The Role of Lipids in Blood Coagulation*

AARON J. MARCUS

Hematology Section, N.Y. Veterans Administration Hospital, New York, N.Y., and the Department of Medicine, Cornell University Medical College, New York, N.Y.

I. Introduction

When a venipuncture is performed on a normal subject and the blood is placed in a glass test tube, it remains fluid for a few minutes,

* Research work mentioned in this report was supported by Grant HE-09070-03, National Institutes of Health, U.S.P.H.S., The Veterans Administration, and The New York Heart Association.

but suddenly a solid mass is seen. The tube can now be completely inverted without loss of its contents. The biochemical and physiological events leading to the formation of this gelatinous mass, or blood clot, have been the subject of intensive research for many years. Recently there has been more agreement on the basic mechanism than ever

Table I[a]

NOMENCLATURE OF BLOOD COAGULATION FACTORS INCLUDING THEIR SYNONYMS

Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Thromboplastin (tissue thromboplastin)
Factor IV	Calcium
Factor V (and VI)	Proaccelerin (accelerin) Accelerator globulin (Ac-globulin)
Factor VII	Proconvertin Serum prothrombin conversion accelerator (SPCA) Autoprothrombin I
Factor VIII	Antihemophilic globulin (AHG) Antihemophilic factor (AHF) Platelet cofactor I Facteur antihémophilique A
Factor IX	Plasma thromboplastin component (PTC) Christmas factor Platelet cofactor II Autoprothrombin II Facteur antihémophilique B
Factor X	Stuart–Prower factor
Factor XI	Plasma thromboplastin antecedent (PTA)
Factor XII	Hageman factor (HF)
Factor XIII	Fibrin-stabilizing factor (FSF) Laki–Lorand (L–L) factor Fibrinase

[a] See Hunter *et al.* (1965).

before, probably because of more refined investigative techniques and increased communication through agencies such as the International Committee on Blood Clotting Factors (Table I). The fibrin clot observed in the test tube resulted from the action of the proteolytic enzyme, thrombin, on the plasma protein, fibrinogen. Thrombin was formed from its precursor, prothrombin—another plasma protein, which can be activated via two pathways: one involving a factor

derived from tissues, and the other originating from components of the intravascular compartment itself (Biggs and Macfarlane, 1962).

It is well-established that lipids are closely associated with the coagulation process. In recent years much has been learned about their contribution to the coagulation sequence, due in large part to advances in lipid methodology (Hanahan, 1960). In this chapter an attempt will be made to summarize and interpret some of the contributions of lipid research to our knowledge of the clotting mechanism. Also, some speculations will be given and personal opinions expressed with which many may disagree. In order to place these in proper perspective, the normal clotting process will be described first. A comprehensive review of this subject has recently been published (Davie and Ratnoff, 1965).

II. Current Theory on the Mechanism of Blood Coagulation

A. Formation of the Intrinsic Prothrombin Activator

When blood comes in contact with a "foreign" surface, such as glass, kaolin, or sodium stearate, a series of interactions are set into motion. This has been termed the "contact phase" of coagulation, recently studied in meticulous detail by Waaler (1959), Iatridis and Ferguson (1962), and Nossel (1964). However, when blood is in contact with normal vascular endothelium or collected in silicone-treated containers or plastic tubing (Spaet *et al.*, 1959), the contact phase is unimportant. Two plasma coagulation factors are involved in this contact stage. The first is Hageman factor, which is in some way converted to an active form by "foreign" surface contact. This may be related to the nonmigratable negative ionic characteristics of surfaces such as glass (Hubbard and Lucas, 1960), but recently a soluble compound, ellagic acid, has also been shown to activate Hageman factor (Botti and Ratnoff, 1964). The difference between active and inactive Hageman factor may be due to actual rearrangement of the protein molecule, but its exact nature is not known (Davie and Ratnoff, 1965). Active Hageman factor, in turn, is responsible for the activation of PTA, and Botti and Ratnoff (1963) have found that this reaction can be accelerated by long-chain saturated fatty acids. The active form of PTA then activates PTC (plasma thromboplastin component), which subsequently interacts with the antihemophilic factor (AHF). From a clinical standpoint, the antihemophilic factor and PTC are the most important coagulation proteins. There is some recent evidence (Macfarlane, 1964; Lundblad

and Davie, 1964) that the activation of AHF requires phospholipid; however, most work on this subject has centered about its participation at a later stage. Activated antihemophilic factor is then responsible for activating factor X. The active form of factor X is probably the same type of material which has been studied in various laboratories and given different names (Milstone, 1964; Seegers, 1964; Spaet, 1964; Ferguson, 1964). In other words, thrombokinase (Milstone, 1964), product I (Bergsagel and Hougie, 1956; Spaet and Cintron, 1963; Zucker-Franklin and Spaet, 1963), and autoprothrombin C (Seegers, 1964) seem to have the same requirements for forming the so-called intrinsic prothrombin activator (also known as "prothrombinase"), that is, factor V, phospholipid, and probably calcium.

It is currently thought that phospholipid exerts a surface catalytic influence on the reaction (Bangham, 1961a; Macfarlane, 1964), and its source is the blood platelet. It is doubtful whether the phosphatide is available as such, but more likely as a lipoprotein complex. Although human plasma will eventually clot in the absence of platelets, the cell-free plasma of lower forms, such as the *Limulus,* does not do so unless amebocytes (a type of cell normally found in its circulation) are added (Levin and Bang, 1964).

B. Formation of the Extrinsic Prothrombin Activator

The important difference between the extrinsic and the intrinsic systems is the involvement of material "extrinsic" to blood vessels. This is referred to as "tissue factor," a lipoprotein moiety especially abundant in such organs as lung, brain, and placenta. It probably plays an important role in situations where the continuity of large areas of tissue is interrupted, as in surgery, extensive trauma, and childbirth. The studies of Williams (1964, 1965), Nemerson and Spaet (1964), and Nemerson (1966) indicate that tissue factor forms a complex with factor VII in the presence of calcium (which may actually be a part of the complex). The material interacts with factor X to produce the active form of this protein which, in the presence of factor V and lipid derived from tissue factor itself, produces the extrinsic prothrombin-activating material. It is of interest that factors V and X are involved in both the extrinsic and intrinsic systems, whereas factor VII appears to play a role in the extrinsic mechanism only. These characteristics are also of diagnostic importance, since the extrinsic activator in a hemophiliac or PTC-deficient patient is normal, but both systems are abnormal in factor V and factor X deficiency. It is emphasized that these

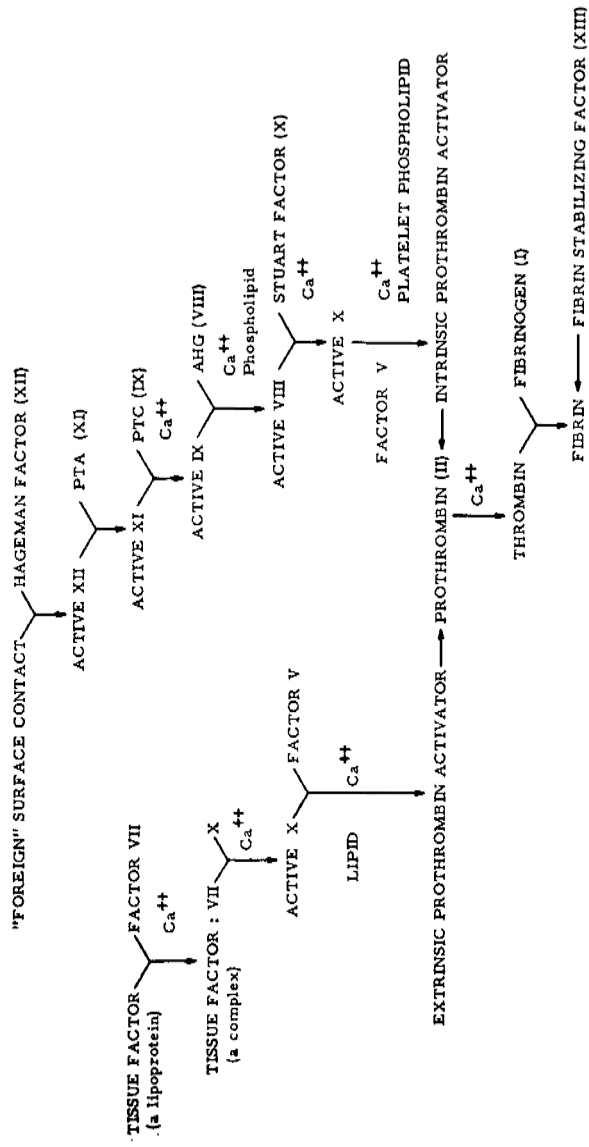

FIG. 1. A simplified diagram showing the two pathways (intrinsic and extrinsic) of prothrombin activation. Note that in the intrinsic pathway several protein interactions take place before lipids play a role. Interesting aspects of this scheme have recently been discussed by Seegers (1965) and Macfarlane (1966).

descriptions of the clotting mechanism are simplified and somewhat diagrammatic. The concepts are constantly changing in the light of new experimental findings and must be considered in that perspective. The extrinsic and intrinsic systems are illustrated in Fig. 1; work in this field has been summarized by Pool (1964).

C. Distinctions between Hemostasis, Thrombosis, and Coagulation

For illustrative purposes these may be considered as separate entities, although there is some evidence for an interrelationship (Mustard *et al.*, 1964a; Horowitz, 1965b). When blood vessels are damaged or cut, the hemostatic mechanism rapidly comes into play. Masses of platelets adhere to the edge of the cut, and a "hemostatic platelet plug" begins to form. There is adhesion of platelets to areas of connective tissue, with further aggregation mediated by adenosine diphosphate (ADP), a finally firm consolidation of these aggregates, in all likelihood mediated by thrombin (Spaet and Zucker, 1964). This mechanism has recently been discussed by Zucker and Marcus (1966). Sawyer *et al.* (1965) have offered an electrochemical explanation for the hemostatic process. They demonstrated irreversible platelet precipitation (in contrast to leucocytes and erythrocytes) on a platinum electrode. The phenomenon showed certain similarities to hemostatic platelet plug formation.

Arterial thrombi in many ways resemble hemostatic platelet plugs. They form in moving blood over distorted endothelial surfaces and are influenced by those factors responsible for platelet adhesion and aggregation. There is little fibrin in the interstices, and the thrombus itself becomes reinforced by a clot at its proximal end. Microscopic sections through the distal portion of arterial thrombi usually reveal a "pure culture" of relatively intact platelets. In addition, their development probably cannot be prevented by the usual doses of anticoagulant drugs. On the other hand, a clot in a vein usually forms in relatively "static" blood, and histologically resembles the clot seen in a test tube. There is a heterogeneous mixture of platelets, red cells, white cells, and fibrin. Furthermore, heparin and coumarin derivatives can prevent their formation (Merskey and Drapkin, 1965). No direct correlation has been found between lipids and hemostasis. It is certainly possible that platelet thrombi may form on the surface of a rough intimal plaque, and the plaque may have originated through some derange-

ment of plasma lipids, but the thrombotic event at its surface probably has no connection with lipid metabolism. Furthermore, in the absence of stasis a thrombus does not appear to be augmented by induced "hypocoagulable" or "hypercoagulable" states (Honour and Russell, 1962; Arakawa and Spaet, 1964).

It is not known whether blood coagulation takes place continuously *in vivo* (Hjort and Hasselback, 1961; Spaet, 1963). The observation that coagulation proteins have a short half-life is frequently cited as evidence in favor of the concept. Although platelets and fibrin have been found on abnormal endothelium, it has never been conclusively shown that they can be deposited on normal endothelial tissue, although Mustard *et al.* (1964b) believe that platelet thrombi can occur on apparently normal endothelium. The question is far from settled, but is of considerable importance since fibrin or platelet masses may form a nidus for atherosclerotic plaques (Duguid, 1954; Mustard *et al.*, 1964b). For further details see O'Brien (1964) and Marcus and Zucker (1965).

D. *In Vitro* Tests for Evaluation of Lipids in Coagulation

One of the tests employed for many years is the clotting time of recalcified platelet-poor plasma. Normally this is about 275 seconds. If platelets or a platelet lipid extract is added along with the calcium, the clotting time ranges between 60 and 125 seconds. Similar results are obtained with phospholipids from other sources. This simple experiment illustrates the importance of lipids in the coagulation process. However, it is important to point out that this is a nonspecific test system, since many types of particulate matter are capable of shortening the recalcified clotting time. The effect of the lipid in question should be compared with platelets or a platelet lipid extract, or lipids extracted from other tissues of known potency. This yields more information than comparison with a buffer blank, where the results are difficult to evaluate, especially when there is only slight improvement. A final dilemma arises when substances with weak clotting activity are compared. For example, how important is the difference between a clotting time of 240 seconds and one of 200 seconds? Certainly the thrombin yield from such materials is approximately the same.

Another useful system is the thromboplastin generation test (TGT) (see Biggs and Macfarlane, 1962, for detailed description). In this

assay, components necessary for forming the intrinsic prothrombin activator are incubated together with platelets or a phospholipid extract (Bell and Alton, 1954), and their ability to form the prothrombin-converting principle is studied. Peak activity in the test is achieved after a 6-minute incubation period, and the clotting times fall below 12 seconds. Differences of a few seconds between test substances are meaningful. Phosphatides which one would theoretically expect to be active in the clotting process on the basis of molecular configuration, surface charge, and particle size are usually found to conform to expectation. The outstanding example of this is the serine phosphoglyceride family (PS) which, at optimal dilution in aqueous media, can function as well as the entire platelet lipid complex. On the other

Table II
THE EFFECT OF WASHING ON THE STYPVEN TIME OF PLATELETS

Test material	Stypven time (sec)
Platelet-rich plasma	32
Platelet-poor plasma	33
Platelets recovered from above plasma and washed	
First wash	23
Second wash	17
Third wash	15
Fourth wash	13
Fifth wash	14

hand, when present in excess, these phosphatides show anticoagulant properties. In clinical practice this test is gradually being replaced by the partial thromboplastin time (PTT) (Rapaport *et al.*, 1961; Nye *et al.*, 1962), but in our hands it has been most useful for evaluating the clot-promoting properties of various phosphatides.

The Russell Viper Venom (Stypven) test has found very wide usage, since it is quite responsive to the presence of "available" lipid in the assay system (Trevan and Macfarlane, 1936; O'Brien, 1959b; Ferguson, 1960; Bradlow, 1961; Billimoria *et al.*, 1965; Macfarlane, 1965). This system, with appropriate modifications, has recently been proposed as a measure of "availability" of platelet lipid or lipoprotein for coagulation (Spaet and Cintron, 1965). For example, unwashed platelets in plasma have a relatively long Stypven time (O'Brien, 1958). If they are washed once, the time shortens and continues to do so with subsequent washes. An example of this phenomenon is shown in Table II (Marcus and Ullman, 1964). Another recently devised test for avail-

ability of platelet factor 3 is the kaolin clotting time of platelet-rich plasma (Hardisty and Hutton, 1965). The shortening of the Stypven time (and recalcified clotting time as well) (Fullerton *et al.*, 1953; Pilkington, 1957a; Merskey and Nossel, 1957) following a fatty meal is probably due to the presence of platelets which have been found to adhere to fat particles in lipemic plasma (Husom, 1961). We have frequently confirmed this observation in our own laboratory. Unfortunately, the Russell Viper Venom time is not particularly useful for distinguishing between clot-promoting properties of different lipids

Table III
STYPVEN TIME OF VARIOUS PHOSPHOLIPIDS[a]

Test substance	Concentration[b]	Clotting time[c]
Cephalin control (1:200)	—	9
Buffer blank	—	39
Cardiolipin	50	11
PE and PS	100	9
PE	100	12
PS	50	13
Sphingomyelin	200	25
Lysolecithin	10	25
Inositol phosphatide[d]	10	20

[a] Cardiolipin, sphingomyelin, and lysolecithin are inactive in almost every standard clotting system. Yet, when compared to a buffer blank, they shorten the Stypven time. These results emphasize the nonspecificity of the Stypven time when it is used to compare the clot-promoting properties of individual phosphatides.

[b] Measured in μg/0.1 ml.

[c] Measured in seconds.

[d] Contaminated specimen.

because it is nonspecific. Lipids which are theoretically and experimentally devoid of clot-promoting properties in other test systems will "shorten" the Stypven time. An experiment illustrating this point is shown in Table III. Nevertheless, as mentioned, this is a most useful test system for detecting the availability of platelet lipid or lipoprotein.

There are many other coagulation tests described in the literature which investigators have used to evaluate the lipid materials. These include the whole blood clotting time in glass or silicone-treated tubes, the thrombin generation test, and the prothrombin consumption test using clotted blood or platelet-free "native" plasma. In general they are modifications or variations of the basic principles involved in the assays mentioned above.

III. Studies on Lipids Derived from Platelets

A. Platelets and Platelet Phosphatides

Blood platelets are believed to originate from cells in the bone marrow known as megakaryocytes, from whose cytoplasm they are shed. Thus, they have no nuclei and very little DNA. During their life span of approximately 8 days, the platelets serve as carriers for metabolic substances such as serotonin; in some unknown way they help preserve vascular integrity, and they function in the hemostatic and coagulation processes. In the formation of arterial thrombi the platelets may be "overplaying" their hemostatic role. Morphologically they do not contain an abundance of cytoplasm or endoplasmic reticulum. The principal intracellular constituents are known as "granules," of which there seem to be about three types. The first is actually a mitochondrion; the second appears to have a homogeneous internal structure; and the third is similar, but contains a somewhat dense body and has been termed a "bull's eye" granule by some observers. These last have a superficial resemblance to microbodies (de Duve, 1965). Granules isolated by continuous sucrose gradient ultracentrifugation contain lysosomal enzymes (Marcus and Zucker-Franklin, 1964b; Marcus *et al.*, 1966)—that is, acid hydrolases, which become activated by cell disruption or cell death.

Many aspects of platelet physiology have recently been reviewed by Marcus and Zucker (1965). Roughly 15% of the dry weight of platelets is lipid (this figure is quite variable due to differences in techniques of extraction). About 80% of the platelet lipid is phosphatide. These figures do not vary radically from those of many other tissues, but there is an important functional difference: this lipid becomes available to the coagulation proteins, probably as a lipoprotein complex in the course of fibrin formation. How this phenomenon comes about is completely unknown. If washed erythrocytes or leucocytes are used in *in vitro* clotting systems, no significant effects are observed. On the other hand, as mentioned, if washed platelets are used the coagulation process proceeds with maximal efficiency. Of interest is a recent study by Hovig (1965) in which the ultrastructure of washed platelets was studied following incubation with phospholipase A (50 μg/ml). There was virtually total loss of internal structure. Only mitochondrial fragments and the outer cell membrane remained. However, the plasma membrane had lost its "unit membrane" appearance.

The advent of techniques for separating lipids into individual classes

(see Rouser *et al.*, 1961; Marinetti, 1962; Hanahan, 1960) was the main impetus to recent research on their role in the coagulation process. One of the early aspirations was to find a single active fraction which might have application *in vivo* as a platelet substitute. This prospect was soon dispelled when it was demonstrated that hemostasis could only be maintained by living platelets—that is, those which can circulate *in vivo*. Although injection of lipid materials *in vivo* has had a temporary beneficial effect on coagulation tests, the hemostatic mechanism was unaffected (for further details see Marcus and Zucker, 1965).

When platelet lipid extracts were fractionated on silicic acid columns (Rouser *et al.*, 1958; Marcus and Spaet, 1958; Barkhan *et al.*, 1961; Speer and Ridgway, 1962; Marcus *et al.*, 1962), or by a combination of silicic acid column and paper chromatography (Troup *et al.*, 1960), or by column and countercurrent distribution techniques (Woodside *et al.*, 1964), it was found that clot-promoting properties were confined to the "cephalin" fractions (the ethanolamine (PE) and serine (PS) phosphoglycerides). The choline-containing phosphatides and the neutral lipids were inert in this regard, although combinations of lecithin with one or more of the cephalins did produce an active clot-promoting substance.

Although they comprise only about 6% of the total platelet lipid (Troup *et al.*, 1960), the serine phosphoglycerides appeared to be the most active of the platelet phosphatides in *in vitro* coagulation (Marcus and Spaet, 1958; Troup *et al.*, 1960; Barkhan and Silver, 1962; Speer and Ridgway, 1962; Woodside *et al.*, 1964). They are capable of about a fivefold increase in activity over comparable total platelet lipid controls, and in our hands have been active in every *in vitro* clotting system thus far studied (see Ferguson *et al.*, 1963). However, it is important to note that on a quantitative basis no platelet lipid or lipid extract from other tissues is as active as platelet lipoprotein. This concept has been well-emphasized by Seegers in his studies of platelet factor 3 (the platelet "thromboplastic" factor) (1961). For example, if platelet membranes or granules are studied in clotting systems, the amount of lipid needed in combination with protein is much less than when the lipid is used by itself (Marcus *et al.*, 1966). Another interesting property of PS is its paradoxical action at high concentrations where it behaves as an anticoagulant. This has been well-demonstrated by Marcus and Spaet (1958), Barkhan *et al.* (1961), and, more recently, Woodside *et al.* (1964).

In contrast, results with the ethanolamine phosphoglycerides have

not always been clear-cut. One explanation may be the difficulties involved in processing this phosphatide once it is isolated. It forms poor emulsions which are unstable, and one must resort to drastic procedures such as sonication or subjecting the preparation to the action of detergents, such as desoxycholate. Unoxidized PE can shorten the recalcified clotting time of platelet-poor plasma, as demonstrated by Rouser *et al.* (1958), Rouser and Schloredt (1958), and Marcus *et al.* (1962), and it is also active in variations of the Stypven test (Ferguson *et al.*, 1963). However, PE appeared to be inactive in the thromboplastin generation test or modifications thereof (Troup *et al.*, 1960; Marcus *et al.*, 1962). In general it appears that the ethanolamine phosphoglycerides are more active in tests like the recalcified clotting time and similar systems which involve long incubation periods. This may be a nonspecific effect, as demonstrated by Horowitz and Marcus (1964), since inert latex particles, if incubated in plasma for a short period of time, will show coagulation properties similar to those of platelets. In our experience, as well as that of Speer and Ridgway (1962), every preparation of platelet PE which showed activity in the thromboplastin generation test was shown to be contaminated with faint traces of PS. This could be shown by taking the preparation in question almost to dryness in a lipid solvent and subjecting it to silicic acid paper or thin-layer chromatography (Marcus *et al.*, 1962) or to a sensitive hydrolysis technique (Slotta and Powers, 1962). Barkhan and associates (1961) reported that their preparations of platelet PE showed clot-promoting properties, but pointed out that they contained traces of PS. Active PE preparations from platelets were also reported by Woodside *et al.* (1964), although the experimental TGT data were not shown in their publication.

It is important to emphasize that these *in vitro* studies on isolated phospholipids are mainly investigative models to further our understanding of the relationship between lipids and the coagulation mechanism. The contribution of blood platelets to the coagulation mechanism (platelet factor 3) is in all likelihood a phospholipoprotein complex consisting of all the platelet lipids in close association with protein (Seegers, 1961). We have recently found that isolated platelet membranes are somewhat more efficient than platelet granules in certain clotting systems (Marcus, 1965; Marcus *et al.*, 1966). This reinforces the original proposal of Surgenor and Wallach (1961) that the reactive site of the platelet in coagulation is oriented toward the plasma, and is perhaps in the plasma membrane of the platelet. However, Horowitz

(1965a) has presented evidence for a soluble form of platelet factor 3 activity following an incubation period as demonstrated by a shortened Stypven time.

B. Fatty Acids and Aldehydes of Platelet Phospholipids

Gas-liquid chromatography (GLC), using ethylene glycol adipate (EGA) and Apiezon L as polar and nonpolar stationary phases, respectively, was carried out by Marcus and associates. They studied the fatty acid and aldehyde composition of platelet PE, PS, PI (+PS), and lecithin (Marcus *et al.*, 1962). The principal saturated fatty acid in the ethanolamine phosphoglyceride group was stearic, and the main unsaturated acid was arachidonic. The fatty aldehydes were confined mainly to the PE fraction. They were of the saturated, straight chain, 16:0 and 18:0 type. The GLC data, in conjunction with biochemical measurements, indicated that 66% of platelet PE was in the plasmalogen form. It has been shown that plasmalogens are not needed for blood coagulation (Rouser *et al.*, 1958; Zilversmit *et al.*, 1961; Speer and Ridgway, 1962). The presence of plasmalogen in platelet PE complicates evaluation of studies on ethanolamine phosphoglycerides from other tissues, and they may not be comparable to results with PE from platelets, since less than half of the platelet PE is in the diester form. It was also of interest that the molar ratio of saturated fatty acids plus fatty aldehydes [which are linked to the α' (C-1 carbon) of glycerol] to unsaturated fatty acids [usually linked to the β (C-2 carbon)] was unity.

The fatty acid composition of the serine phosphoglycerides was relatively simple (Marcus *et al.*, 1962). The main saturated fatty acid was stearic, which was present in amounts equal to the sum of the unsaturated fatty acids, oleic and arachidonic. There was very little plasmalogen in the serine phosphoglycerides, as was also noted by Speer and Ridgway (1962). The fatty acid and aldehyde composition of inositol phosphatide (PI) was quite similar to that of PS. There were trace amounts of serine phosphoglycerides in the PI fractions, but it was felt that they did not contribute to the overall pattern. In contrast to the cephalin fractions, the lecithins contained large quantities of palmitic and oleic acids. As with platelet PS, the plasmalogen component in the lecithin fractions was small.

It had been previously shown by Rouser and Schloredt (1958) that the degree of unsaturation of fatty acids in phosphatides might be directly correlated with clot-promoting activity. Thus it was possible

that the efficiency of PS in clotting could be accounted for if it contained predominantly unsaturated fatty acids. However, this was not borne out, since PS, as well as the other phosphoglycerides in the platelet, showed an approximately equal distribution of saturated and unsaturated fatty acids. The possibility that there are groups of PS molecules with diunsaturated fatty acids could not be ruled out by these studies. Nevertheless it was concluded that the coagulation properties of the platelet phosphatides did not depend wholly upon the degree of unsaturation, since the overall distribution of saturated and unsaturated fatty chains in an inactive phosphatide, such as lecithin, was the same as that of PE or PS. Other investigators have proposed that the degree of unsaturation of a phospholipid was not a primary determinant of clotting activity (Troup *et al.*, 1960; Williams, 1961).

It is of considerable interest that human erythrocyte phosphoglycerides have a similar distribution of plasmalogens and fatty acids to that of platelets (Farquhar, 1962). Although the fatty acid composition of PS in the red cell resembles that of the platelet, there is about twice as much PS in the erythrocyte. This may account for the marked clot-promoting effects of lipids and lipoproteins derived from red cells after damage or hemolysis (O'Brien, 1959a; Shinowara, 1951, 1957, 1961).

IV. Experiments with Lipids from Sources Other than Platelets

A. Early Studies

One of the first attempts to relate phospholipid structure to coagulation activity was that of Rouser and associates (1958) and Rouser and Schloredt (1958). Using mainly the recalcified clotting time of platelet-poor plasma for assay, they made a number of interesting observations. Among them were the following. Neutral lipids, such as cholesterol and triglycerides, were inactive, as were cerebrosides and cerebron. Saturated fatty acids containing at least sixteen carbon atoms showed slight activity, and a variety of nonlipid, water-soluble substances which may have been present in the phospholipid extracts were inert. Finally, a series of hydrolysis products of phospholipids also was devoid of any clot-promoting properties.

Phosphatides such as lecithin, sphingomyelin, PI, synthetic preparations, and surfactants with structural similarities to phosphatides showed no activity in the recalcified clotting time. Synthetic PE and

PS, the surfactants BPE and TR (esters of sulfosuccinic acid), as well as liver monophosphoinositide, shortened the Russell Viper Venom time, but were inactive in the recalcified clotting test. The authors then isolated ethanolamine phosphatide fractions from rabbit appendix, leucocytes, soy beans, and human platelets. Positive results were obtained with all these preparations, but none was as active as PE derived from platelets. It was also shown that the plasmalogen form of PE was not essential for coagulation, since activity persisted after acetic acid hydrolysis. If the amino group was removed or blocked with ninhydrin, acetic anhydride, or succinic anhydride, the clotting activity disappeared. When the PE was hydroxylated with performic acid which produced a soluble product, it was devoid of clotting activity. This was evidence that a phosphatide was inactive in an aqueous medium unless it existed as a finely dispersed, colloidal particle. The negative charge on the phosphate group of PE was also deemed important. However, it might be mentioned that PS contains an additional negative charge on the carboxyl group which, as will be discussed, favors its clot-promoting properties. Rouser and Schloredt (1958) then studied the fatty acid composition of PE preparations from various sources by paper chromatography and showed that activity in the recalcified clotting time was directly correlated with the degree of unsaturation of fatty acids on the PE molecules. It was pointed out that phosphatide compounds with unsaturated fatty acids were physically shorter and greater in diameter than those containing mainly saturated fatty acids, and the degree of unsaturation would contribute to a more spherical shape of the molecule. The active complex in coagulation was visualized as a linkage via divalent calcium ion between an acidic phosphatide and a negatively charged acidic group from a plasma protein. At that time the PE, rather than PS, preparations were most active, and many biochemical studies were carried out on this substance. Later Rouser and co-workers (1961) devised a technique for a more effective separation of PE from PS, which we verified and used extensively in studies of brain and platelet phosphatides (Marcus *et al.*, 1962). With this separation procedure we found the serine phosphoglycerides to be the most active group of phospholipids. However, Rouser did not report coagulation studies on phosphatides separated by his newer methods.

The important work of Wallach *et al.* (1959) further explored the relationship between the colloidal state of purified phosphatides and their clot-promoting properties and extended the observations of

Rouser's group. The authors treated lyophilized egg yolks with acetone, followed by chloroform-methanol (2:1) extraction. The lipid extract was further purified and then subjected to silicic acid column chromatography. There were two important advantages in studying egg yolk lipids. First, there is no PS in this material; and second, it is devoid of plasmalogen. Thus it is quite different from what we find in platelets, erythrocytes, and brain, but very advantageous for studying the biochemistry of coagulation. The initial PE fractions recovered from the columns were more unsaturated than the final ones (the iodine numbers dropped from 157 to 77 in the course of collection). These PE preparations were assayed in a coagulation system consisting of purified antihemophilic globulin, purified factor V, calcium, magnesium, and the lipid in question. Use of this system instead of a "standard" one limited the usefulness of these studies for other laboratories working in the field. Purified coagulation factors are relatively unstable and difficult for most investigators to obtain. However, the system probably gave results analogous to what would be found in a recalcified clotting time assay. Column eluates with differing degrees of saturation were studied under a large variety of chemical and physical circumstances. Aqueous suspensions were prepared by injecting ether solutions of the phosphatide into the medium, with subsequent removal of the ether by bubbling nitrogen through the suspension. The more unsaturated preparations formed stable suspensions at neutral pH. A pH of 8 was required to prepare a stable emulsion of the more saturated material, and synthetic dimyristoyl phosphatidyl ethanolamine could be suspended only at a pH of 10. When preparations were frozen and thawed at different temperatures, there was a decrease in turbidity with an increase in temperature. With a rise in the pH of the suspending medium there was a gradual increase in clotting activity to a pH of 8, followed by marked potency up to a pH of 10, where the entire assay system became inhibited. Thus, these PE fractions showed a wide spectrum of clot-promoting properties which appeared to depend mainly upon physical characteristics of the emulsion. This in turn was shown to be governed by the degree of unsaturation of the constituent fatty acids, temperature, pH, and ionic composition of the aqueous medium in which it was suspended. It was concluded that clot-promoting PE preparations were micelles in the form of bimolecular leaflets of a definite size, thickness, and a unique surface configuration. The work of these investigators and that of Rouser and associates were significant contributions.

B. Experiments on Lipids from Various Tissues

As more and more workers became interested in this field, there was an increasing divergence in results reported. There are at least partial explanations for some of the differences, a few of which will be mentioned. The method of lipid fractionation is an important determinant of coagulation results. Folch-type procedures are probably less useful for coagulation work than they are for other purposes. There is a slight degree of overlapping in Folch fractions, which is significant enough to influence coagulation tests. An illustrative example is the study of Biggs and Bidwell (1957).

Oxidized phospholipids give variable results, also related to the assay system. They are frequently inert in the thromboplastin generation test, but under certain conditions may show an increase in activity in a thrombin-generating system (Wallach *et al.*, 1959). Isolated lipids do not withstand storage well, even under the most careful conditions, and this influences results of coagulation assays. Partially hydrolyzed phosphatides can show clot-promoting properties which may have been absent in the initial preparation (Slotta and Deutsch, 1960). Nonlipid contaminants which remain with the phosphatides throughout the extraction procedure may exert an inhibitory effect (Woodside *et al.*, 1964). As already mentioned, the colloidal state of the phosphatide is of great importance. Results will vary depending on whether the phospholipid has been suspended in a protein medium or an aqueous environment. If ancillary procedures, such as solubilization with a detergent, sonication, homogenization, etc., are used to prepare the lipid for testing, the biochemical properties of the phospholipid may be so altered as to render the results misleading. Some of these difficulties could have been obviated by performance of more than one type of lipid extraction and the use of different types of coagulation systems to assay the lipids (Ferguson, 1960; Marcus *et al.*, 1962; Silver *et al.*, 1963; Ferguson *et al.*, 1963). It seems unrewarding for an investigator to carry out a careful lipid extraction, separation, and analysis, only to examine the purified materials in a single coagulation test. Finally, since the ultimate purpose in many investigations is to compare the results with those obtained with platelets or platelet lipid, it appears more logical to us to study purified lipids in a manner similar to that conventionally used for platelets—suspension in an aqueous medium at physiological pH and ionic strength.

A comment should also be made regarding the criteria for clotting

activity. A common practice has been to compare the test lipid with a buffer blank, and if the clotting times were more rapid than the blank the material was said to be active in clotting. This may merely have been a nonspecific effect of adding particulate matter to the system. It is more appropriate to compare the test lipid with a control which gives maximal activity, such as a total lipid extract from brain (Bell and Alton, 1954), platelets, erythrocytes, or even washed whole platelets. Another problem in methodology which has produced conflicting results is the practice of using test lipids in one or two concentrations. It is important to examine the material in highly concentrated form first, and then to study its *in vitro* behavior as it is serially diluted.

With this background information in mind, the results of coagulation tests performed on natural and synthetic phosphatides are presented in Table IV. We have attempted to present the main points of information about each study, but the reader is advised to consult the individual publications for greater detail.

C. Lipid Anticoagulants

The concept that lipids may play a role as inhibitors of the blood coagulation process has been pursued most extensively by Tocantins and co-workers (Tocantins *et al.*, 1948; Silver *et al.*, 1957). The subject was recently reviewed by Silver and associates (1959). Most of the recent work has been carried out with the serine phosphoglycerides derived from natural and synthetic sources. In fact, these investigators have termed phosphatidyl serine "phospholipid antithromboplastin." In their experiments the sodium and potassium salts of naturally occurring PS, as well as synthetic preparations (see Table IV), seemed to be responsible for the anticoagulant activity found, and it appeared to be directed against the early stages of coagulation. In order to demonstrate this property, the phosphatides must be solubilized and tested as a water-clear preparation. If the same material is suspended in an aqueous medium such as buffered saline, it usually shows clot-promoting properties, especially at low concentrations. Sodium desoxycholate has been the most frequently used compound for solubilization, although recently serum albumin has also been employed (Silver *et al.*, 1963). Of further interest were the results obtained when purified serine phosphoglyceride fractions were injected intravenously into dogs (Silver *et al.*, 1957). The whole blood clotting time was prolonged, and did not return to the normal preinjection range until 72 hours had elapsed. In agreement with these findings are the experiments of Mus-

Table IV
In Vitro COAGULATION TESTS ON PHOSPHATIDES FROM VARIOUS SOURCES

Lipid[a]	Source	Type of extraction and separation	Test system	Results	Reference
PS, PE	Human brain	Folch	TGT, antithrombo-plastin test, Stypven test	PE active; PS inhibitory	Barkhan *et al.* (1956)
PE, PS, PI, lecithin	Brain, egg yolk	Folch	Thrombin generation	PE active; PS, PI, lecithin inactive	Robinson and Poole (1956)
PI, PS, PE, PE plasmalogen, cardiolipin, lecithin, sphingosine, sphingo-myelin	Various investigators' laboratories	Mostly Folch	Coagulation time, TGT, prothrombin consumption, thrombin generation, Stypven	PI, PS, PE plasmalogen, PE active; egg lecithin, sphingo-sine, sphingomyelin inactive	O'Brien (1957c)
PE	Human brain	Folch	TGT	PE could replace platelets in the test	Garrett (1956)
All "cephalin" fractions	Human brain	Folch (and modification)	TGT, recalcified clotting time, prothrombin conversion test	PE inactive in TGT; PS active in recalcified clotting time; all had some activity; no single active lipid found	Biggs and Bidwell (1957)

[a] In most cases these were the principal lipids used in the studies.

Table IV (*Continued*)

Lipid[a]	Source	Type of extraction and separation	Test system	Results	Reference
PS, lecithin	Beef brain	Folch and counter-current distribution	Recalcified clotting time of rabbit plasma, modified TGT	No appreciable activity unless dissolved together in chloroform and retested	Therriault *et al.* (1958)
PE, PS, lecithin	Beef brain, egg, synthetic	Folch and others	TGT	PE and lecithin inactive; both became active in presence of 10% PS	Slotta (1960)
PE, PI, lecithin	Ethanol-insoluble fraction of soy bean phospholipid	Folch fractionation and silicic acid chromatography	TGT, recalcified clotting time	PE and lecithin inactive in TGT; PE shortened recalcified clotting time; lecithin inert. PI fractions with variable amounts of PS and PE as contaminants—active; activity may have been due to presence of PS. PI fractions mixed with PE or lecithin—very effective	Williams (1961)

Table IV (*Continued*)

Lipid[a]	Source	Type of extraction and separation	Test system	Results	Reference
PE, PS, lecithin, synthetic phosphatides	Ox brain cephalin, egg yolk	Folch	Recalcification time of human and chicken plasma	PS active at low and inhibitory at high concentrations; lecithin inactive; maximal activity with combination of PS and PE; PS and lecithin almost as active; combinations of synthetic phosphatides active	Hecht and Slotta (1962)
PE, PS	Human brain	Rouser *et al.*, (1961)	TGT, recalcified clotting time, Stypven	PE inactive in TGT, active in recalcified clotting time and Stypven; PS most active in all systems	Marcus *et al.* (1961)
PE, PS, lecithin	Beef and pork brain, synthetic, egg	Folch fractionation, purification, silicic acid chromatography	Hicks–Pitney, (1957); TGT, recalcification time, antithromboplastin test, Stypven	PS—anticoagulant effect when solubilized in albumin or desoxycholate; PE active in Hicks–Pitney and Stypven; synthetic PE active in Hicks–Pitney; lecithin inert	Silver *et al.* (1963)

Table IV (*Continued*)

Lipid[a]	Source	Type of extraction and separation	Test system	Results	Reference
PE	Egg yolk, synthetic	Egg—Folch and DEAE column; synthetic—dioleoyl PE synthesized in investigator's laboratory	TGT and Hicks–Pitney	Active in Hicks–Pitney; slow in TGT when compared to platelet control	Turner *et al.* (1963)
PS	Synthetic	Oleoyl stearoyl, oleoyl palmitoyl, dioleoyl PS—synthesized in author's laboratory	Preparations in Na desoxycholate, albumin, and buffered saline; Hicks–Pitney, antithromboplastin, and recalcification tests	Solubilized preparations showed anticoagulant effect; those in buffered saline had weak to moderate activity	Turner *et al.* (1964)

Table IV (*Continued*)

Lipid[a]	Source	Type of extraction and separation	Test system	Results	Reference
PE, PS, lecithin, phosphatidic acid	Synthetic	Phosphatides with various types of fatty acids synthesized in author's laboratory	Recalcified clotting time and TGT	*Recalcified clotting time:* no activity with individual phospholipids; PS inhibitory at high concentrations; lecithin + PE inactive; lecithin + PS active; PE + phosphatidic acid active; PE + PS—marked clot-promoting properties. *TGT:* Lecithin inactive; phosphatidic acid inactive; PS weak; PE weak; PE + 5-20% PS—excellent clot-promoting properties; some preparations of lecithin + PS very active; PE + cardiolipin —some preparations showed maximal activity. Author concluded that all individual phospholipids showed poor clotting activity when compared to combinations. In any active combination a negative charge from an acidic phosphatide was required	Daemen (1963); Daemen *et al.* (1965)

tard *et al.* (1962) and of Nishizawa (1965), who injected preparations rich in PS into dogs and found the whole blood clotting time in glass and silicone-treated tubes to be prolonged after the infusion. In addition, the prothrombin time lengthened and prothrombin consumption became worse. In striking contrast to the other *in vitro* coagulation tests, the Russell Viper Venom test became shortened.

The overall significance of lipid anticoagulants is difficult to assess, but perhaps in the future they might prove therapeutically useful as anticoagulants. One would assume that the phosphatides of blood platelets, which probably act in the form of lipoproteins, function primarily as clot-promoting substances, since their absence leads to a prolongation of the coagulation time. Nevertheless, we do not know the exact physical state of phosphatides in their natural environment, and the transfer of *in vitro* information to *in vivo* situations is still highly speculative.

D. Fatty Acids in Coagulation and Thrombosis

Recent research on the free fatty acids of plasma (Fredrickson and Gordon, 1958) has stimulated interest in the possibility that this group of compounds are involved in coagulation and thrombosis. In addition to the unsaturated fatty acid component, saturated fatty acids of chain length C_{10}–C_{18} have been identified in the free fatty acids of plasma, and these long-chain acids are capable of shortening the recalcified clotting time (Poole, 1955; Pilkington, 1957b; Margolis, 1962). A cause and effect relationship has thus been assumed, but concrete evidence is lacking. The free fatty acids are bound by plasma proteins, especially albumin, and erythrocytes as well (Goodman, 1958). In order to hypothesize a relationship to intravascular thrombosis, one must infer that situations exist in which the binding capacity of plasma protein is exceeded, and the offending fatty acids are circulating in an unbound form. As yet this has not been demonstrated.

The *in vitro* and *in vivo* effects of salts (sodium or potassium) of various fatty acids have been investigated by Connor and associates (summarized by Connor *et al.*, 1965). The *in vitro* work was carried out by injecting the blood (after 1 minute of incubation with test substance) into plastic tubing which was subsequently placed on a rotating wheel in the form of a loop.* After recalcification, the time taken for solidification of the column of blood was termed the "throm-

* Chandler, 1958.

bus-formation time." Microscopic examination of the solidified material revealed a white head of platelets and leukocytes followed by a fibrin tail with trapped erythrocytes. Thus, although it has been called a thrombus, some would take issue with this interpretation and propose that the material is a clot, not a thrombus, and that the procedure is basically a combination of platelet clumping and a recalcified clotting time. When long-chain saturated fatty acids were tested, the thrombus time was about 3 minutes, as compared to a finding of 8 minutes when a saline blank was added. It is important to mention that substances such as bentonite, kaolin, and quartz glass produced results identical to those of stearate (Connor, 1962). Other points of interest were that: a period of incubation of the fatty acid with the blood specimen (1 minute) was necessary for maximal activity; and the fatty acids were used in a concentration of 0.1%. The highly soluble salts of long-chain unsaturated fatty acids, soy bean phosphatides, and short-chain saturated fatty acids had little or no effect. Finally, it was postulated that the effect of long-chain saturated fatty acids was due to activation of Hageman factor since it did not occur in duck (Hageman-deficient) blood. In agreement with this were the findings of Margolis (1962), who also demonstrated the release of plasma kinins, along with the clot-promoting properties of the long-chain saturated fatty acids. Similar results were reported by Didisheim and Mibashan (1963). The experiments of Botti and Ratnoff (1963) did not support the finding that long-chain saturated fatty acids activated Hageman factor, but that they probably accelerated the formation of activated PTA (factor XI), which is also deficient in duck blood. In addition, the clot-promoting effect of long-chain saturated fatty acids did not occur when they were added to "native" plasma (no anticoagulant); this was taken as an indication that the importance of free fatty acids in the development of intravascular thrombi was doubtful. On the other hand, Connor *et al.* (1965) reported that the clotting of "native" plasma was markedly shortened by the addition of stearate, but the experimental conditions may not have been strictly comparable to those of Botti and Ratnoff (1963). Nossel (1964) had initially concluded that stearate accelerated coagulation by activating both Hageman and PTA factors; but more recently (1965), on the basis of experiments with congenitally deficient Hageman and PTA plasmas, he is of the opinion that the stearate effect is on Hageman factor.

A further complicating problem is the direct effect of fatty acids on platelets. Haslam (1964) showed that the addition of long-chain

saturated fatty acids to washed platelets caused ADP release and prompt aggregation in the presence of calcium; and Shore and Alpers (1963) demonstrated histamine and serotonin release from rabbit platelets after the addition of various long-chain saturated fatty acids. Soloff and Wiedeman (1963) produced vasoconstriction and platelet thrombosis following intra-arterial injection of sodium stearate into the bat wing. Finally, long-chain saturated fatty acids have been shown to cause thrombocytopenia after intravenous injection into rabbits (Zbinden, 1964). This effect could be greatly reduced by preincubation of the fatty acids with serum.

When long-chain saturated fatty acids were administered intravenously to dogs (Connor *et al.*, 1963; Hoak *et al.*, 1964), the animals died within a 10-minute period. This was not observed with prolonged infusion times, low concentrations of the saturated fatty acids, or unsaturated fatty acids of comparable chain lengths. The long-chain unsaturated fatty acids did, however, induce "hypercoagulability" and jugular segment thrombi. Thrombi (clots?) were observed in the heart and larger veins. An important additional effect of the fatty acids was profound myocardial damage, as evidenced by electrocardiographic changes and a clinical picture of shock. This was found in animals who died with no evidence of thrombosis, as well as in those who received heparin or warfarin, which prevented thrombosis but not myocardial failure. If the fatty acids were preincubated with albumin or the injections were made into the portal circulation, the animals did not develop thrombosis or die, although in some thrombi were noticed in isolated segments of the jugular veins (Wessler *et al.*, 1959; Wessler, 1963). This type of *in vivo* study was carried a step further by Hoak and associates (1963). They injected ACTH or anterior pituitary extracts into rabbits, which produced a five- to sevenfold rise in the plasma-free fatty acids, the animals became toxic, some died, and many developed thrombi, especially in the lungs. Isolated segments of jugular veins in many of the animals contained thrombi as well. This finding, in combination with a shortening of the coagulation time in silicone-treated tubes, was interpreted as a hypercoagulable state. Various clinical conditions, which are characterized by elevated levels of plasma-free fatty acids (acute stress, diabetic acidosis, rigid weight reduction, starvation, heavy smoking, pregnancy—week 36–40), are known to be associated with thromboembolic phenomena. However, as already mentioned, one must assume that the free fatty acids under these circumstances circulate in an unbound form; and even if they do, stearate

would only represent 13% of the total. Fifty-six percent of this total is unsaturated, and these may very well have a "solubilizing effect" on the saturated components. Furthermore, it has been shown that blood coagulation "intermediates" are rapidly cleared from the circulation by the reticuloendothelial system (Spaet *et al.*, 1961).

Also of interest is a recent study by Tompkins and Dayton (1965) in which it was demonstrated that fluctuations in free fatty acid concentrations within physiological limits did not produce significant changes in coagulation. Saturated free fatty acid concentrations and whole blood clotting times were measured in nine subjects after fasting, after a high carbohydrate intake, and after ingestion of a large amount of water. The whole blood clotting times remained the same under all these conditions. The free fatty acid levels after carbohydrate ingestion were one-half the fasting value, but there was no change in the clotting time. The effects of adding sodium salts of saturated fatty acids to blood may thus be quite different from those produced by physiological variations in free fatty acid levels.

We are therefore left with tempting clinical speculations for which concrete evidence is totally lacking.

V. Biophysical Aspects of Coagulation

The Importance of Charge

As is well-known, phospholipid molecules have a nonpolar region consisting of long-chain fatty acids, and a polar component which contains groups responsible for the charge on the molecule. For example, PS possesses a carboxyl and primary amino group, PE contains a primary amino group, and lecithin and sphingomyelin a quaternary ammonium group. The nonpolar area tends to orient itself away from water or other polar environments, but will attract nonpolar portions of other molecules which happen to be in the environment, such as protein or another lipid. The polar part of the molecule is hydrophilic and orients itself in the direction of water or polar regions of other molecules, and it can also combine with protein or lipid by mechanisms such as hydrogen bonding or electrostatic forces (Rossiter, 1963; Gurd, 1960).

The structural arrangement of platelet phospholipids which take part in the coagulation process is not known. If they are initially present as lipoprotein complexes the lipids may exist as a series of parallel groups surrounded on both sides by protein. The polar portion of the molecule may be directed toward protein, and the nonpolar areas

directed toward each other (Salem, 1962). On the other hand, the phosphatides may be present, with the polar groups pointing toward the plasma atmosphere and the hydrocarbon chains bound to cell protein. In a preliminary report, Marcus and Zucker-Franklin (1964a) found that subcellular platelet particles contained a high proportion of long-chain, highly unsaturated fatty acids. This may be a characteristic of "loose" lipoprotein membranes, and partially explains the dynamic role of platelets in coagulation. If one assumes a platelet lipoprotein complex to begin with, it could be speculated that during the early stages of coagulation a mechanism (proteolytic?) exists which is capable of removing the outer layer of protein, thereby exposing the polar portion of the platelet phospholipids with their negative charge, making them "available" for interaction with less negatively or positively charged activated coagulation proteins. Whatever this process is, it does not appear to affect erythrocytes and leukocytes. Admittedly the evidence for such a speculation is scant. Actually, we do not know whether the polar or nonpolar portion of the phosphatide is more functional in the coagulation process. There is good evidence that both parts of the molecule are important. Vroman (1964) has shown that a role can be found for both the negatively charged polar sites and the apolar hydrophobic fatty acid chains. The latter may combine with a hydrophobic site on one of the coagulation proteins, such as factor V.

In the early studies of Rouser and Schloredt (1958) and Wallach *et al.* (1959), attention was directed primarily toward the nonpolar (hydrophobic) portion of the phosphatides. The work of Bangham (1961b) focused attention on the polar region of phospholipids in relation to coagulation. The observations stemmed from earlier investigations on the ability of amphipathic molecules to affect the charge of lipids. Egg lecithin was mixed with varying amounts of the amphipath dicetyl phosphoric acid in chloroform solution; this was taken to dryness and emulsified in tris buffer. The zeta potential of these mixtures was correlated with their activity in the Russell Viper Venom test. When the electrophoretic mobility of various emulsions used in the test system was measured, it was found that the more negatively charged suspensions produced shorter substrate clotting times. There was excellent correlation between the activity of lecithin: dicetyl phosphoric acid mixtures and other known clot-promoting materials. For example, the electrophoretic mobility of a brain lipid extract (Bell and Alton, 1954) resembled the active lecithin:dicetyl phosphoric acid mixture, and both gave a clotting time of 8 seconds.

Platelets and a lecithin:PE mixture behaved in a similar fashion. When the negative charge of the lecithin:dicetyl phosphoric acid combination was neutralized by the addition of C_{22}-pyridinium bromide, the clotting time increased to 34 seconds.

These observations were confirmed and extended by Papahadjopoulos *et al.* (1962). Working with phospholipids prepared from beef brain and egg yolk, they studied the effects of surface charge on clot-promoting properties of aqueous emulsions of these phosphatides. The assay system consisted of purified reagents and was essentially the same as the "product I" system of Bergsagel and Hougie (1956) and Horowitz and Spaet (1961), in that it responded to the presence of clot-promoting phospholipids in a sensitive manner. Mixtures of the separated phosphatides were prepared, and the electrophoretic mobility of each was measured. Four combinations were used in a wide range of concentrations—PS in PE, PS in lecithin, phosphatidic acid in lecithin, and PE in lecithin. Maximal clot-promoting properties (the lowest concentration of lipid needed for a 10-second clotting time) were found with mixtures which showed an electrophoretic mobility value of -4.5×10^{-4} cm^2 sec^{-1} volt^{-1}. Thus, there was an optimal negative surface charge at which phospholipids exhibited maximal clot-promoting properties and activity diminished as the charge became more or less negative. For example, if PS alone was emulsified at a pH of 7.4, its electrophoretic mobility was too far in the negative range and activity was low. On the other hand, when it was emulsified in water at a pH of 5, the charge was closer to the optimal range, and clotting activity was higher. If the suboptimal negative charge of PE was increased by the addition of PS, maximal activity was observed. Therefore, the clot-promoting properties of various phospholipids and mixtures thereof were clearly correlated with the surface charge of the micelles they formed in aqueous media. The results of this important paper may also explain the findings of investigators cited in Table IV, as well as those of an early paper by Rapport (1956) which gave impetus to much of the work on phospholipid mixtures in coagulation.

In a later publication, Papahadjopoulos and associates (1964) invoked the concept of charge to explain the affinity of purified factor X for phospholipids. Preparations of factor X were activated by treatment with trypsin, Russell Viper Venom, or "spontaneously." Activation by trypsin and venom (but not "spontaneous") produced a change in the electrical charge of this clotting factor from negative to neutral or positive. The activated materials were incubated with a mixture of PS

and lecithin in the presence of calcium, and then chromatographed on Sephadex G-200 (also with the addition of calcium). A highly active complex was formed, as had been previously demonstrated by centrifugal methods (Spaet and Cintron, 1963). The affinity of activated factor X for the negatively charged phospholipid micelles was attributed to an alteration of charge on the factor X molecule resulting from the activation process. However, the spontaneously activated factor X preparations also showed this affinity for the phospholipid emulsion, despite retention of the negative charge.

Vroman (1964) has studied the properties of coagulation factors before and after adsorption onto hydrophobic and hydrophilic surfaces. Quantitative studies on the amount and rate of adsorption at a solid/liquid interface were made with a recording ellipsometer. On the basis of his own experimental observations and evidence from the literature, he visualizes the clotting process as a series of changes in the molecular shape of coagulation proteins in a manner which exposes hydrophobic sites. This initiates changes in other clotting proteins, which result in mutual "hydrophobic adsorption." Eventually serial complexes are formed [possibly similar to the "cascade" (Macfarlane, 1964) or "waterfall" (Davie and Ratnoff, 1964)] which terminate in fibrin formation. Calcium attaches to activated factor X (positively charged), giving a total of three positive charges, which then interact with polar groups from three negatively charged phosphatides. This exposes hydrophobic fatty acid chains to the aqueous medium, and they bind to hydrophobic sites on factor V, completing formation of the intrinsic prothrombin activator. One of the interesting aspects of this concept is that it invokes a function for both the polar and apolar portions of the phospholipid molecule. However, it contains many assumptions which we hope will eventually be confirmed by more experimental data.

VI. Hypercoagulability, Thrombosis, and Atherosclerosis as Related to Lipids

In parallel with interest in phospholipids as participants in the normal coagulation process, additional lines of investigation have been pursued in an attempt to relate lipids to such entities as a hypercoagulable state, thrombosis, atherosclerosis, and intravascular coagulation. A number of reviews on these subjects have been published (O'Brien, 1957a; Merskey and Lackner, 1959; Spaet, 1963, 1966; Merskey and Marcus, 1963; Mustard *et al.*, 1964b). Therefore, only a few salient points will be mentioned here.

There are clinical and experimental situations in which lipid or lipoprotein has been postulated to become "available" as a result of hemolysis (Shinowara, 1961), exposure to endotoxin (Horowitz *et al.*, 1962; McKay, 1965), pancreatitis (Shinowara *et al.*, 1963), or antigen-antibody complexes (Robbins and Stetson, 1959). In these studies, phospholipid availability has been thought to bring about a hypercoagulable state resulting in intravascular coagulation. Although the hypothesis is attractive, this sequence of events is as yet unproven. As indicated in the clotting scheme (Fig. 1), other protein interactions occur prior to the participation of phospholipid. There is no conclusive evidence that coagulation is initiated by lipids or lipid availability.

Although scattered reports still appear in the affirmative, those who have examined the evidence critically and objectively (Merskey and Lackner, 1959; O'Brien, 1960; Spaet, 1963; Bronte-Stewart, 1965) agree that the thrombotic complications of atherosclerosis do not represent disorders of lipid metabolism or a "hyperactive" coagulation mechanism. Although alimentary lipemia in human subjects (Fullerton *et al.*, 1953; O'Brien, 1957b) and "thrombogenic" diets in animals (Hartroft and Thomas, 1957; Thomas and Hartroft, 1959; Gresham and Howard, 1963) can produce changes in *in vitro* coagulation tests, the correlation between results of these tests and the presence or absence of thrombosis and atherosclerosis is poor (O'Brien, 1958; Merskey and Wohl, 1964). The many studies on lipid and coagulation parameters in patients with and without coronary artery disease have yielded results which are inconclusive and frequently misleading. It is an unfortunate fact that methods for preventing occlusive vascular disease still elude us. Currently available evidence would indicate that a common link between blood coagulation, the hemostatic mechanism, thrombosis, and atherosclerosis remains to be demonstrated.

VII. Summary

Lipids play an essential role in both the intrinsic and extrinsic pathways of prothrombin activation. They enter the coagulation sequence after it has been initiated by surface contact and other protein interactions. There is no evidence that they initiate the coagulation process. The lipid is normally derived from the blood platelet where it probably exists as lipoprotein. It exerts a surface catalytic action on the plasma coagulation proteins with which it may bind in the presence of calcium. The resulting complex could contain platelet liproprotein or its lipid moiety alone and may mediate prothrombin activation.

Isolated platelet phosphatides, especially the serine phosphoglyceride family, can replace the entire platelet in *in vitro* coagulation tests; but the lipid itself is not as efficient as lipoprotein particles derived from platelet membranes or granules. Although the molar ratio of saturated to unsaturated fatty acids in platelet phosphatides is unity, there are large amounts of long-chain, highly unsaturated fatty acids present.

In addition to their role in coagulation, platelets are of the utmost importance in the hemostatic and thrombotic processes. The structure of an arterial thrombus resembles that of a hemostatic platelet plug.

Much work has been carried out on the properties of isolated lipids in *in vitro* coagulation systems. Conflicting results were inevitable because of problems inherent in lipid research, such as incomplete fractionations, oxidative changes, breakdown during storage, and questionable comparisons. These problems were then superimposed upon already existing difficulties in the field of coagulation research. Nevertheless, as experimental data accumulated, the reasons for conflicting results became more apparent. The same phosphatide may show paradoxical behavior under differing conditions of suspension, solubilization, or concentration. In addition, it is important to remember that some aspects of *in vitro* coagulation research may be more closely related to colloid chemistry than to the *in vivo* coagulation process, and sweeping conclusions based on *in vitro* evidence should be avoided. The importance of a negative charge on the polar portion of the phospholipid molecule has been stressed, and this is even more pertinent when phospholipid mixtures are studied. As to the hydrophobic fatty acid chains, it appears that optimal coagulation activity is achieved when at least one of the two fatty acids is unsaturated and of the long-chain variety.

The tendency to equate lipids with a hypercoagulable state and intravascular coagulation should be undertaken with caution pending further, more convincing evidence. Although platelets are a major component of arterial thrombi, their presence in these lesions is probably related to their role in hemostasis rather than coagulation.

References

Arakawa, T., and Spaet, T. H. (1964). *Proc. Soc. Exptl. Biol. Med.* **116**, 1034.

Bangham, A. D. (1961a). *Nature* **192**, 807.

Bangham, A. D. (1961b). *Nature* **192**, 1197.

Barkhan, P., and Silver, M. J. (1962). *In* "Progress in Hematology" (L. M. Tocantins, ed.), Vol. III, p. 170. Grune & Stratton, New York.

Barkhan, P., Newlands, M. J., and Wild, F. (1956). *Lancet* **271**, 234.
Barkhan, P., Silver, M. J., and O'Keefe, L. M. (1961). *In* "Blood Platelets" (S. A. Johnson, R. W. Monto, J. W. Rebuck, and R. C. Horn, Jr., eds.), p. 303. Little, Brown, Boston, Massachusetts.
Bell, W. N., and Alton, H. G. (1954). *Nature* **174**, 880.
Bergsagel, D. E., and Hougie, C. (1956). *Brit. J. Haematol.* **2**, 113.
Biggs, R., and Bidwell, E. (1957). *Brit. J. Haematol.* **3**, 387.
Biggs, R., and Macfarlane, R. G. (1962). "Human Blood Coagulation and Its Disorders." Davis, Philadelphia, Pennsylvania.
Billimoria, J. D., Irani, V. J., and Maclagan, N. F. (1965). *J. Atherosclerosis Res.* **5**, 90.
Botti, R. E., and Ratnoff, O. D. (1963). *J. Clin. Invest.* **42**, 1569.
Botti, R. E., and Ratnoff, O. D. (1964). *J. Lab. Clin. Med.* **64**, 385.
Bradlow, B. A. (1961). *Brit. J. Haematol.* **7**, 476.
Bronte-Stewart, B. (1965). *Practitioner* **194**, 195.
Chandler, A. B. (1958). *Lab. Invest.* **7**, 110.
Connor, W. E. (1962). *J. Clin. Invest.* **41**, 1199.
Connor, W. E., Hoak, J. C., and Warner, E. D. (1963). *J. Clin. Invest.* **42**, 860.
Connor, W. E., Hoak, J. C., and Warner, E. D. (1965). *Thromb. Diath. Haemorrh. Suppl.* **17**, 89.
Daemen, F. J. M. (1963). "Syntheses of Phosphatidyl Ethanolamines and Application of Synthetic Phosphatides in Studies on Blood Coagulation." Drukkerij Elinkwijk, Utrecht.
Daemen, F. J. M., van Arkel, C., Hart, H. Ch., van der Drift, C., and van Deenen, L. L. M. (1965). *Thromb. Diath. Haemorrh.* **13**, 194.
Davie, E. W., and Ratnoff, O. D. (1964). *Science* **145**, 1310.
Davie, E. W., and Ratnoff, O. D. (1965). *In* "The Proteins: Composition, Structure and Function" (H. Neurath, ed.), Vol. III, p. 359. Academic Press, New York.
de Duve, C. (1965). *In* "The Harvey Lectures," pp. 49-87. Academic Press, New York.
Didisheim, P., and Mibashan, R. S. (1963). *Thromb. Diath. Haemorrh.* **9**, 346.
Duguid, J. B. (1954). *Lancet* **266**, 891.
Farquhar, J. W. (1962). *Biochim. Biophys. Acta* **60**, 80.
Ferguson, J. H. (1960). "Lipoids and Blood Platelets." Univ. of North Carolina Press, Chapel Hill, North Carolina.
Ferguson, J. H. (1964). *Federation Proc.* **23**, 762.
Ferguson, J. H., Marcus, A. J., and Robinson, A. J. (1963). *Blood* **22**, 19.
Fredrickson, D. S., and Gordon, R. S., Jr. (1958). *Physiol. Rev.* **38**, 585.
Fullerton, H. W., Davie, W. J. A., and Anastasopoulos, G. (1953). *Brit. Med. J.* **2**, 250.
Garrett, J. V. (1956). *J. Lab. Clin. Med.* **47**, 752.
Goodman, D. S. (1958). *J. Am. Chem. Soc.* **80**, 3892.
Gresham, G. A., and Howard, A. N. (1963). *Federation Proc.* **22**, 1371.
Gurd, F. R. N. (1960). *In* "Lipide Chemistry" (D. J. Hanahan, ed.), p. 208. Wiley, New York.
Hanahan, D. J. (1960). "Lipide Chemistry." Wiley, New York.
Hardisty, R. M., and Hutton, R. A. (1965). *Brit. J. Haematol.* **11**, 258.

Hartroft, W. S., and Thomas, W. A. (1957). *J. Am. Med. Assoc.* **164**, 1899.
Haslam, R. J. (1964). *Nature* **202**, 765.
Hecht, E., and Slotta, K. H. (1962). *Am. J. Clin. Pathol.* **37**, 126.
Hicks, N. D., and Pitney, W. R. (1957). *Brit. J. Haemat.* **3**, 227.
Hjort, P. F., and Hasselback, R. (1961). *Thromb. Diath. Haemorrh.* **6**, 580.
Hoak, J. C., Poole, J. C. F., and Robinson, D. S. (1963). *Am. J. Pathol.* **43**, 987.
Hoak, J. C., Connor, W. E., Eckstein, J. W., and Warner, E. D. (1964). *J. Lab. Clin. Med.* **63**, 791.
Honour, A. J., and Russell, R. W. (1962). *Brit. J. Exptl. Pathol.* **43**, 350.
Horowitz, H. I. (1965a). *Thromb. Diath. Haemorrh. Suppl.* **17**, 243.
Horowitz, H. I. (1965b). Personal communication.
Horowitz, H. I., and Marcus, A. J. (1964). *Blood* **23**, 178.
Horowitz, H. I., and Spaet, T. H. (1961). *J. Appl. Physiol.* **16**, 112.
Horowitz, H. I., DesPrez, R. M., and Hook, E. W. (1962). *J. Exptl. Med.* **116**, 619.
Hovig, T. (1965). *Thromb. Diath. Haemorrh.* **13**, 84.
Hubbard, D., and Lucas, G. L. (1960). *J. Appl. Physiol.* **15**, 265.
Hunter, R. B., Wright, I. S., Koller, F., and Streuli, F., eds. (1965). "Genetics and the Interaction of Blood Clotting Factors." Schattauer, Stuttgart. *Thromb. Diath. Haemorrh. Suppl.* **17**, 343.
Husom, O. (1961). *Scand. J. Clin. Lab. Invest.* **13**, 619.
Iatridis, S. G., and Ferguson, J. H. (1962). *J. Clin. Invest.* **41**, 1277.
Levin, J., and Bang, F. B. (1964). *Bull. Johns Hopkins Hosp.* **115**, 337.
Lundblad, R. L., and Davie, E. W. (1964). *Biochemistry* **3**, 1720.
Macfarlane, R. G. (1964). *Nature* **202**, 498.
Macfarlane, R. G. (1965). *Oxford Med. Sch. Gaz.* **17**, 100.
Macfarlane, R. G. (1966). *Thromb. Diath. Haemorrh.* **15**, 591.
McKay, D. G. (1965). "Disseminated Intravascular Coagulation: An Intermediary Mechanism of Disease." Harper, New York.
Marcus, A. J. (1965). *Thromb. Diath. Haemorrh. Suppl.* **17**, 85.
Marcus, A. J., and Spaet, T. H. (1958). *J. Clin. Invest.* **37**, 1836.
Marcus, A. J., and Ullman, H. L. (1964). Unpublished observations.
Marcus, A. J., and Zucker, M. B. (1965). "The Physiology of Blood Platelets: Recent Biochemical, Morphologic and Clinical Research." Grune & Stratton, New York.
Marcus, A. J., and Zucker-Franklin, D. (1964a). *Blood* **23**, 389.
Marcus, A. J., and Zucker-Franklin, D. (1964b). *J. Clin. Invest.* **43**, 1241.
Marcus, A. J., Ullman, H. L., Safier, L., and Ballard, H. S. (1961). Unpublished observations.
Marcus, A. J., Ullman, H. L., Safier, L. B., and Ballard, H. S. (1962). *J. Clin. Invest.* **41**, 2198.
Marcus, A. J., Zucker-Franklin, D., Safier, L. B., and Ullman, H. L. (1966). *J. Clin. Invest.* **45**, 14.
Margolis, J. (1962). *Australian J. Exptl. Biol. Med. Sci.* **40**, 505.
Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
Merskey, C., and Drapkin, A. (1965). *Blood* **25**, 567.
Merskey, C., and Lackner, H. (1959). *Postgrad. Med. J.* **35**, 203.
Merskey, C., and Marcus, A. J. (1963). *Ann. Rev. Med.* **14**, 323.
Merskey, C., and Nossel, H. L. (1957). *Lancet* **1**, 806.

Merskey, C., and Wohl, H. (1964). *Thromb. Diath. Haemorrh.* **10**, 295.
Milstone, J. H. (1964). *Federation Proc.* **23**, 742.
Mustard, J. F., Medway, W., Downie, H. G., and Rowsell, H. C. (1962). *Nature* **196**, 1063.
Mustard, J. F., Hegardt, B., Rowsell, H. C., and MacMillan, R. L. (1964a). *J. Lab. Clin. Med.* **64**, 548.
Mustard, J. F., Murphy, E. A., Rowsell, H. C., and Downie, H. G. (1964b). *J. Atherosclerosis Res.* **4**, 1.
Nemerson, Y. (1966). *Biochemistry* **5**, 601.
Nemerson, Y., and Spaet, T. H. (1964). *Blood* **23**, 657.
Nishizawa, E. (1965). *Federation Proc.* **24**, 154 (Abstract 147).
Nossel, H. L. (1964). "The Contact Phase of Blood Coagulation." Davis, Philadelphia, Pennsylvania.
Nossel, H. L. (1965). Personal communication.
Nye, S. W., Graham, J. B., and Brinkhous, K. M. (1962). *Am. J. Med. Sci.* **243**, 279.
O'Brien, J. R. (1957a). *Am. J. Med. Sci.* **234**, 373.
O'Brien, J. R. (1957b). *Lancet* **272**, 1213.
O'Brien, J. R. (1957c). *Brit. J. Exptl. Pathol.* **38**, 529.
O'Brien, J. R. (1958). *Nature* **181**, 420.
O'Brien, J. R. (1959a). *J. Clin. Pathol.* **12**, 45.
O'Brien, J. R. (1959b). *Nature* **184**, 1580.
O'Brien, J. R. (1960). *Postgrad. Med. J.* **36**, 558.
O'Brien, J. R. (1964). *Blood* **24**, 309.
Papahadjopoulos, D., Hougie, C., and Hanahan, D. J. (1962). *Proc. Soc. Exptl. Biol. Med.* **111**, 412.
Papahadjopoulos, D., Hougie, C., and Hanahan, D. J. (1964). *Biochemistry* **3**, 264.
Pilkington, T. R. E. (1957a). *Clin. Sci.* **16**, 261.
Pilkington, T. R. E. (1957b). *Clin. Sci.* **16**, 269.
Pool, J. G. (1964). *Ann. Rev. Med.* **15**, 215.
Poole, J. C. F. (1955). *Brit. J. Exptl. Pathol.* **36**, 248.
Rapaport, S. I., Schiffman, S., Patch, M. J., and Ware, A. G. (1961). *J. Lab. Clin. Med.* **57**, 771.
Rapport, M. M. (1956). *Nature* **178**, 591.
Robbins, J., and Stetson, C. A., Jr. (1959). *J. Exptl. Med.* **109**, 1.
Robinson, D. S., and Poole, J. C. F. (1956). *Quart. J. Exptl. Physiol.* **41**, 36.
Rossiter, R. J. (1963). *Trans. Roy. Soc. Can.* **1** (*Ser. IV, Sect. III*), 377.
Rouser, G., and Schloredt, D. (1958). *Biochim. Biophys. Acta* **28**, 81.
Rouser, G., White, S. G., and Schloredt, D. (1958). *Biochim. Biophys. Acta* **28**, 71.
Rouser, G., O'Brien, J., and Heller, D. (1961). *J. Am. Oil Chemists' Soc.* **38**, 14.
Salem, L. (1962). *Can. J. Biochem. Physiol.* **40**, 1287.
Sawyer, P. N., Reardon, J. H., and Ogoniak, J. C. (1965). *Proc. Natl. Acad. Sci. U.S.* **53**, 200.
Seegers, W. H. (1961). *In* "Blood Platelets" (S. A. Johnson, R. W. Monto, J. W. Rebuck, and R. C. Horn, Jr., eds.), p. 241. Little, Brown, Boston, Massachusetts.
Seegers, W. H. (1964). *Federation Proc.* **23**, 749.

Seegers, W. H. (1965). *Thromb. Diath. Haemorrh.* **14**, 213.
Shinowara, G. Y. (1951). *J. Lab. Clin. Med.* **38**, 11, 23.
Shinowara, G. Y. (1957). *J. Biol. Chem.* **225**, 63.
Shinowara, G. Y. (1961). *Nippon Ketsueki Gakkai Zasshi* **24**, 716.
Shinowara, G. Y., Stutman, L. J., Walters, M. I., Ruth, M. E., and Walker, E. J. (1963). *Am. J. Surg.* **105**, 714.
Shore, P. A., and Alpers, H. S. (1963). *Nature* **200**, 1331.
Silver, M. J., Turner, D. L., and Tocantins, L. M. (1957). *Am. J. Physiol.* **190**, 8.
Silver, M. J., Turner, D. L., and Tocantins, L. M. (1959). *In* "Progress in Hematology" (L. M. Tocantins, ed.), Vol. II, p. 264. Grune & Stratton, New York.
Silver, M. J., Turner, D. L., Rodalewicz, I., Giordano, N., Holburn, R., Herb, S. F., and Luddy, F. E. (1963). *Thromb. Diath. Haemorrh.* **10**, 164.
Slotta, K. H. (1960). *Proc. Soc. Exptl. Biol. Med.* **103**, 53.
Slotta, K. H., and Deutsch, E. (1960). *Thromb. Diath. Haemorrh.* **4**, 283.
Slotta, K. H., and Powers, J. K. (1962). *Anal. Biochem.* **4**, 69.
Soloff, L. A., and Wiedeman, M. P. (1963). *Nature* **199**, 495.
Spaet, T. H. (1963). *In* "Progress in the Chemistry of Fats and Other Lipids" (R. T. Holman, W. O. Lundberg and T. Malkin, eds.), Vol. VI, p. 173. Macmillan (Pergamon), New York.
Spaet, T. H. (1964). *Federation Proc.* **23**, 757.
Spaet, T. H. (1966). *Blood* **28**, 112.
Spaet, T. H., and Cintron, J. (1963). *Blood* **21**, 745.
Spaet, T. H., and Cintron, J. (1965). *Brit. J. Haematol.* **11**, 269.
Spaet, T. H., and Zucker, M. B. (1964). *Am. J. Physiol.* **206**, 1267.
Spaet, T. H., Cintron, J., and Kropatkin, M. (1959). *J. Lab. Clin. Med.* **54**, 467.
Spaet, T. H., Horowitz, H. I., Zucker-Franklin, D., Cintron, J., and Biezenski, J. J. (1961). *Blood* **17**, 196.
Speer, R. J., and Ridgway, H. (1962). *Proc. 9th Congr. Intern. Soc. Hematol.*, Vol. II, p. 175. U. Nacional Autonoma de Mexico, Mexico City.
Surgenor, D. M., and Wallach, D. F. H. (1961). *In* "Blood Platelets" (S. A. Johnson, R. W. Monto, J. W. Rebuck, and R. C. Horn, Jr., eds.), p. 289. Little, Brown, Boston, Massachusetts.
Therriault, D., Nichols, T., and Jensen, H. (1958). *J. Biol. Chem.* **233**, 1061.
Thomas, W. A., and Hartroft, W. S. (1959). *Circulation* **19**, 65.
Tocantins, L. M., Carroll, R. T., and McBride, T. J. (1948). *Proc. Soc. Exptl. Biol. Med.* **68**, 110.
Tompkins, M. J., and Dayton, S. (1965). *Proc. Soc. Exptl. Biol. Med.* **119**, 588.
Trevan, J. W., and Macfarlane, R. G. (1936). *Med. Res. Council Ann. Rept., 1936–37*, p. 143. H. M. Stationery Office, London.
Troup, S. B., Reed, C. F., Marinetti, G. V., and Swisher, S. N. (1960). *J. Clin. Invest.* **39**, 342.
Turner, D. L., Holburn, R. R., DeSipin, M., Silver, M. J., and Tocantins, L. M. (1963). *J. Lipid Res.* **4**, 52.
Turner, D. L., Silver, M. J., Baczynski, N. G., and Rodalewicz, I. (1964). *J. Lipid Res.* **5**, 616.
Vroman, L. (1964). *Thromb. Diath. Haemorrh.* **10**, 455.
Waaler, B. A. (1959). "Contact Activation in the Intrinsic Blood Clotting System." Oslo Univ. Press, Oslo.

Wallach, D. F. H., Maurice, P. A., Steele, B. B., and Surgenor, D. M. (1959). *J. Biol. Chem.* **234**, 2829.
Wessler, S. (1963). *Federation Proc.* **22**, 1366.
Wessler, S., Reimer, S. M., and Sheps, M. C. (1959). *J. Appl. Physiol.* **14**, 943.
Williams, W. J. (1961). *In* "Blood Platelets" (S. A. Johnson, R. W. Monto, J. W. Rebuck, and R. C. Horn, Jr., eds.), p. 277. Little, Brown, Boston, Massachusetts.
Williams, W. J. (1964). *J. Biol. Chem.* **239**, 933.
Williams, W. J. (1965). *In* "Biophysical Mechanisms in Vascular Homeostasis and Intravascular Thrombosis" (P. N. Sawyer, ed.). Appleton-Century-Crofts, New York, p. 192.
Woodside, E. E., Therriault, D. G., and Kocholaty, W. (1964). *Blood* **24**, 76.
Zbinden, G. (1964). *J. Lipid Res.* **5**, 378.
Zilversmit, R. D., Marcus, A. J., and Ullman, H. L. (1961). *J. Biol. Chem.* **236**, 47.
Zucker, M. B., and Marcus, A. J. (1966). *Nouvelle Rev. Franc. Hematol.* **6**, 345.
Zucker-Franklin, D., and Spaet, T. H. (1963) *Am. J. Physiol.* **205**, 341.