ethylmaleimide after the ingestion of one 600-mg dose of aspirin. Platelet lipid peroxidation (nanomoles of malondialdehyde per 10⁶ platelets) is markedly inhibited, with a linear return to normal production as the acetylated platelets disappear. Half-life values in both normal persons and thrombocytopenic patients agree closely with those obtained by using ⁵¹Cr, and the technique is considerably less cumbersome than injection of radioisotope-labeled autologous platelets. Uncontrolled aspirin-taking must be excluded for 10 days before and during the study period. Roncucci et al. have automated the method, enabling multiple replicate determinations of malondialdehyde to be made when necessary (127).
A recent report has compared the plasma concentration of thromboxane B₂ in Prinzmetal's angina (variant angina) and clinical angina pectoris (128, 129). These investigators concluded that spontaneous thromboxane generation in variant angina is associated with coronary artery spasm and may play a key role in anginal and myocardial infarction. Thromboxane B₂ is a stable metabolite of thromboxane A₂ and has a much longer half-life. A reliable radioimmunoassay for thromboxane has been previously described (130). Undoubtedly, the measurement of prostaglandins and their metabolites by various conventional methods will aid in the diagnosis of coagulation and vessel defects. Some of the newer immunological methods will be extensively applied in this area. The antiplatelet action of various drugs can also be assessed by using techniques to measure prostaglandins and their metabolites.

Assessment of coagulation function is no longer restricted to the measurement of clot formation by such methods as the bleeding, clotting, prothrombin, and partial thromboplastin times. Newer tests based on biochemical, pharmacological, immunological, chemical, physiological, and radiochemical principles have emerged to identify the locale and nature of the hemostatic defect in a given disorder. With the introduction of synthetic chromogenic and fluorogenic tripeptide substrates, it has become possible to assay for specific coagulation enzymes, their activators, and inhibitors. The concept of coagulation profiling has emerged, and many panels are being developed. Newer therapeutic agents have necessitated the development of fast and reliable diagnostic tests to measure therapeutic efficacy and effect on the overall coagulation status of a patient. The continued development of newer methods to evaluate the hemostatic function and to understand the mechanisms involved is made possible by an overwhelming interest among basic and clinical scientists in the study of hemostasis. Because of the increased understanding of the biochemistry and pathophysiology of thrombotic and hemostatic disorders, the introduction of synthetic peptides and immunochemical methods, and the application of automated analyzers, we project a growing role for clinical chemists in this developing area of laboratory medicine. In the coming years the testing of hemostatic functions will be affected by these dramatic developments; clinical chemists, immunochemists, and pharmacologists will play an important role in implementing many valuable tests for diagnosing disorders related to the hemostatic system.

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