Effect of Trypsin on the Exposed Polypeptides and Glycoproteins in the Human Platelet Membrane†

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ABSTRACT: The polypeptides and glycoproteins in the human platelet plasma membrane were analyzed by polyacrylamide disc gel electrophoresis containing sodium dodecyl sulfate. Protein stains of these gels showed that this membrane is composed of polypeptides of molecular weights varying from more than 200,000 to 13,000. It was also shown that this membrane contains three major glycoproteins. The molecular weights of these glycoproteins were determined to be 150,000, 118,000, and 92,000, respectively. The membrane proteins that have exposure on the membrane surface were determined by the lactoperoxidase iodination technique. Use of this technique will iodinate only those membrane proteins which exist on the exposed surface of the plasma membrane. It was shown that at least seven different membrane polypeptides were labeled and are therefore exposed to the outside of the platelet. These labeled polypeptides correspond to the three major glycoproteins in the membrane in addition to four polypeptides with lower molecular weights (68,000, 34,000, 18,000, and 13,000, respectively). Alterations induced by trypsin on the plasma membrane surface were also investigated. Of the iodinated surface components, only the molecular weights of the three glycoproteins were decreased by this proteolytic enzyme, suggesting that the primary target for proteolytic enzymes on the membrane is glycoprotein. The glycopeptide hydrolytic products that remain with the membrane all contain iodine indicating that the iodinatable tyrosine residue on all three glycoproteins is on the membrane side of the trypsin-sensitive bond. Iodination of trypsin-treated platelets showed that the glycopeptide hydrolytic products are no longer in an iodinatable position in the membrane, demonstrating that hydrolysis of exposed glycoprotein will induce a conformational change in the plasma membrane. Trypsin hydrolysis was also shown to decrease the concentration of a polypeptide with a molecular weight of 150,000. However, this polypeptide does not appear to be a plasma membrane component since it is not isolated with the membrane fraction nor iodinated by lactoperoxidase. It therefore appears that it is released from within the platelet as a result of glycoprotein hydrolysis on the membrane surface.

The specialized functions of blood platelets are primarily concerned with hemostasis and thrombosis. In these functions the plasma membrane plays a central role. In hemostasis platelets adhere almost instantly at the site of vascular injury (Tranzer and Baumgartner, 1967; Zucker and Borrelli, 1961), the platelet membrane interacting with connective tissue, particularly collagen (Spaet *et al.*, 1962; Hovig, 1963). Other agents such as epinephrine, serotonin, thrombin and other proteases, particular matter, and adenosine 5′-diphosphate can also induce aggregation of platelets (Mills and Roberts, 1967; Mitchell and Sharp, 1969; Davey and Lüscher, 1967; Haslam, 1967). To determine the mechanism involved in hemostasis and thrombosis, many questions concerning the platelet membrane composition and structure remain to be answered. Since platelet-specific reactions in hemostasis and thrombosis involve interactions on the outer surface of the membrane, this face of the membrane becomes of particular interest.

The protein composition of the platelet membrane surface has been examined primarily by the susceptibility of surface proteins to proteolytic enzymes. The proteolytic enzymes thrombin, trypsin, papain, Pronase, and chymotrypsin all act on the intact platelet (Davey and Lüscher, 1967). Although many proteins have been demonstrated to be hydrolyzed by these proteolytic enzymes, they may not be located on the membrane surface. Proteolytic enzymes, including thrombin, will hydrolyze many platelet proteins and can potentially remove several "layers" from the membrane, releasing or digesting substrates not actually located on the surface. Proteolysis can also induce alterations in platelet membrane structure, bringing buried proteins to the surface for further proteolysis, thus destroying the spatial arrangement of membrane components. Most importantly, proteolytic action induces the release reaction so that it is not clear if altered components are on the membrane surface or derived from within the platelet. For these reasons, use of proteolysis to determine the surface structure of the platelet membrane is subject to ambiguity. Thrombosthenin (Booyse *et al.*, 1971) and fibrinogen (Nachman *et al.*, 1967), however, have been localized on the membrane surface by antigen–antibody reactions. The use of antigen–antibody reactions of this type to determine the total protein composition on the membrane surface has not been accomplished. Since the platelet membrane is composed of at least 20 polypeptide chains (Nachman and Ferris, 1970; Barber and Jamieson, 1971; Phillips, 1972), this would be an extremely difficult task. To date, the total protein composition on the platelet membrane surface has not been determined.

A prerequisite to studying the total surface structure of the platelet membrane is the development of techniques to determine the composition of a membrane surface. The requirements of a technique to determine the surface *protein* composition are: (1) that it is specific for proteins on the membrane surface; (2) that it modifies surface proteins in an easily de-

† From the Laboratories of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38101. *Received August 7, 1972.* This work was supported by Public Health Service Grants No. GM-15913 and CA-08480 and by Grant DRG-1132 from the Damon Runyon Memorial Fund for Cancer Research, Inc. A preliminary account of this work was presented at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N. J., April 1972 (Phillips, 1972).

tectable manner; (3) that the modifications induced are sufficiently small so that biological properties have not been altered; and (4) that the modifying reactions can be accomplished under physiological conditions. Recently, a mild technique has been developed in this laboratory which fulfills these requirements (Phillips and Morrison, 1970, 1971a,b). This technique, the lactoperoxidase iodination technique, has been employed to specifically iodinate proteins on membrane surfaces. Use of this technique on the human erythrocyte membrane has demonstrated the asymmetry of this membrane with only two protein components exposed to the outside, while most membrane polypeptides have exposure to the inside of the cell.

The purpose of this report is to describe the polypeptide and glycoprotein composition of the platelet membrane, to determine which membrane proteins have exposure on the membrane surface using the lactoperoxidase iodination technique, and to show the alterations induced on this surface by a representative of one class of the aggregating agents, namely trypsin.

Materials and Methods

All chemicals used in these experiments were reagent grade. Radioactive ^{125}I was purchased from New England Nuclear. Lactoperoxidase was isolated from bovine milk by the method of Morrison and Hultquist (1963). Trypsin (type III), soybean trypsin inhibitor (type I-S), bovine serum albumin, carbonic anhydrase, and myoglobin were purchased from Sigma Chemical Co. Phosphorylase *a* and β-glucuronidase were obtained from Worthington Biochemical Corp.

Preparation of Platelets and Platelet Membranes. Platelet concentrates prepared for clinical transfusions were used in less than 3 hr after venipuncture. The anticoagulant employed was acid–citrate–dextrose. Unless otherwise indicated, all manipulations were performed at 0–4°. Platelets from one unit of blood were separated from the plasma by centrifugation at 2000*g* for 10 min and resuspended in 20 ml of isolation media. The isolation media contained 0.0965 M NaCl, 0.0857 M glucose, 0.0011 M Na_2EDTA, and 0.00858 M Tris (pH 7.4). The suspension was then centrifuged at 120*g* for 7 min to remove residual white and red blood cells. The supernatant was carefully decanted and centrifuged at 2000*g* for 10 min to sediment the platelets. This procedure was repeated to further remove plasma proteins and contaminating cells. Platelets were then suspended in isolation media to a concentration of 10^9/ml and used immediately.

Platelet membranes were isolated from washed platelets by the glycerol lysis method. The plasma membrane fraction obtained from the discontinuous sucrose gradient centrifugation was employed in these studies (Barber and Jamieson, 1970).

Trypsin Hydrolysis of Platelets. Trypsin at a final concentration of 1 mg of protein/ml was added to the platelet suspension and incubated at 37° for 30 min in a shaker bath. At the end of the reaction, inhibitor was added to a final concentration of 3 mg of protein/ml. The solution was cooled to 0–4° in an ice bath and centrifuged at 2000*g* for 10 min. The platelets were washed by resuspending in the original volume of isolation media and centrifuging the platelets to free them of the trypsin–inhibitor complex and trypsin hydrolytic products. This procedure was repeated twice.

Iodination of Platelets. Lactoperoxidase-catalyzed iodination of platelet surface proteins was performed by a modification of the procedure previously reported (Phillips and Morrison, 1971a). A typical iodination reaction was prepared as follows. Ten milliliters of a platelet suspension containing 10^9 platelets/ml was gently stirred by a Teflon-coated magnetic stirring bar. Lactoperoxidase (10 μl of 1.4×10^{-4} M) and carrier-free $Na^{125}I$ (0.6 mCi) were then added. The iodination reaction was then initiated by adding five 10-μl aliquots of 10^{-2} M H_2O_2 in isolation media at 30-sec intervals. This time period of additions is sufficient for all the hydrogen peroxide to be consumed before the next aliquot is added (Morrison *et al.*, 1970). At the end of the reaction, the platelets are centrifuged at 2000*g* for 10 min and washed two times by resuspending in the original volume of isolation media and centrifuging to remove unincorporated iodide. Control solutions lacking lactoperoxidase routinely showed no incorporation of iodine. Examination of the platelets by electron microscopy after iodination demonstrated that this procedure did not induce any morphological alterations in the platelet.

Gel Electrophoresis. Isolated platelets and platelet plasma membrane fractions were prepared for electrophoresis by solubilizing in 3% sodium dodecyl sulfate. It was found essential to first make a suspension of the platelet fraction and then add 10% sodium dodecyl sulfate to a final concentration of 3% to obtain complete solubilization.These solutions were immediately immersed in a boiling bath for 5 min to minimize any effect of autolysis by platelet proteolytic enzymes which may be activated by solubilization. The samples were then frozen and stored at −20°. Immediately prior to electrophoresis, 100-μl samples containing 300 μg of protein were made to 1% mercaptoethanol. The solution was then immersed in a boiling bath for 3 min and electrophoresed on 5% gels containing 0.1% sodium dodecyl sulfate as previously described (Lenard, 1970). Identical gels were stained for protein with Coomassie Brilliant Blue according to Shapiro *et al.* (1967), and for carbohydrate by the fuchsin sulfate method (Clarke, 1964). The radioactive distribution in the gel was determined by slicing unstained gels laterally to 2.0-mm sections and counting the γ emissions in each slice.

Molecular Weight Determinations. The protein standards employed in these studies for molecular weight determinations were β-glucuronidase (130,000), phosphorylase *a* (94,000), bovine serum albumin (68,000), carbonic anhydrase (29,000), and myoglobin (17,600). These standards yielded a linear line when distance migrated was plotted *vs.* log molecular weight. The molecular weight of the iodinated membrane components was determined by applying 100-μg samples of the iodinated fractions and standard proteins to the same gel, and staining the gel for protein with Coomassie Brilliant Blue. The position of the standards was noted and the gel was sliced to determine the relative position of the iodinated components. This technique enables the molecular weight of the iodinated components to be determined from the same gel that contains the molecular weight standards and eliminates any difference in mobility between gels. Identical gels containing 300 μg of platelet sample which had been stained for protein and carbohydrate in the absence of standards were also sliced to confirm the molecular weights of the platelet polypeptides and glycoproteins.

Other Procedures. Protein concentrations were determined by the fluorescent method described by Fairbanks *et al.* (1971). Hydrogen peroxide concentrations were determined from the optical density at 230 nm using a molar extinction coefficient of 72.4. The concentration of lactoperoxidase was determined from the millimolar extinction of 114 at 412 nm (Morrison *et al.*, 1957).

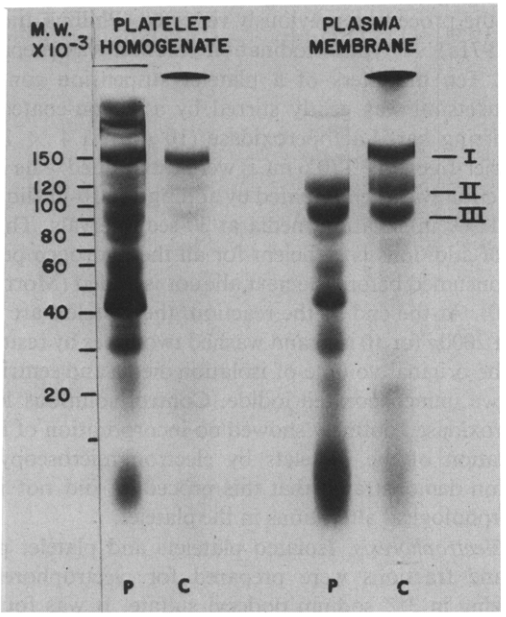

FIGURE 1: Separation of platelet membrane proteins and glycoproteins. Whole platelet homogenates and isolated membranes were solubilized in sodium dodecyl sulfate and electrophoresed as described in Methods. Identical gels were stained for protein (labeled P) and for carbohydrates (labeled C). Molecular weights were determined as described in Methods.

Results

Polypeptide and Glycoprotein Subunits of the Platelet Plasma Membrane. In Figure 1 are presented acrylamide gel electrophoretic patterns of polypeptides and glycoproteins for whole platelet homogenates and platelet plasma membranes. Gels labeled P have been stained for protein with Coomassie Brilliant Blue and those labeled C were stained for carbohydrate with fuchsin sulfate. These specific stains show heterogeneity of polypeptides in the platelet homogenate with molecular weights ranging from 13,000 to more than 200,000. Three major carbohydrate bands are also visible in this fraction with molecular weights corresponding to 150,000, 118,000, and 93,000, bands I, II, and III, respectively. A comparison of these gels to the plasma membrane gels shows gross differences in the protein composition of the two fractions. In contrast, the three glycoproteins in the isolated plasma membrane show a marked increase in concentration when compared to the platelet homogenate. It thus appears that these glycoproteins are membrane components and that they constitute the major glycoproteins in the platelet plasma membrane. The molecular weight of these glycoproteins was not affected by increasing the concentrations of sodium dodecyl sulfate or mercaptoethanol in the sample, or by increasing the time of incubation in sodium dodecyl sulfate prior to electrophoresis. A protein band that has the same apparent molecular weight as a carbohydrate band in this electrophoretic system does not necessarily mean that the two bands are due to the same material. A good example of this is the intense protein band at 150,000 and the highest molecular weight glycoprotein in the platelet homogenate (band I). Although these two bands share similar electrophoretic mobilities, they cannot be the same since band I glycoprotein is increased in concentration in the membrane fraction while the polypeptide of 150,000 is greatly decreased in concentration.

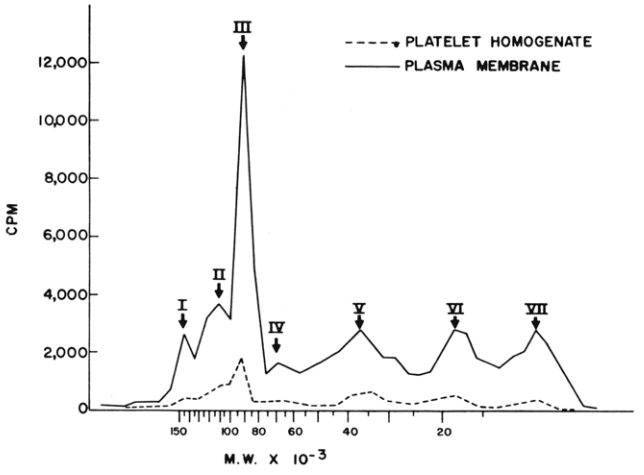

FIGURE 2: Distribution of iodine label in platelet plasma membrane polypeptides. Intact platelets were subjected to lactoperoxidase-catalyzed iodination and the plasma membranes isolated. The iodinated platelets and membranes were solubilized in 2–3% sodium dodecyl sulfate and electrophoresed on 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Each gel contains an equal amount of protein. The solid line represents the iodine distribution into the membrane polypeptides while the dotted line was obtained from the whole platelet homogenate.

Exposed Protein on the Platelet Plasma Membrane. The lactoperoxidase iodination technique was employed to iodinate proteins on the exposed surface of the platelet membrane. These results are presented in Figure 2. The dotted line represents the iodine distribution into the polypeptides of whole platelets after iodination by lactoperoxidase. The plasma membranes from iodinated platelets were also isolated and fractionated by disc gel electrophoresis, shown by the solid line in this figure. Almost a fivefold increase in specific activity of the membrane fraction is observed. The specific activities of the whole platelet homogenate and the isolated membrane were 24,000 and 102,000 cpm per mg of protein for the two fractions, respectively. The molecular weights of the iodinated polypeptides were determined by coelectrophoresing standard proteins of known molecular weight with radioactive samples as described in Methods. The three highest molecular weight components have molecular weights of 150,000, 118,000, and 93,000 and correspond to the molecular weights of the three major glycoproteins in the platelet membrane, bands I, II, and III. The most labeled component is the 93,000 glycoprotein. Four other labeled components are routinely observed which have molecular weights of 68,000, 34,000, 18,000, and 13,000, bands IV, V, VI, and VII, respectively, in Figure 2. These later components do not show a positive stain with fuchsin sulfate on these gels. A comparison of the two tracings in Figure 2 shows that the same labeled polypeptides and glycoproteins are present in the whole platelet homogenate and the plasma membrane fraction isolated from it. In addition, the labeled components are present in the same ratios in the two fractions.

Effect of Trypsin on Platelet Polypeptides and Glycoprotein. Figure 3 shows the susceptibility of platelet polypeptides and glycoproteins to trypsin hydrolysis. Comparison of the protein-stained gels shows that the only polypeptide that is decreased in concentration as a result of trypsin hydrolysis is the one with a molecular weight of 150,000. However, since this polypeptide is neither isolated with the plasma membrane fraction (Figure 1) nor iodinated by lactoperoxidase, it is probably derived from within the platelet. The carbohydrate stained gels, however, reveal that trypsin has a

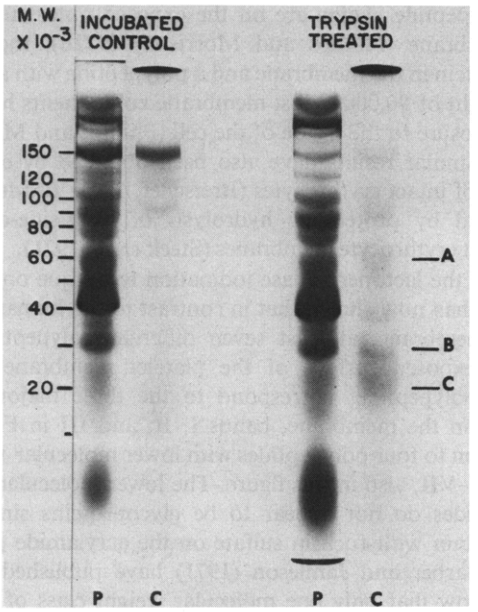

FIGURE 3: Separation of platelet proteins and glycoproteins from normal and trypsin-treated platelets. Trypsin-treated platelets were solubilized in sodium dodecyl sulfate and electrophoresed as described in Methods. Identical gels were stained for protein (labeled P) and for carbohydrate (labeled C). Incubated control gels represent the platelets incubated in the absence of trypsin. Molecular weights were determined as described in Methods.

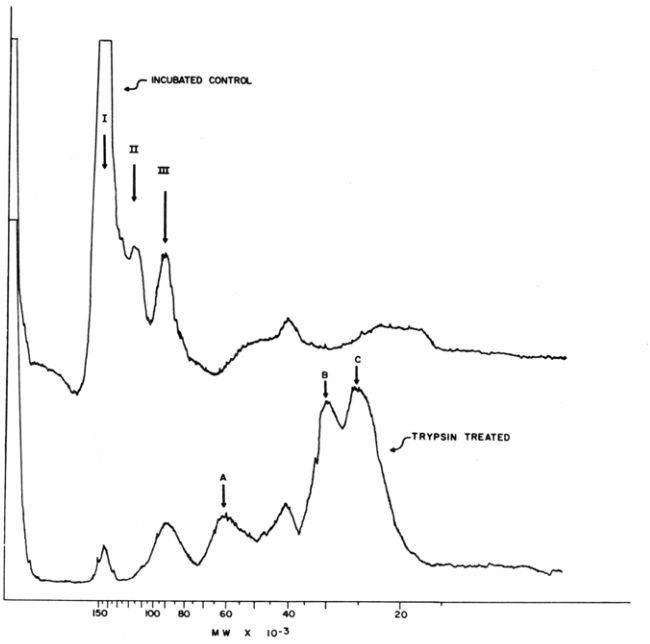

FIGURE 4: Densitometer tracings of glycoproteins from platelets incubated in the presence and absence of trypsin. The carbohydrate-stained gels from Figure 3 were scanned for density of fuchsin sulfate positive material.

greater effect than can be detected by the protein stain. Band II glycoprotein is completely removed while the 150,000 and 93,000 glycoproteins, bands I and III, respectively, are reduced in intensity. Concomitant to the decrease in concentrations of the three major glycoproteins is the appearance of three new glycopeptides with reduced molecular weights. The molecular weights of these glycopeptide hydrolytic products are 60,000, 31,000, and 26,000, bands A, B, and C, respectively. These trypsin-induced alterations in the glycoprotein composition of the platelet membrane are better illustrated by the densitometer tracings of the fuchsin sulfate stained gels, shown in Figure 4. The upper line shows the normal glycoprotein composition of the platelets, while the lower line demonstrates the alterations resulting from trypsin hydrolysis of membrane glycoproteins. These hydrolytic products, bands A, B, and C, are tightly bound to the platelet and cannot be removed by further washings.

Effect of Trypsin on the Surface Protein of the Platelet Membrane. Trypsin hydrolysis of platelets which have been iodinated does not result in the release of appreciable amounts of iodinated material. The specific activities of the iodinated platelets are 24,000 and 22,000 cpm per mg of protein before and after trypsin hydrolysis, respectively. Trypsin does, however, affect the molecular weights of the iodinated glycoproteins as is shown in Figure 5. The dotted line shows the normal distribution into the platelet polypeptides, as described in Figure 2. The solid line shows the effect of trypsin on these components. The iodinated glycoproteins, bands I, II, and III, are greatly reduced in concentration. This coincides with the reduction of fuchsin sulfate staining material with these molecular weights, shown in Figures 3 and 4. Concomitant with this loss in high molecular weight material is the appearance of three new molecular weight species which contain iodide. The molecular weights of these components are 60,000, 31,000, and 26,000, bands A, B, and C, respectively. The molecular weights of these iodinated hydrolytic products are coincident to the molecular weights of the newly generated glycopeptides shown in Figures 3 and 4.

Trypsin treatment prior to iodination greatly reduces the iodinatable sites on the membrane surface. The relative specific activities for platelets iodinated after incubation in the absence or presence of trypsin are 644,000 and 185,000 cpm per mg of protein, respectively. Incubated platelets routinely incorporated more iodine than platelets iodinated immediately upon isolation, even though the iodination patterns were qualitatively similar. No explanation for this observation is apparent at this time.

Platelets that have been iodinated after trypsin hydrolysis were also subjected to disc gel electrophoresis. The dotted

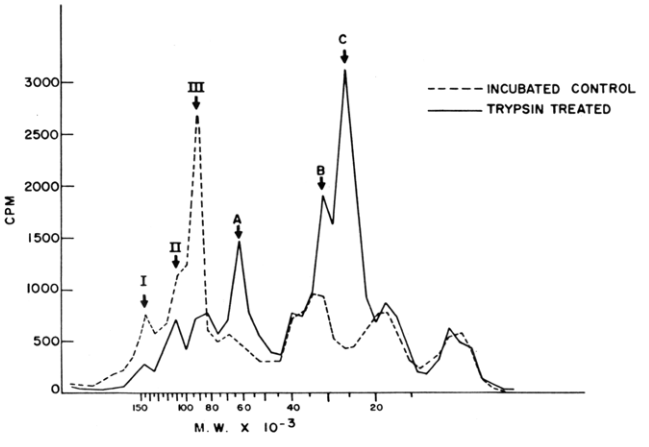

FIGURE 5: Effect of trypsin on iodinated platelets. Iodinated platelets were incubated either in isolation media or in isolation media containing trypsin, solubilized in sodium dodecyl sulfate, and electrophoresed on polyacrylamide gels as described in Methods. The dotted line is the control while the solid line represents the preparation after treatment with trypsin.

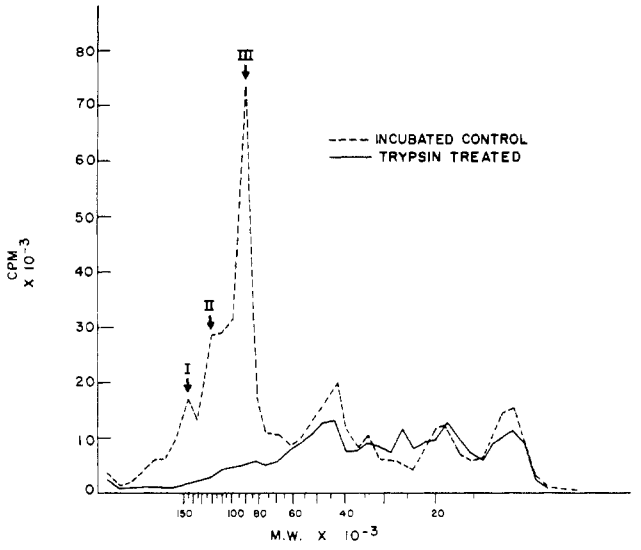

FIGURE 6: Effect of trypsin on the iodinatable sites on the platelet membrane surface. Platelets were incubated in the presence and absence of trypsin, iodinated by lactoperoxidase, solubilized in sodium dodecyl sulfate and electrophoresed on polyacrylamide gels. The iodine distribution into the platelet membrane polypeptides from the incubated control is shown by the dotted line while the solid line was obtained from trypsin-treated platelets.

line in Figure 6 is the control, *i.e.*, lactoperoxidase-catalyzed iodination of platelets which were incubated in the absence of trypsin. The solid line is the pattern obtained when trypsin-treated platelets are iodinated. Bands, I, II, and III, the three glycoproteins, have been reduced in concentration by the trypsin hydrolysis, and indeed are no longer present to be iodinated. Bands IV–VII, however, were not affected by trypsin hydrolysis (Figure 5) and are still in an iodinatable position on the platelet membrane. Most importantly, however, is the observation that the trypsin products, glycopeptides A, B, and C, are no longer in an iodinatable position on the membrane. Only band C appears to incorporate a significant amount of the label.

Discussion

The present results show that the platelet plasma membrane is composed of polypeptides of molecular weights varying from more than 200,000–13,000. There are also three major glycoproteins in this membrane (I, II, and III in Figure 1), with molecular weights of 150,000, 118,000, and 92,000, respectively. It has been shown that molecular weight determinations of glycoproteins by disc gel electrophoresis in sodium dodecyl sulfate can be erroneous with the apparent molecular weight decreasing with increasing cross-linking of the gel (Segrest *et al.*, 1971). The molecular weights determined by this system must therefore be considered as an upper limit for their true molecular weight, even though significant alterations were not observed when 10% gels were employed. The presence of these glycoproteins in the platelet membrane has not been previously demonstrated, and it appears that they constitute the major glycoproteins in the platelet plasma membrane.

The lactoperoxidase iodination technique employed in this study specifically iodinates only those plasma membrane proteins which exist on the exposed surface. Initial studies employing this technique on the human erythrocyte membrane have demonstrated the asymmetry of this membrane. Only two polypeptide chains are on the exposed outer surface of the membrane (Phillips and Morrison, 1971b), the major glycoprotein in the membrane and a polypeptide with a molecular weight of 90,000. Most membrane components however, have exposure to the inside of the cell (Phillips and Morrison, 1971a). Similar results have also been obtained by chemical labeling of intact erythrocytes (Bretscher, 1971; Bender *et al.*, 1971) and by proteolytic hydrolysis of right-side-out and inside-out erythrocyte membranes (Steck *et al.*, 1971).

Use of the lactoperoxidase iodination technique on human platelets has now shown that in contrast to the human erythrocyte membrane, at least seven different polypeptides are on the exposed surface of the platelet membrane. These labeled polypeptides correspond to the three major glycoproteins in the membrane, bands I, II, and III in Figure 2, in addition to four polypeptides with lower molecular weights, bands IV–VII, also in this figure. The lower molecular weight polypeptides do not appear to be glycoproteins since they do not stain with fuchsin sulfate on the acrylamide gel. Recently, Barber and Jamieson (1971) have published results which show that only one molecular weight class of protein with an unreported molecular weight was labeled when human platelets were subjected to lactoperoxidase-catalyzed iodination. The results reported here show that at least seven molecular weight classes are labeled. The source of the difference between these results and those published by Barber and Jamieson is not clear. A likely explanation can be found by examining the specific activities of the iodinated platelet membrane fractions. Gel slices analyzed by Barber and Jamieson contained 110 cpm/slice in the most radioactive slices, while in the present study up to 12,000 cpm/slice were observed. The increased specific activity employed here might enable more sensitive analysis of labeled membrane components.

The difference in the protein composition on the surfaces of the human erythrocyte and platelet membranes as determined by the lactoperoxidase iodination technique is striking. Functional groups observed on the human erythrocyte membrane surface are limited. Only the antigenic properties of glycoprotein (Winzler, 1969) and the activity of acetylcholinesterase (Firkin *et al.*, 1963; Herz *et al.*, 1963) have clearly been ascribed to this membrane surface. The other activities which have been examined appear to be localized on the membrane's inner surface (Green *et al.*, 1965; Nilsson and Ronquist, 1969; Marchesi and Palade, 1967). The outer surface of the platelet membrane, however, performs many more functions. In addition to antigenic properties and acetylcholinesterase activity (Saba and Mason, 1970), platelets have an "ecto" ATPase (Chambers *et al.*, 1967), a surface to enhance coagulation (Marcus, 1965), glycosyl transferase activity (Jamieson *et al.*, 1971; Bosmann, 1971), glycosidases (Bosmann, 1972), and factors involved in aggregation which may be some of the above. The finding that lactoperoxidase will label a wide spectrum of glycoproteins and polypeptide chains on the platelet, and only two on the erythrocyte, is consistent with the difference in the number of functions performed by the two membrane surfaces.

Membrane isolation techniques have been developed for many cell types and intracellular organelles. Often it is difficult to assess the purity of the isolated membrane fraction since a suitable assay is not readily apparent. In the present study, however, the results show that the lactoperoxidase iodination technique, when used on intact cells, can be used to evaluate purity of the isolated plasma membrane fraction (Morrison and Gates, 1972). All iodinated components

in the intact platelet were isolated in the membrane fraction and in the same ratio as that present in the intact platelet. This demonstrates that surface proteins are not selectively removed when plasma membranes are isolated by this method. The increased specific activity of the membrane fraction over the platelet homogenate (almost fivefold) agrees favorably with enzyme markers previously assayed for this membrane preparation (Barber and Jamieson, 1971).

The proteolytic activity of trypsin has been employed to mimic thrombin in many aggregation studies. The alterations induced on the membrane surface by this enzyme have been the subject of several investigations. Behnke has shown that trypsin will remove ruthenium red-stainable material from the membrane surface but that it has no effect on colloidal iron and Thorotrast staining (Behnke, 1968). It has also been demonstrated that proteolysis will allow platelets to be more closely packed by centrifugation (Hovig, 1962). These observations indicate that material is being removed from the membrane surface. Pepper and Jamieson have examined the material released by trypsin and have isolated three glycopeptide fragments with reported molecular weights of 120,000, 20,000, and 5000. From the nature of these products it appears that each glycopeptide fragment released is derived from a different glycoprotein on the membrane surface (Pepper and Jamieson, 1970).

The present studies define more clearly the membrane alterations induced by trypsin. Of the seven surface components labeled by lactoperoxidase, only the three glycoproteins are hydrolyzed by trypsin, while the four polypeptides are unaffected. Since this result was obtained when all surface proteins that have exposed tyrosine were labeled, it strongly suggests that the primary target on the membrane for proteolytic enzymes is glycoprotein.

The polypeptide with a molecular weight of 150,000 that is decreased in concentration by trypsin has a striking resemblance to thrombin-sensitive protein described by Baenziger *et al.* (1971). This polypeptide, however, does not appear to be a plasma membrane component since it is not isolated with the membrane fraction. It is possible that this component could be weakly bound to the membrane surface, accessible to proteolytic hydrolysis, and removed by membrane isolation. If this occurred, this polypeptide should be readily iodinated by lactoperoxidase. The finding that all iodinated components are isolated with the membrane fraction while the 150,000 polypeptide is not, however, demonstrates that it is not iodinated by lactoperoxidase. It therefore appears that this polypeptide is an intracellular component and that it is released from within the platelet in response to glycoprotein hydrolysis on the membrane surface.

The iodinated tyrosine residues on the three major glycoproteins are on the membrane side of the trypsin-sensitive bond. This conclusion is obtained from two observations. First, the specific activity of iodinated platelets is virtually unaltered by trypsin treatment, even though the three major glycoproteins have been hydrolyzed and glycopeptides have been released from the platelet surface. Second, the loss of radioactivity from the molecular weight region of bands I, II, and III is retained on the platelet in the form of glycopeptide hydrolytic products. Since this hydrolysis results in the loss of ruthenium red-staining material from the membrane surface (Behnke, 1968), it appears likely that the glycopeptides released from the membrane surface by trypsin may constitute in part the fuzzy coat of the membrane while the fragment remaining which contains iodine is used to anchor the glycoprotein to the membrane.

Assuming that each glycoprotein gives rise to a single glycopeptide after trypsin hydrolysis, one can arrive at the following relationships. Glycoprotein I gives rise to glycopeptide B; glycoprotein II gives rise to glycopeptide A, and glycoprotein III gives rise to glycopeptide C. These relationships are based on the following observations. Band C glycopeptide in Figure 5 contains approximately the same amount of radioactivity as band III glycoprotein, suggesting that the former is derived from the latter. The maximum size of a hydrolytic product that could be released would therefore be 119,000 (subtracting band B, 31,000 from band I, 150,000). Since a glycopeptide of 120,000 has been observed after trypsin hydrolysis of platelets (Pepper and Jamieson, 1970), it would thus appear that this glycopeptide is derived from glycoprotein I. Glycopeptide B is the part of this glycoprotein that remains with the platelet. Glycopeptide A would therefore be obtained from glycoprotein II.

The results on the iodination of trypsin-treated platelets indicate that proteolysis induces a conformational change in the platelet membrane. The iodinatable portion of the glycoproteins remains with the membrane after trypsin treatment; however, when the iodination reaction is initiated after proteolysis, the iodinatable residue is no longer accessible to lactoperoxidase. It thus appears that the glycopeptide fragments that remain with the membrane have undergone a change in orientation or that proteolysis has induced other membrane components to alter the accessibility of the glycopeptides. Changes in the surface properties of the platelet plasma membrane after proteolysis have been shown to occur by other techniques. The alterations in ruthenium red-staining material (Behnke, 1968) and the tighter packing of platelets after trypsin treatment (Hovig, 1962) have been previously discussed. Trypsin hydrolysis also results in decreased sialic acid on the membrane surface (Barber and Jamieson, 1971). Most importantly, trypsin-treated platelets are "sticky" and will aggregate (Davey and Lüscher, 1967). These effects have now been correlated directly to a conformational change in the membrane. Studies are now in progress to determine what effect this conformational change, *i.e.*, glycoprotein accessibility on the membrane surface, has on the aggregation process.

Acknowledgments

I acknowledge the superb technical assistance of Miss Patricia Poh and Mrs. Joan Metcalfe. In addition, I would like to thank Dr. Harold Edwards for electron micrographs of iodinated platelets. Finally, special thanks are due to Dr. Martin Morrison for advice and encouragement during the course of this work.

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Denaturation of Rat Liver Ribosomal Ribonucleic Acid with Dimethyl Sulfoxide†

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ABSTRACT: Dimethyl sulfoxide is an effective denaturing agent for rRNA. However, this solvent can also promote *aggregation* of RNA, presumably at the time its concentration is lowered. The phenomenon has been investigated and this has led to the development of empirical methods which circumvent the problem.

High molecular weight RNA molecules, such as rRNA, possess some degree of secondary and tertiary structure which is stabilized by hydrogen bonds and by base stacking and other less well-defined interactions (Spirin, 1963; Cox, 1970; Attardi and Amaldi, 1970). As well as influencing the sedimentation and electrophoretic properties of an RNA molecule, these forces may hold together separate polynucleotide chains (*e.g.*, the replicative form of RNA viruses) or fragments of a larger molecule which arise due to "hidden" breaks. It has been possible to disrupt noncovalent bonds and dissociate chains and fragments using high concentrations of

† From the Biology and Health Physics Division, Atomic Energy of Canada Limited, Chalk River, Ontario, Canada. *Received May 9, 1972.*