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Human Platelet Lipids and Their Relationship to Blood Coagulation

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Abstract

There is now reasonable agreement on the sequence of physiological and biochemical events leading to fibrin formation, and phospholipids are an important part of this process. The phosphatides are ordinarily provided by platelets, and it appears that a lipoprotein complex is responsible for this activity. The anatomic site of this complex is not known, but evidence is presented that it may be a property of the platelet membrane. Methods for the study of platelet lipids including fatty acids and aldehydes are described, and include silicic acid column and paper chromatography, as well as thin-layer and gas-liquid chromatographic procedures. These are also being utilized in studies of subcellular platelet particles, where only limited amts of biological material are available for study. It is stressed that experimental results obtained from studies on isolated lipids should be interpreted with a certain degree of caution. It is unlikely that they are available as such in in vivo coagulation, and the drastic procedures used for their extraction and isolation may alter their basic physiological properties.

IN RECENT YEARS, the role of lipids in blood coagulation has been better understood, and there appear to be two main reasons for this increase in our knowledge. First, the sequence of protein interactions leading to the formation of a fibrin clot has been further elucidated (1). Secondly, advances in research have enabled us to characterize the specific lipids involved in the coagulation mechanism. Our thinking has probably been further clarified by the realization that there are three physiological events that should be dealt with separately—at least for the time being. They are: a) blood coagulation; b) hemostasis; c) thrombosis. The tendency in the past to think of these as a single entity has led to confusion. For example, an increase in plasma lipids or postprandial lipemia was equated with a "hypercoagulable" state. This was presumed to be directly related to thrombosis. Arterial thrombi histologically resemble hemostatic platelet plugs in that there is a white "head" consisting of relatively intact platelets. Venous thrombi, on the other hand, more closely resemble clots as formed in the test tube. That is, they consist of a mixture of fibrin, entrapped red cells and leukocytes, as well as platelets. These are the so-called "red" thrombi. Thus, it is important to realize that lipids may not play a role in thrombosis and hemostasis, but may only be important for coagulation. These concepts have been discussed in detail in recent reviews of the subject (2–4).

Current Theory of Coagulation

Figure 1 shows the sequence of events leading to the formation of fibrin. Contact with a foreign surface, such as damaged endothelium or the cut edge of a blood vessel appears to activate Hageman factor. This in turn activates PTA and a pattern of biochemical transformations appears to ensue, which involves the activation of a previously inactive coagulation

protein (1,31). Note that the lipid contribution appears relatively late in the reactions of the intrinsic prothrombin activator system. Current thinking is that the lipid is derived from platelets which have already arrived at the site of blood vessel damage because of their role in hemostasis. Classical theories of platelet involvement in clotting propose that granules are discharged into the surrounding medium, which make their lipid available to the coagulation process. Recent data in our own laboratory indicate that the platelet membrane has somewhat better clot-promoting capabilities than do the granules. We have proposed that the platelet granules are "storehouses" for metabolic substances concerned with other platelet functions, and the membrane lipid acts as a surface catalyst for coagulation. It is emphasized that these are merely theoretical proposals, and further investigation will be necessary before they can be considered unequivocal (5).

Conclusions drawn from studies on isolated platelet lipids must be considered in the light of their in vitro activity only. For example, phospholipids, especially of the ethanolamine and serine groups, are the most active in the test tube. However, in in vivo coagulation, all the platelet lipids are probably available as a lipoprotein complex. In addition, when platelet homogenates are made, a soluble lipoprotein appears which possesses clot-promoting properties. It is not known whether this represents an artefact of the disruption procedure or whether the platelet actually contains soluble lipoprotein in an unbound form.

With these preliminary comments in mind, we would like to describe some of the methods utilized in our laboratory for the past ten years in the investigation of human platelet lipids.

Methods and Materials

Methods of Collection and Lipid Extraction of Platelets

Platelets were isolated from freshly collected blood (6). The appearance of platelets as seen by electron microscopy is illustrated in Fig. 2. They were washed and frozen within a period of 5 hr. Ordinarily, they were maintained at −20C for periods up to 3 months prior to extraction. The subcellular platelet particles had a tendency to deteriorate at −20C, as evidenced by discoloration and putrefaction. Therefore, the subcellular materials are now being stored at −85 C. Following venisection, all operations involved in platelet processing were carried out in a cold room at 4C. Two main extraction procedures have been used. The first was based on the procedure of Bell and Alton (7). This involves suspension in four times the original volume of acetone for 30 min, following which the acetone was removed by centrifugation. This was repeated 4–6 times. The acetone-insoluble material was then extracted with ten times its volume of chloroform, and this was repeated twice, the third extraction being allowed to take place overnight. The yields appeared to be the same whether the procedure was carried out at 4C or at room temp. This extraction procedure yielded less of the neutral lipid fraction and is still our procedure of choice for preparation of platelet or brain "cephalin" for use in blood coagulation tests as platelet substitutes.

In later studies, chloroform:methanol 2:1 was used. The procedures were always carried out under highly purified nitrogen, and the solvents were deoxygenated immediately before use (8). In this method, extraction of 1 g (wet weight) of platelet material yielded about 31 mg of crude lipid. The starting material

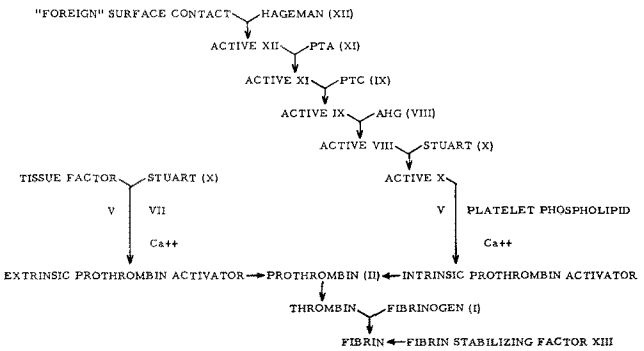

FIG. 1. Diagrammatic scheme of blood coagulation mechanism as currently accepted by most investigators.

for each column separation was approximately 400 mg of lipid. Approx 13 g (wet weight) of platelets was homogenized in 150 ml chloroform-methanol in a Waring Blendor under an atmosphere of nitrogen. The insoluble material was removed by filtration through a sintered glass filter and finally the filtrate was passed through fat-free sharkskin filter paper. The filtrate was then dried under reduced pressure and nitrogen in an Erlenmeyer flask at 30C. It was found useful to add aldehyde-free absolute ethanol to the flask, which formed a water-ethanol azeotrope, thus aiding in the removal of last traces of water (9). The lipid material was stored under nitrogen in C:M 2:1 for a max of 12 hr at −20C prior to column chromatography.

Column Chromatography

In earlier work (6,10), the procedures of Lea, Rhodes and Stoll (11) were used, as well as those of Hirsch and Ahrens (9). Although the methods themselves were excellent, they did not serve our purpose as well as the procedures later developed by Rouser and associates. The reason for this was that there was an unfortunate overlapping between the ethanolamine and serine phosphoglycerides, and only small amts of serine phosphoglycerides could be obtained. The clot-promoting properties of almost any combination of PE and PS were quite impressive, but we were mainly interested in the activity of *individual* phosphatides. Thus, we turned our attention to the techniques of Rouser (12,13) in our later studies. Although we have tried others, Mallinckrodt silicic acid 100 mesh has always appeared to be the most suitable. Usually 60 g of the material was washed as described by Rouser (12). The washed silicic acid was heated for 12–15 hr at 120C under negative pressure and nitrogen in a

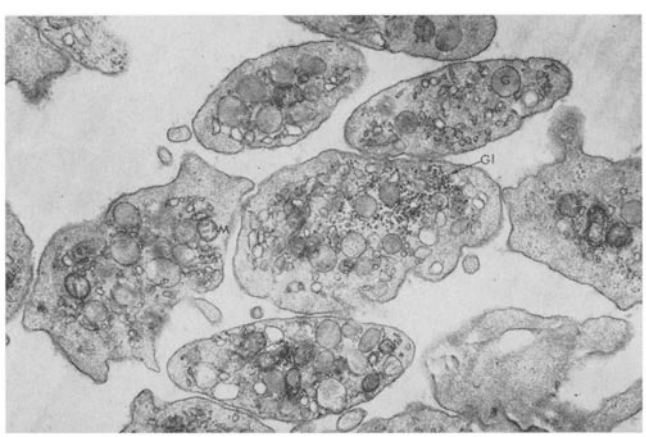

FIG. 2. Electron micrograph of human blood platelets. G: granule; Gl: glycogen particles; M: mitochondrion. Magnification: x 32,000.

1,000 ml three-necked flask. This was allowed to cool, and 200 ml chloroform:methanol 4:1 was added to make a slurry. The slurry was poured into a glass column 2.5 × 40 cm. In such a column, 60 gm of silicic acid extended to a height of 20 cm after the application of nitrogen pressure. The platelet lipid was applied to the column in a small volume of chloroform:methanol 4:1, and the chromatography was carried out at 10C by allowing cold water to be pumped through a jacket surrounding the column. Fractions of 10 ml were collected by hand and examined by means of Rouser's rapid ninhydrin test (12). About every tenth tube was studied in greater detail by thin-layer and silicic acid paper chromatography.

A rough quantitative index was also obtained by adding 1 ml of eluate to 1 ml 5 *N* sulfuric acid in a 30 ml Kjeldahl flask and heating at 320C for 5–7 min. The intensity of charring was an index of the amount of organic material present.

In the first silicic acid column separation, the eluting solvent was chloroform:methanol 4:1 until the fractions became negative to the ninhydrin reagent. Before storing the samples, 0.1 ml concd ammonia was added to each tube, which retarded plasmalogen breakdown. After the ninhydrin-positive fractions from the first run were verified as a mixture of PE and PS by thin-layer and silicic acid paper chromatography, they were all pooled and evaporated down to 5 ml. Twelve hours later, the PE and PS were separated from each other on a silicic acid-silicate-water column, which was prepared by allowing a mixture of chloroform:aqueous ammonia to pass through the previously prepared silicic acid column. As Rouser has clearly shown, the PS remains bound to the column while PE is eluted first with chloroform:methanol 4:1. The PS is finally eluted with methanol.

Paper Chromatography

We still use paper chromatographic procedures to supplement our thin-layer techniques. The systems devised by Marinetti et al. have been most useful (14). Examination of the phosphatides under ultraviolet light is occasionally more profitable than the identification procedures for thin-layer chromatography (TLC).

Thin-Layer Chromatography

This technique has been most useful to us for identifying lipids in individual fractions. It has been less valuable for total lipid extracts, mainly because we have been interested in complete separation of PE from PS and PI. In systems currently available, such as those of Skipski and associates (15–17), PI and PS run very close to each other. Thus, for thin-layer examination of column fraction we have used a solvent system consisting of chloroform-methanol:water:concd ammonia 75:25:4:1 (18). The chromatographic plates were dried in air and sprayed with 40% sulfuric acid followed by charring on a hot plate. If the separated phosphatides were oxidized or broken down, these phenomena could also be detected on thin-layer plates (8).

Gas-Liquid Chromatography

For this we have employed a Barber-Colman model 10 apparatus, equipped with two strontium^{90} detector cells. U-shaped glass columns, 6 ft in overall length, were most frequently used. It was important to check the linearity of detector response to mass with the fatty acid standards provided by the National Heart Institute. The range of error using all of the mixtures, A through D, was never more than 3%. Methyl esters and dimethyl acetals were prepared by the method of Stoffel, Chu and Ahrens (19). It was found that sublimation following esterification was not necessary.

A. *Nonpolar Columns.* Gas-Chrom P (Applied Science Laboratories, State College, Pa.) and Anakrom AB (Analabs, Hamden, Conn.) were found to be satisfactory stationary supports. Usually the stationary supports were washed with acid and alkali, and then brought back to neutrality. However, if this was done, precise quantification of the dimethyl acetals was not possible. After the alkali wash it was necessary to leave the support material in an alkaline state. The manner in which this was done is as follows: The support was washed in 1% methanolic potassium hydroxide which was removed by filtration through a Buchner funnel. After drying in a convection oven for 12 hr at 120C, the pH was tested by suspending 1 mg of powder in 1 ml of distilled water. The pH of the support material was 7.4 It was then coated with 10–15% Apiezon L or M (20). This material is now commercially available from Applied Science Laboratories, State College, Pa. Before use, the columns were conditioned for 12–24 hr at temps slightly above that used for normal operation. It was important not to connect the columns to the detector cells during the conditioning period (21). During this time, large loads of mixed methyl esters were applied to the column for a "soaking" effect (22). Detector responses were most linear if they were operated at a voltage of 350. The amplifier setting was usually 3×10^{-8}a. Column temp was 197C, the detector was maintained at 220C, and flash heater at 197C.

B. *Polar Columns.* Stationary support was the same as used for nonpolar columns and pretreatment with alkali was helpful. The "soaking" procedure was also used here. Supports were coated with 10–15% ethylene glycol adipate (EGA). The cell voltage was about 400, amplifier setting 3×10^{-8}a, column temp 173C, detector 220C, and flash heater 173C.

We have found it advantageous to compare quantitative and qualitative results with another laboratory engaged in GLC. Aliquots of methyl esters prepared in our laboratory were analyzed by Dr. J. W. Farquhar at the Rockefeller Institute, New York. Our results were in excellent agreement.

C. *Fatty Acids of PE and PS.* The ethanolamine phosphoglycerides in human platelets had stearic acid as the main saturated fatty acid. The principle unsaturated acid was arachidonic. As in the red cell (18), the fatty aldehydes were confined mainly to PE. Almost all were of the saturated straight-chain 16:0 and 18:0 type. In PS, the main saturated fatty acid was also stearic, but the principle unsaturated acids were oleic and arachidonic. Only a small amt of plasmalogen was noted in PS. Platelet lecithin contained large amts of palmitic acid. The major unsaturated fatty acid was oleic. The plasmalogen content of PE was also measured by iodine addition (23) and was found to be 65%, which was in close agreement with the finding by GLC, which was 66%. If we assume that the aldehydogenic group and the saturated fatty chains are linked to the alpha carbon atom, and the unsaturated fatty acids are linked to the beta carbon as acyl esters, then the ratio of saturated to unsaturated fatty acids in the platelet diacyl phosphatides is approx 1.

Methods for Testing Lipids in in Vitro Coagulation Systems

Many of the differences found in various laboratories working in the field are attributable to a lack of agreement on which coagulation system to use for testing lipids they have isolated. When whole plate-

lets are tested, they are suspended in saline or buffer and introduced into the test system in this manner. We have followed the same procedure with platelet lipids. That is, suspension in buffer, so that they are in the same medium as the platelets from which they were derived. This was carried out on unoxidized lipids as they came off the column, as well as after short periods of storage. No attempts were made to alter the surface charge of the phosphatides before testing. On the other hand, Silver et al. preferred to study lipids in a solubilized state (24). This gives rise to results which are different from those obtained with lipid emulsions. We will confine our discussion to results obtained with platelet lipids in a few standard clotting systems.

A. *Thromboplastin Generation Test* (TGT). In this system, platelet lipids are incubated with plasma and serum reagents containing clotting proteins necessary for forming the intrinsic prothrombin activator. At given intervals, aliquots are removed and tested for their ability to convert prothrombin to thrombin. PS is the most active lipid in this test. In our hands, PE has been inactive, but in other laboratories clot-promoting activity has been found (25,26). The other phosphatides and neutral platelet lipids are inert in this test.

B. *Recalcified Clotting Time.* PS is again the most active platelet lipid in this test. In contrast to the TGT, PE is able to shorten the recalcified clotting time when compared to a buffer blank. These findings are also true with lipids from other sources.

C. *Other Clotting Systems.* In the two-stage system developed by Ferguson, both PE and PS were active (25). These phosphatides also shorten the "Stypven" time, but this is a nonspecific test. All of the platelet phosphatides can shorten the Stypven time (27).

In testing individual lipids, whenever possible it is important to compare the activity of the test substance with that obtained by whole platelets, crude platelet lipid extracts, or crude brain cephalin. In other words, what is the capability of the lipid in question as compared to what is attained by materials of known activity? There is a tendency to compare lipids with buffer blanks, which in our opinion may reflect a nonspecific response.

Again it is emphasized that studies on isolated lipids may bear little relationship to in vivo coagulation. It is unlikely that single phosphatides exist as such in the platelet, nor is it plausible that lipid per se plays a role in the clotting process in vivo.

Recent Studies on Subcellular Platelet Particles

We have initiated a study to correlate the structure, enzyme content, and clotting functions of subcellular platelet particles. An attempt has been made to compare the platelet membrane with the platelet granules with regard to the above characteristics. By differential centrifugation, we were able to recover predominantly granule fractions. However, they were contaminated with membranes (28). Nevertheless, interesting information was obtained when the fatty acids in the phosphatides from these granule preparations were studied. In the ethanolamine phosphoglycerides there was more 22:5 fatty acid than found with whole platelets, and there was also more 22:6 than in the PE from whole platelets. This gave us the impetus to attempt further separation which was accomplished by the use of sucrose gradient ultracentrifugation. In this manner, pure membrane preparations, as well as highly purified granule fractions, were obtained.

In order to obtain adequate separation, it was necessary to use a continuous sucrose gradient of from 30–65%. When the particles were recovered and the lipids extracted with chloroform:methanol, large amts of sucrose appeared in the lipid extract, and quantitative studies were not possible. In order to overcome this problem, the method of Folch, Lees and Sloane Stanley (29) was modified for extraction of small amts of tissue, and will be described in detail:

Pure solvents upper phase consisted of chloroform: methanol:water (3:48:47 v/v), and contained 0.02% calcium chloride. A rinsing fluid was prepared for the filter papers and flasks after filtration of the initial extract consisting of 19 volumes of chloroform: methanol 2:1 to 0.8 volume of water.

Five-milliliter Lusteroid tubes containing the desired fractions were filled with 0.25 M sucrose (0.001 M EDTA) and mixed by inversion. The fractions were then brought down as a pellet by centrifugation in a Spinco Model L ultracentrifuge for one hour at 39,400 rpm. Centrifugation was more effective if the SW 39 (swinging bucket) head was used. The supernatants were decanted, and each pellet was suspended in 1 ml 0.25 molar sucrose (0.001 M EDTA) with a small Teflon plunger. All pellets were pooled in a graduated conical centrifuge tube and immediately after mixing on a vortex agitator, aliquots were removed for protein analysis.

Fractions were transferred by Pasteur pipet from the graduated centrifuge tubes to glass-stoppered Erlenmeyer flasks. Tubes and pipets were rinsed with part of the 19 volumes of C:M 2:1 and extraction was performed. Filtration was then carried out through solvent-washed Whatman No. 1 filter paper into glass-stoppered graduated cylinders. After completion of filtration, the flasks and filter papers were washed with fresh solvent (19 parts of C:M 2:1 to 0.8 parts of water). A volume of 0.05% calcium chloride, equivalent to 0.2 of the final volume of filtrate, plus washings in the cylinder, was added and the cylinder stoppered and shaken. The respective phases were separated by centrifugation at 5C for 15 min at 1200 rpm. For this purpose, 50 ml conical centrifuge tubes covered with aluminum foil were used. The aqueous top layer was removed by suction and Pasteur pipet as far down as possible, and discarded. The remaining top layer was rinsed off by carefully layering 3 ml of pure solvents upper phase containing 0.02% calcium chloride and gently swirling and removing the top layer. The lower phases were then recombined into graduated cylinders and washed 3 times with pure solvents upper phase containing 0.02% calcium chloride by adding 40 parts of fresh solvent to 60 parts of lower phase, shaking, centrifuging, discarding the aqueous layer and rinsing the interphase. After the last wash, the interphase was rinsed 3 times and all aqueous layer removed as completely as possible. Corresponding lower phases in the centrifuge tubes were pooled into glass-stoppered graduated centrifuge tubes and taken to dryness under nitrogen at 37C. The dried lipid residues dissolved completely in C:M 2:1.

Total lipid was determined by weighing aliquots measured with Lang-Levy pipets on the Cahn electrobalance after evaporating the solvent under an infrared lamp. Weights were carried out in duplicate. Because of the small amt of lipid being processed, correction was made for residual calcium chloride in the lower phase of the Folch extraction procedure. A calculated value of 4.13 μg. calcium chloride per milliliter lower phase was used.

The amt of phospholipid lost in the initial aqueous phase of the Folch wash was also investigated (29). First, some visible remaining drops of lower phase at the bottom of the flasks were removed by Pasteur pipet. The aqueous layers were taken to dryness under vacuum at 37C. The residues were dissolved in 10 ml distilled water and dialysed in the cold overnight against 2 liters of distilled water. Dialysis was continued for about 3½ hr longer with 2 liters of fresh distilled water. The material remaining in the dialysis bag was taken to dryness, the residue dissolved and washed into tubes for phosphorus assay with about 3 ml C:M 2:1. Less than 1% of the lipid phosphorus recovered from the entire extraction was found in the undialysable portion of the first aqueous phase.

Qualitatively, by TLC, the lipids in the granules and membranes were identical, but when the particles as such were tested in clotting systems, the membranes were more active than the granules. On the other hand, lipids extracted from both the granules and membranes behaved similarly in various clotting systems. It appeared that the lipid (or lipoprotein) in the platelet membrane was more "available" for interaction with the plasma clotting factors than was the lipid from the granules. In addition, the granules appeared to be the major site of acid phosphatase, beta-glucuronidase, and cathepsin activity, which was consistent with the hypothesis that they may be lysosomes. Some of the granules are mitochondria. As shown by Fleischer (30), these are rich in phospholipid, but we would suggest that such phospholipid does not ordinarily play a role in blood coagulation.

An approach to the study of platelet lipids and their role in in vitro coagulation has been described. It is stressed that correlations with in vivo coagulation mechanisms be made with the realization that these situations may not be comparable.

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Arachidonic, 5, 11, 14, 17-Eicosatetraenoic and Related Acids in Plants—Identification of Unsaturated Fatty Acids

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Abstract

Arachidonic and related fatty acids which normally are found only in animals or microorganisms have been isolated and identified from several mosses and ferns. Fatty acids with a double bond in position 5, separated by more than one methylene group from other double bonds, have been found in *Ginkgo biloba* and *Equisetum*. Analyses of fatty acids from numerous plants, in particular their chlorophyll containing parts, are listed according to components.

The experimental part gives details on structure determination of the usual methylene-interrupted fatty acids by ozonization-hydrogenation-GLC. Alkaline isomerization combined with these procedures was applied to determine the unusual double bond structures. The method permits positional identification of an internal double bond.

Introduction

TENTATIVE IDENTIFICATIONS of unsaturated fatty acid methyl esters are often made by comparison of retention times in gas-liquid chromatography (GLC) of the unknown with an authentic ester. Preferably, their equivalent chain lengths (ECL) (38,63) in GLC over different phases are compared. Interpolation of ECL may be helpful in characterizing a sample when an authentic ester of the expected structure is not accessible but when similar type esters of different chain length are available. Obviously, esters of different chain length cannot have identical double bond structures. Systematic studies have been reported by Ackman (1–5) where relative retention times are correlated with the location of methylene-interrupted double bond systems. As such data are accumulated, they contribute further to tentative identifications of fatty esters with a minimum of effort and substance.

The composition of mixtures may be such that resolution by GLC is unsatisfactory, that coordination of peaks from different phases is uncertain, or that ECL's merely indicate the presence of esters which are different from any reference compound. Another reason for isolation and investigation of structure by chemical means is to verify the tentative identification of an ester that is well known but is unexpected in