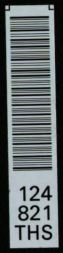
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DETERMINATION OF EXTERNAL MEMBRANE PROTEINS IN RESTING AND STIMULATED PLATELETS: A Study Designed to Determine if Actin and/or Myosin Are External Components

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Manfred L. Schwarz

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Joan C. Mattson, DVM
Major professor

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DETERMINATION OF EXTERNAL MEMBRANE PROTEINS IN RESTING AND STIMULATED PLATELETS:

A Study Designed to Determine if Actin and/or Myosin Are External Components

By

Manfred L. Schwarz

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ABSTRACT

DETERMINATION OF EXTERNAL MEMBRANE PROTEINS IN

RESTING AND STIMULATED PLATELETS:

A Study Designed to Determine if Actin and/or Myosin

Are External Components

By

Manfred L. Schwarz

The localization of contractile proteins in non-muscle cells is necessary if we are to understand how the contractile proteins function in these cells. Although there is agreement on the presence of the contractile proteins actin and myosin inside of cells and possibly also on the inside of the plasma membrane, there is much controversy about the external location of these proteins. There has been considerable interest in the contractile proteins of platelets since the contractile proteins appear to play a major role in the functions of this important blood cell.

Using ^{125}I lactoperoxidase labeling of intact platelets the external proteins can be radioactively labeled. The separation of the platelet proteins by molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) helps in identifying actin and myosin. With this technique it was found that platelet myosin was not radioactively labeled although actin was, indicating that myosin may not be an external component of the platelet membrane while actin is. The isotopic labeling of external actin appears to increase if the platelets are weakly activated with adenosine diphosphate (ADP).

DEDICATION

To my family, especially my parents whose support made my graduate studies possible.

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INTRODUCTION

The platelet is the major element in the blood responsible for the primary arrest of bleeding. The unique properties of platelets which allow them to adhere to surfaces, aggregate with each other, and contract are necessary for their hemostatic function (1). When platelets do not stick to each other (aggregation) or to damaged vessels (adhesion) bleeding results. The precise mechanism of platelet adhesion and aggregation is not known.

To propose an accurate mechanism of platelet adhesion and aggregation the structure of the platelet must be better understood. This is particularly true of the platelet membrane. The platelet plasma membrane is the primary site of stimulus recognition and therefore plays a central role in hemostatic processes involving the platelet. It is probable that many of the unique specialized functions of platelets are intimately related to specific plasma-oriented phenomena. Thus a more precise insight into the architectural biochemistry of the platelet surface may help clarify the physiological mechanisms involved in hemostasis and the pathological aberrations associated with early thrombotic disease (2).

During hemostatic processes platelets change shape, secrete intracellular contents (3), and induce retraction of fibrin clots. These are contractile, energy requiring processes attributed to the contractile proteins actin and myosin (4). Platelets contain large quantities of these contractile proteins compared to other

non-muscle cells. The contractile protein myosin is of particular interest since it is the energy transducing enzyme of the cellular contractile system.

Efforts to localize myosin in platelets have not been successful. The presence of myosin on the surface of the platelet is disputed. Since the surface of the platelet plays such a major role in platelet function further evidence on the surface location of myosin would be of great importance.

LITERATURE REVIEW

The contractile proteins are composed mainly of actin and myosin. Actin is a small (45,000 molecular weight) globular protein that can polymerize to form long filaments. Myosin is a protein composed of a globular head possessing actin binding and ATPase activity, and a filamentous rod region. These proteins are arranged in skeletal muscle so that the myosin can convert the chemical energy of ATP into mechanical energy by pulling on the actin filaments. Two accessory proteins, tropomyosin and troponin are responsible for regulating the contractile action primarily by calcium ion regulation (5). In striated muscle the arrangement of actin and myosin is regulated and accounts for the tremendous contractile force that can be generated by these muscles (6). Non-muscle cells such as WBC's, fibroblasts, brain cells, platelets, and others also have actin and myosin present. Platelets have a particularly high content of these proteins with actin making up 20% and myosin 1.5% of the total platelet proteins (4). These proteins were first discovered in the platelet by Bettex-Galland and Lüscher in 1959 (7) while investigating the possibility that clot retraction might be the result of a muscle-like substance. Their isolation of actomyosin was rather crude and they termed it thrombosthenin. They demonstrated that this protein complex could be separated into thrombosthenin M, a myosin-like fraction, and thrombosthenin A, an actin-like fraction. Thrombosthenin was believed to be responsible for the contractile activities of the

platelet such as pseudopod formation, cell movement, phagocytic activity, fluid transport, expulsion of materials from platelets, clot retraction, and platelet aggregation (4, 8).

Determining the arrangement of actin and myosin in the muscle cell was necessary to understanding contraction in these cells. Similarly, determination of the arrangement of contractile proteins in the platelet will also be necessary in order to understand how these contractile proteins function in non-muscle cells. The contractile proteins of non-muscle cells are often compared with those of muscle cells even though the muscle cell is highly specialized, containing contractile proteins that are also highly specialized. Even so, there are many similarities in the contractile proteins of muscle and those found in the platelet. Both contain myosin that has actin activated ATPase activity and bind actin, although the molecular weights and amino acid sequences are slightly different. Both contain the regulatory proteins troponin and tropomyosin (4) although the role of these accessory proteins in regulating contraction has not been established in the platelet.

Actin Localization

There are three major techniques used to identify actin in a cell. The heavy meromyosin labeling technique has been widely used for the identification of actin filaments. This technique utilizes heavy meromyosin, a trypsin digest of myosin containing the head or globular region and part of the rod or filamentous region. Since the globular head of myosin binds to actin in a specific way, actin filaments can be identified by electron microscopy by the typical "arrowhead" distribution of the heavy meromyosin along the actin

filaments. The major drawback with this procedure is the need to glycerinate the cells to facilitate entry of heavy meromyosin into the cell. The actin filaments in some cells do not survive glycerination well (5).

Another procedure used to identify actin is by immunofluorescence techniques (9). A possible advantage of these techniques is that they can reveal monomers or small oligomers of actin in a cell which would be missed by both electron microscopy where only fully polymerized filaments can be visualized, and heavy meromyosin staining where polymerized filaments are required for heavy meromyosin binding.

A third approach is the isolation of the various cell fractions and the determination of the content of actin in these fractions. The problem with this approach is the possibility of actin contamination of cell fractions during cell disruption or fractionation.

Using all of these techniques it has been shown that actin in non-muscle cells lack the highly organized distribution seen in muscle cells. Actin is believed to make up the 60 Å microfilaments of the platelet. These microfilaments are found only in activated platelets; they are present around the canaliculi or open channel system which connects to the platelet surface. Platelets and certain other non-muscle cells have some microfilaments in the cortical region just inside the plasma membrane. A bundle of these cortical actin filaments extend into the microvilli on the surface of gut epithelial cells as well as into the pseudopodia of platelets where they appear to support these thin processes (10). Actin filaments appear to be attached to the plasma membrane in many cells. Although

the function of this plasma membrane attachment has not been proven, it is possible that at the sites where the plasma membrane makes contact with other cells or with substrate, the filaments associated with the membrane would serve as an anchor against which the contractile apparatus could exert force for movement (5).

Perdue (11) has presented ultrastructural evidence for the idea that actin filaments may even penetrate the plasma membrane of fibroblasts. Bouvier et al. (9) using autoimmune anti-actin antibodies found no binding of these antibodies to resting platelets as seen by indirect immunofluorescence. Incubation of ADP stimulated platelets with anti-actin antibody showed small but definite binding. This suggests either increased plasma membrane permeability with leakage of actin into the surrounding medium or unmasking actin at the surface of the cell allowing the binding of anti-actin antibodies to the plasma membrane.

Myosin Localization

Little is known about the intracellular distribution of myosin in non-muscle cells compared with the progress that has been made on the localization of cytoplasmic actin. In most cells demonstrable myosin filaments are either absent or not preserved by fixation, so only actin filaments are usually identified.

Nachman et al. (12) extracted thrombosthenin (actomyosin) from isolated platelets and from purified platelet membranes, suggesting that actomyosin might be present in the membrane as well as the cytoplasm. However he explains that actomyosin made available after platelet homogenization could denature, precipitate and nonspecifically sediment in the sucrose gradient. Chambers et al. (13)

indicated that the properties of an "ecto-ATPase" (e.g., membrane ATPase) of intact platelets was very similar if not identical to platelet thrombosthenin. This "ecto-ATPase" was competitively inhibited by ADP. Since ADP also causes platelet aggregation, externally located thrombosthenin could play a role in the regulation of ADP aggregation.

Another approach to the localization of myosin is the use of specific antisera. Salzman et al. (14) showed that thrombosthenin antisera inhibited platelet "ecto-ATPase" activity. Nachman et al. (12) also made an antibody to thrombosthenin. They found that it inhibited the ATPase of actomyosin and inhibited clot retraction. Booyse and Rafelson (8) developed a contractile model for platelet aggregation using the ATPase inhibitory effect of ADP. To prove this theory it was essential that actomyosin be found on the platelet surface. To verify this they used an unlabeled antibody peroxidase staining technique to localize actomyosin in both intact and ultrathin sections of platelets. The antibody staining of the ultrathin sections showed membrane associated and cytoplasmic actomyosin. Antibody staining of intact cells showed staining on the external coat of the platelet (15). While this study showed that the antisera reacted with platelet actomyosin, it did not exclude that the antisera also contained antibodies directed against platelet membrane components.

Puszkin et al. (16) produced an antibody to platelet myosin rod. With this antisera they were able to inhibit clot retraction, aggregation with ADP, epinephrine, and collagen, and to induce shape change. The antisera did not inhibit ATPase activity of platelet

actomyosin. Platelets take up serotonin from the plasma and release it when they are activated. Platelets incubated with (^{14}C) serotonin and antisera showed inhibition of (^{14}C) serotonin release after exposure to epinephrine or collagen. Indirect immunofluorescent staining of the platelet surface by anti-platelet myosin rod was positive but not very intense even with high concentrations of antibody.

Fugiwara and Pollard (18) using antimyosin rod antibody observed staining of the cytoplasm but no fluorescent staining of the platelet surface. They explain the difference in their results from previous antimyosin work mainly on the basis of purer antisera. The antigen used by Fugiwara and Pollard was chromatographically purified human platelet myosin as compared to the antigen prepared by Puszkin et al. (16) in which the preparatory steps stopped short of chromatography. Fugiwara and Pollard postulate that contamination by plasma membrane components, which may not be detected by polyacrylamide gel electrophoresis stained with protein dye, are removed by KI-gel filtration on 4% agarose. The presence of plasma membrane fragments may elicit antibodies against membrane components. To avoid a response to the small amount of low molecular weight contaminants that could not be completely removed, the antigen was injected in small amounts in adjuvant at multiple sites. Pollard et al. (12), in contrast with the results of Puszkin et al. (16) found that platelet antimyosin rod did not effect ADP-induced aggregation and the release of (^{14}C) serotonin was not inhibited by the antisera after ADP activation of the platelet.

MATERIALS AND METHODS

Ficoll-Hypaque Separation of Platelets

All plastic or siliconized labware is utilized during the isolation of platelets to prevent glass activation. All steps are performed at room temperature. Ten milliliters of whole blood is collected in 1.6 ml of ACD. The acid citrate dextrose (ACD) solution contains 2.5 g of Na_3 citrate · $2H_2O$, 2 g of glucose, and 1.5 g of citric acid in 100 ml of H_2O. The platelets are separated from cellular constituents by a modification of the ficoll separation (29) (Sigma Company Bulletin). Four milliliters of a mixture of equal volumes whole blood and balanced salt solution containing 0.1% D glucose, 5 μM $CaCl_2$, 0.098 mM $MgCl_2$, 0.54 mM KCl, 0.02 M Tris, pH 7.3, and 0.8% NaCl, are carefully layered on ficoll-hypaque (Sigma Chemical Co., St. Louis, MO). The tubes are centrifuged in a swinging bucket at 200 x g for 10 minutes. The upper platelet layer is removed leaving about 2 mm of plasma above the ficoll-hypaque layer. A sample of this suspension is checked in a counting chamber to ensure that contamination by leukocytes has not occurred. The platelets are sedimented by centrifugation at 2000 x g for 10 minutes. The supernatant is removed and the pellet is resuspended with gentle pipette aspiration in 10 ml of isolation media. This media consists of 0.9% NaCl and 0.3% citrate, pH 6.5. This wash is repeated one more time to remove plasma proteins. Platelets are pelleted at 2000 x g and suspended in the appropriate solution to give 1 x 10^9 platelets/ml.

The resulting solution is better than 99.9% platelets as determined by light microscopy.

Iodination of Platelets

The method of Phillips and Poh Agin (28) is used for the $Na^{125}I$ lactoperoxidase iodination of whole platelets. All reactions are carried out at room temperature. The platelets are isolated by ficoll-hypaque as previously described. One-half milliliter of 1×10^9 platelets/ml in a suspension buffer consisting of 0.01 M Tris, 0.9% NaCl, 0.3% Na_3 citrate, pH 7.4, or an EDTA suspension buffer (0.001 M EDTA is substituted for 0.3% Na_3 citrate) is placed in a siliconized tube. One-half millicurie of ^{125}I (carrier-free, Schwarz) is added to the stirring platelets, followed by the addition of 5 µl of 0.1 M NaH_2PO_4, pH 7.4, containing 2.5 nM of lactoperoxidase (Sigma Chemical Co.). The iodination was initiated by adding 5 aliquots (5 µl each) of freshly prepared hydrogen peroxide solution (1 mM hydrogen peroxide, Sigma Chemical Co., in Na_3 citrate or EDTA suspension buffer) at 10 second intervals. The platelets are washed 2 times with 10 ml of the appropriate suspension buffer and pelleted by centrifugation at 800 x g for 10 minutes. The platelets are then rapidly suspended in 0.2 ml of deaired water and solubilized with 0.2 ml SDS sample buffer containing 2% SDS, 10 mM tris-glycine, 2% 2-mercaptoethanol, 20% glycerol, and .17 mg/ml Pyronin Y (Eastman Kodak Co., Rochester, NY).

SDS Polyacrylamide Gel Electrophoresis

The SDS polyacrylamide gel electrophoresis is performed on 10% acrylamide (Bio-Rad Laboratories, Richmond, CA) disc gels according to the method of Porzio and Pearson (27) with the following

modifications. Samples are solubilized by mixing equal parts sample and sample buffer and heating in a boiling water bath 1 to 3 minutes. Protein samples (40-100 µg in up to 100 µl) are loaded on the gel and entry of the gel initiated by applying a current of 1 mA per gel. After the dye has completely entered the gel the current is increased to 0.4 watts per gel tube until the dye front is within 3 mm of the end of the tube. After electrophoresis is complete the gel is removed from its tube and the dye front is marked by stabbing with a needle dipped in India ink. The gels are fixed for several hours in a solution of 25% isopropanol, 10% acetic acid, and 0.03% Coomassie Brilliant Blue R250 (Sigma Chemical Co.). The gels are destained in 10% acetic acid, 5% methanol until the background is clear. Non-radioactive gels are stored in 7.5% acetic acid at 4 C in the dark. Radioactive gels are processed for autoradiography or gamma counting.

Molecular Weight Determination

Coomassie Brilliant Blue stained proteins on polyacrylamide gels are scanned on an ISCO gel scanner (Instrument Specialities Co., Lincoln, NE) at 546 nanometers. The relative mobilities of the individual proteins are calculated from the scans as:

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}} \quad (19)$$

Protein standards employed in the molecular weight determinations are phosphoraylase a (93,000 daltons), bovine albumin (68,000 daltons), alcohol dehydrogenase (37,000 daltons), and rabbit skeletal muscle myosin (200,000 daltons). These standards yield a straight line when mobility is plotted against the log of the molecular weight (Figure 2).

Autoradiography

One millimeter thick slices through the length of stained polyacrylamide disc gels are dried onto filter paper under vacuum and heated over boiling water bath using a slab gel drying apparatus (Bio-Rad Laboratories, Richmond, CA). The dried gels are stored next to Kodak NS-5T x-ray film for 1 to 3 weeks. The exposed film is developed for 5 minutes with Kodak developer-replenisher (146-5327) rinsed 1 minute with filtered water at 68 F, fixed 4 minutes with Kodak fixer (146-4106), washed with water for 1 hour, and air dried.

Radiolabeling patterns are also determined by slicing frozen stained gels into 1 mm discs with an electrophoresis gel slicer (Bio-Rad Laboratories, Richmond, CA). The individual sections are counted on a gamma spectrophotometer (Biogamma II, Beckman Instruments, Irvine, CA). The slice containing actin or myosin, determined by migration and staining, is recorded before counting to serve as a check on the molecular weight determinations and give a more precise location of these proteins in the isotopic labeling pattern.

Lactic Dehydrogenase

Lactic dehydrogenase determinations were performed on the supernatant from washed platelets, washed platelets following iodination, and from platelets lysed in 0.2% Triton X-100 (Research Products International, Elk Grove Village, IL).

Lactic dehydrogenase catalyses the reaction:

$$\text{Lactate} + NAD^+ \rightleftharpoons \text{Pyruvate} + NADH + H^+$$

The equilibrium is far to the side of the nicotinamide adenine dinucleotide (NAD). The LDH activity is measured by the rate of

consumption of pyruvate and reduced NADH. The oxidation of NADH causes a decrease in the optical density at 340 nanometers.

In the procedure by Bergeyer et al. (26), 0.1 ml of sample is added to 2.9 ml of a solution containing 0.05 M phosphate buffer, pH 7.5, 0.31 mM Na_3 pyruvate, and 8 mM NADH (Sigma Chemical Co.). The change in optical density at room temperature is recorded in half minute intervals for at least 3 minutes. The LDH is calculated in Worblewski units (ΔE_{340}/min. x 10,000 = LDH units/ml sample).

Adenosine Diphosphate Platelet Activation

One-half milliliter of 3.8% Na_3 citrate was added to 4.5 ml whole blood. The platelets were separated by ficoll-hypaque. A 0.5 ml sample containing 150,000 platelets/mm^3 of ficoll-hypaque separated platelets in a plasma-balanced salt solution were activated by the addition of 0.02 ml 2.5 x 10^{-5} M ADP. The aggregation reaction was recorded on a Chrono-Log aggregometer (Chrono-Log Corporation, Bromall, PA) while stirring at 37 C for 3 minutes. One-hundred-eighty microliters of 2.5 x 10^{-5} M ADP was added to a 4.5 ml aliquot of platelets from which the recorded sample was taken. This platelet suspension was washed 2 times in isolation media as previously described and finally resuspended to give approximately 0.5 ml of platelets at a concentration of 1 x 10^9 platelets/ml. The activated platelets were then iodinated as previously described.

Coelectrophoresis with Purified Myosin

To a 15 µl (20 µg) sample of normal platelets, solubilized in SDS sample buffer, is added 75 µl (10 µg) purified human platelet myosin (gift from Joan Mattson). The mixture is then electrophoresed as previously described.

RESULTS

Identification of Actin and Myosin

Actin and myosin were identified in the SDS gels of whole platelet homogenates by their co-migration with purified human platelet actin and myosin standards (gift from Joan Mattson) (Figure 3). The molecular weight of human platelet actin was calculated to be 41,000 ± 1,000 daltons by its relative motility in SDS gels (Figure 2). Scans of gels showed that actin was the major platelet protein present in homogenates of whole platelets (Figure 4 and 5). Myosin in SDS-PAGE is reduced by 2-mercaptoethanol into its heavy and light chain components (Figure 3). Human platelet myosin heavy chains were calculated to have a molecular weight of 196,000 ± 11,000 and the two myosin light chains, 22,000 dalton and 16,000 dalton molecular weights (Figure 2).

Since there are numerous protein bands present in the high molecular weight region in SDS gels of platelet homogenates, an enhancement study was performed to further document that the 196,000 molecular weight band was myosin heavy chain. The addition of purified myosin to whole platelet homogenates enhanced the 196,000 dalton band but not other higher molecular weight bands, substantiating that this band is comprised of myosin heavy chain (Figure 1).

Effect of EDTA on Platelet Surface Proteins

Ethylenediamine-tetraacetic acid (EDTA) is commonly used in the preparation of platelets for radioiodination (28, 30). Based on

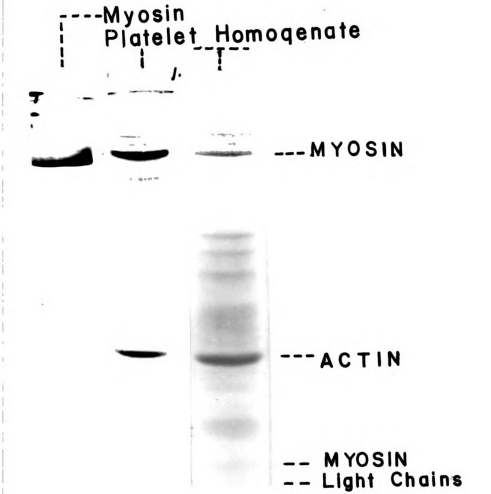


Figure 1. Identification of myosin by enhancement of myosin heavy chains in whole platelet homogenates as detected by SDS-PAGE.

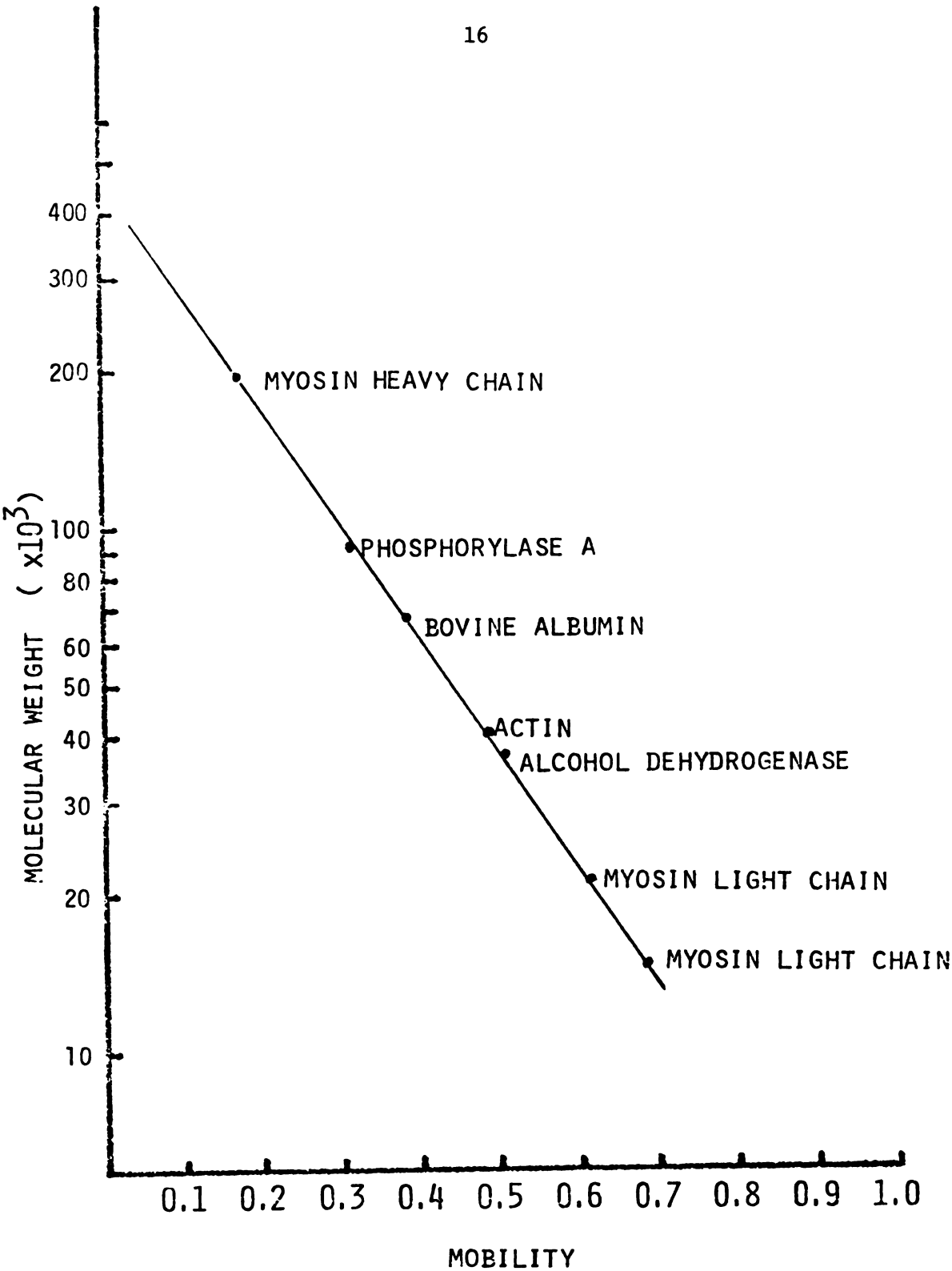


Figure 2. Molecular weight determination by relative motility in 10% SDS-PAGE. Comigration of myosin and actin in whole platelet homogenates with purified myosin and actin standards.

Figure 3. Comigration of myosin and actin in whole platelet homogenates with purified myosin and actin standards.

evidence that the contractile proteins can be dissociated from the cell membrane in the presence of EDTA (15) experiments were performed to see if EDTA causes loss of proteins from the outer face of the platelet membrane.

Washing platelets with EDTA altered at least 2 bands seen on SDS-PAGE. A protein band of 180,000 molecular weight, only seen when an excessive amount of sample (160 µg), was applied to gels, appeared diminished in the presence of EDTA (Figure 4). A second protein of 69,000 molecular weight appeared slightly enhanced in the presence of EDTA. This protein was best seen with less sample (80 µg) (Figure 5). There appeared to be no difference in the quantity of actin or myosin present as determined by peak heights in gel scans, of EDTA or Na_3 citrate prepared platelet homogenates. Based on the evidence from these studies that EDTA causes loss of a major membrane protein, all latter studies were performed with sodium citrate instead of EDTA.

No difference was observed in the ^{125}I lactoperoxidase labeled proteins on autoradiographs of EDTA or Na_3 citrate washed platelets (Figure 6). Less sample was used in the autoradiograph of EDTA washed platelets and therefore less radioactivity is detected. In the unactivated platelet autoradiograph of Figure 6, numerous labeled proteins can be identified. A protein of 255,000 MW was strongly labeled. A protein of 215,000 MW slightly larger than myosin heavy chain 196,000 MW was labeled but no radioactivity was seen in the myosin heavy chain. Glycoproteins I, II, and III of molecular weights of 150,000, 118,000, and 92,000 daltons respectively were labeled. Glycoprotein III has the most activity seen on the autoradiograph.

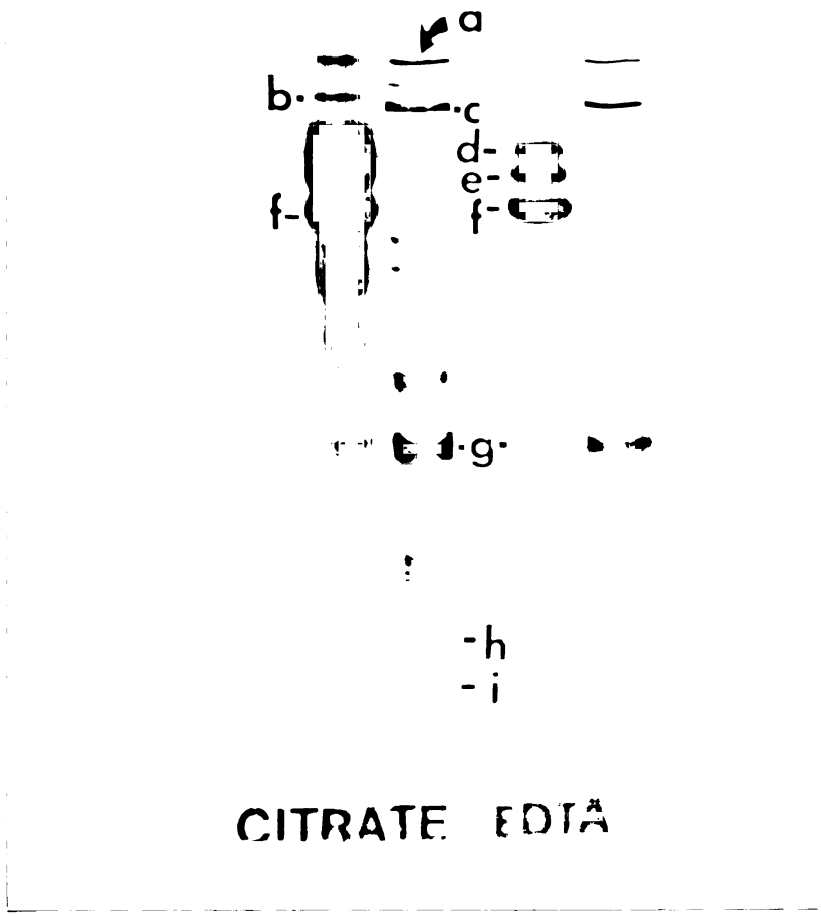


Figure 4. Determination of platelet proteins in the presence and absence of EDTA as determined by scans of Coomassie Blue stained SDS-polyacrylamide gels. 160 µg total platelet protein. a, 180,000 MW; b, myosin heavy chain; c, 69,000 MW; d, actin.

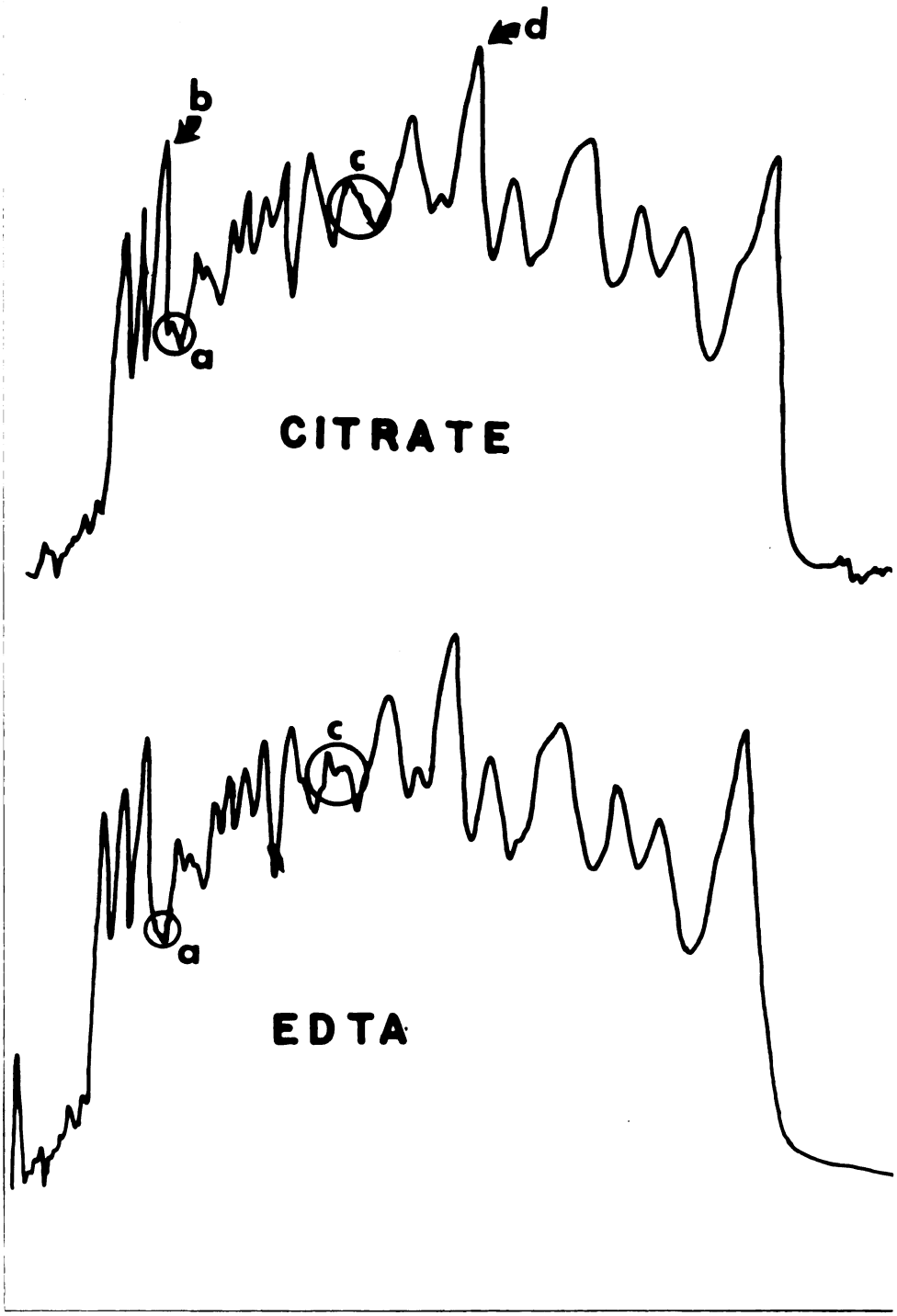


Figure 5. Determination of platelet proteins in the presence and absence of EDTA as determined by scans of Coomassie Blue stained SDS-polyacrylamide gels. 80 µg total platelet protein. a, 180,000 MW; b, myosin heavy chain; c, 69,000 MW; d, actin.

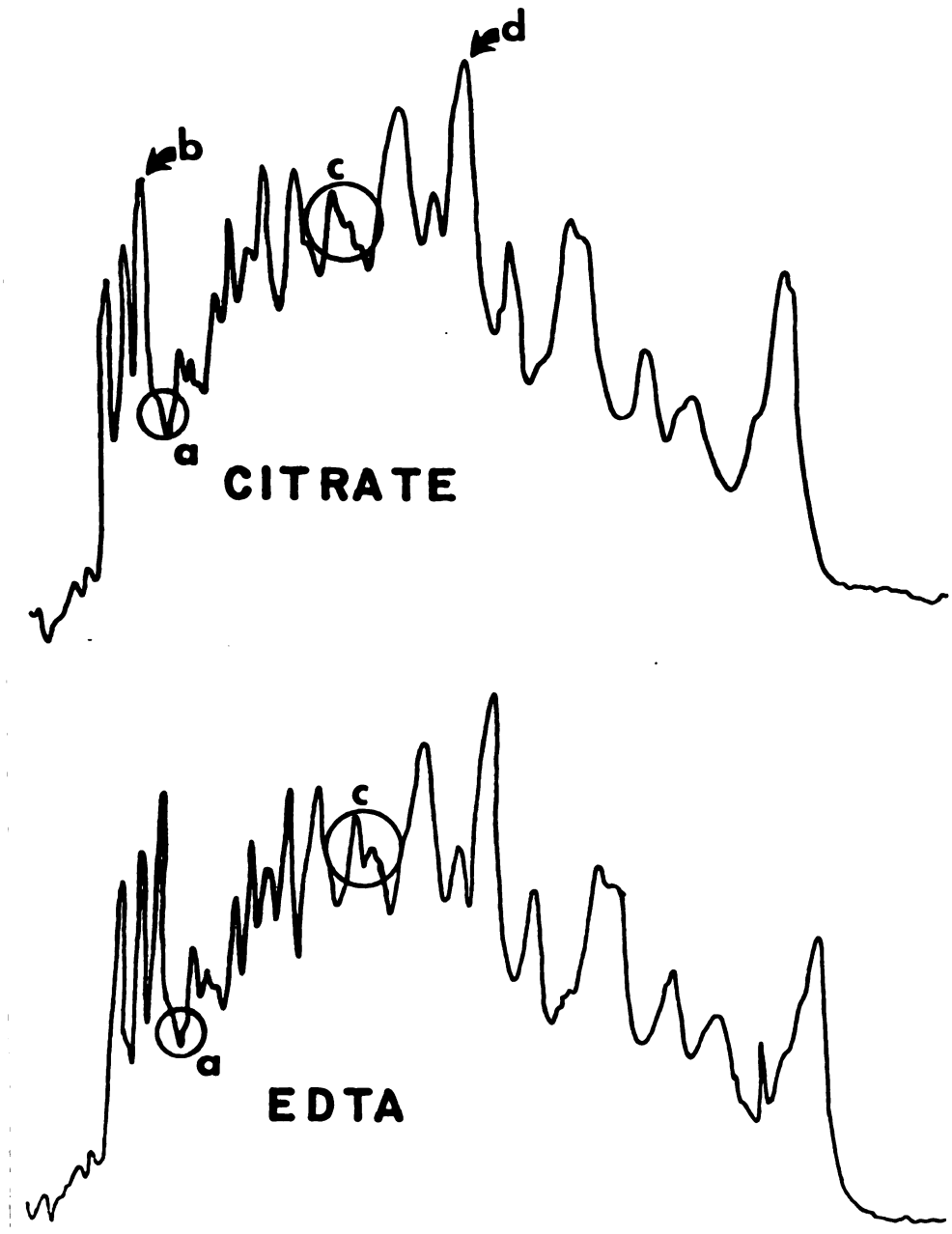


Figure 6. Comparison of ^{125}I labeled surface proteins of EDTA and Na_3 citrate washed platelets as detected by autoradiography. a, 255,000 MW; b, 215,000 MW; c, myosin heavy chain (196,000 MW); d, Glycoprotein I (150,000 MW); e, Glycoprotein II (118,000 MW); f, Glycoprotein III (92,000 MW); g, actin (41,000 MW); h, myosin light chain (22,000 MW); i, myosin light chain (16,000 MW).

Actin was slightly labeled and several other proteins of molecular weights between 92,000 and 41,000 daltons are labeled. Some proteins below actin were labeled although the myosin light chains are not labeled.

Isotopic Labeling of Activated Platelets

The SDS-PAGE Coomassie Brilliant Blue stained protein pattern of platelets activated with 1 x 10^{-6} M ADP was essentially identical to that of normal unactivated platelets (Figure 7). This concentration of ADP was chosen because it was sufficient to elicit a primary aggregation response with no secondary aggregation (Figure 8). Using gamma counts of 1 mm sections of frozen stained gels, the 3 major platelet membrane glycoproteins I (150,000 MW), II (118,000 MW), III (92,000 MW) (20) were labeled in both preparations (Figure 7). A 255,000 molecular weight protein showed enhanced labeling in both resting and activated platelets. There appeared to be greater labeling of actin in activated platelets. If the ratio of the actin peak is compared to that of the glycoprotein III peak, unstimulated platelets have an actin/glycoprotein III ratio of 1:9 versus a ratio of 1:3 in activated platelets. The glycoprotein II/glycoprotein III ratio remains 1:3 in both preparations (Figure 7).

Lactic Dehydrogenase Activity

Lactic dehydrogenase activity in the incubation medium was measured to monitor leakage of cytoplasmic constituents during the iodination procedure. Three separate samples of washed platelets were iodinated with no increase in LDH activity in the incubation medium as compared to washed non-iodinated platelets. Platelets solubilized with Triton X-100 to release cytoplasmic LDH showed that the platelets

contain considerable LDH. Normal human serum LDH was run as a control (Figure 9).

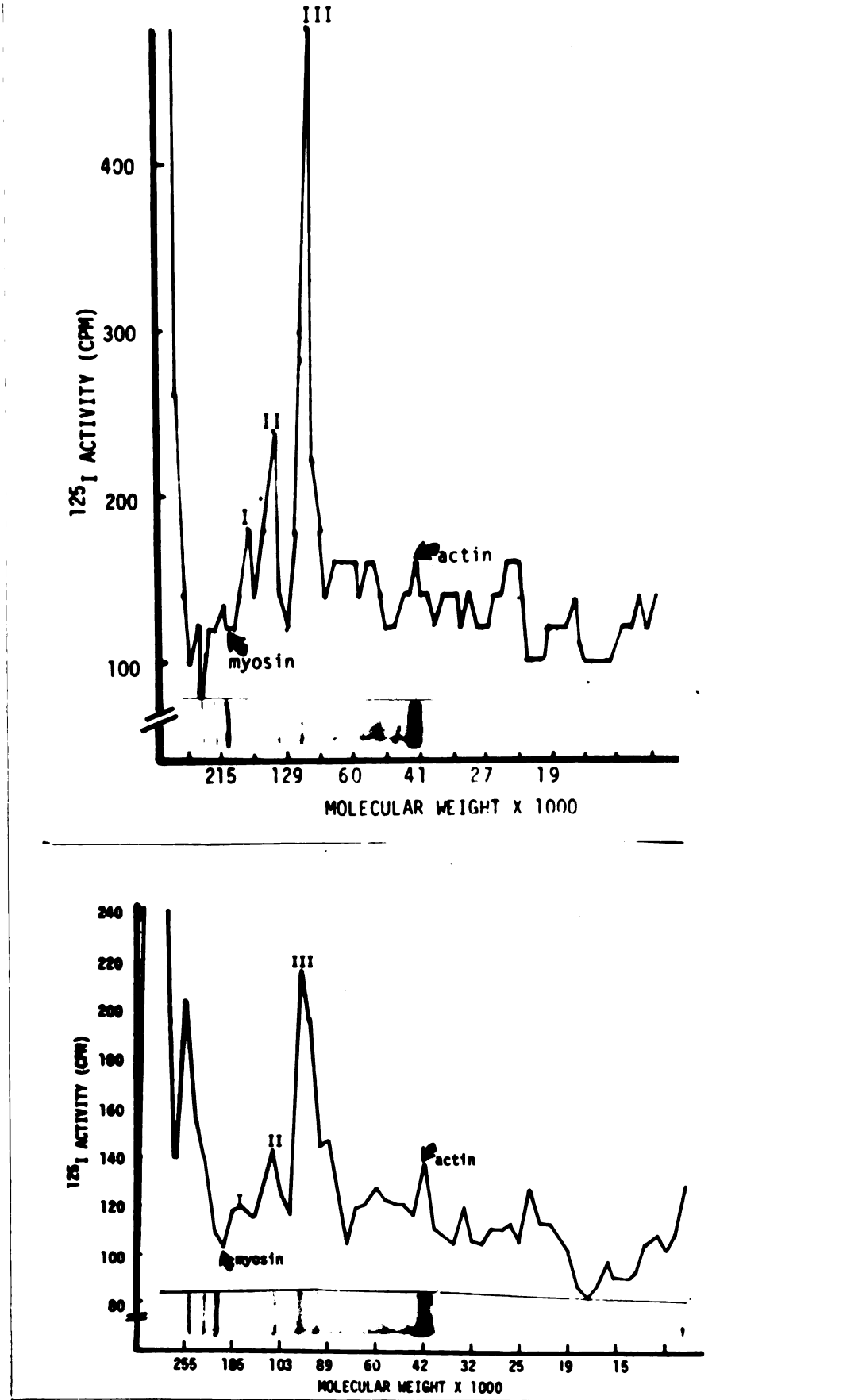


Figure 7. Radioiodination patterns of normal (top) and ADP activated platelets (bottom) comparing SDS-PAGE migration with gamma counts.

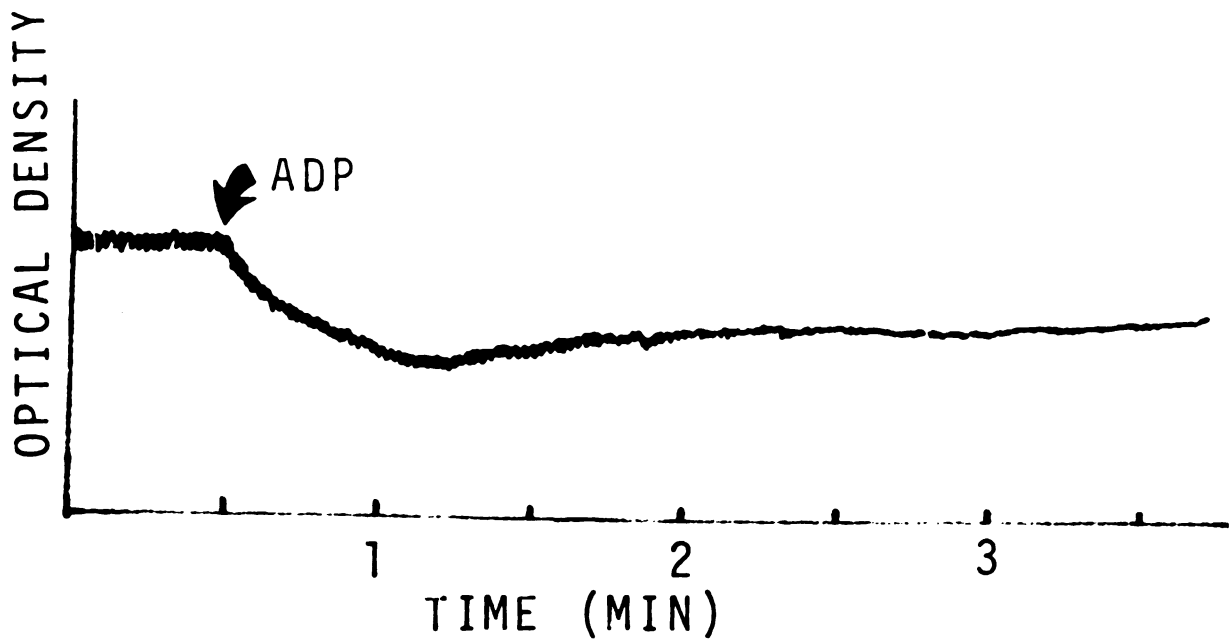


Figure 8. Aggregometer tracing of platelets activated by 1×10^{-6} M ADP (final concentration).

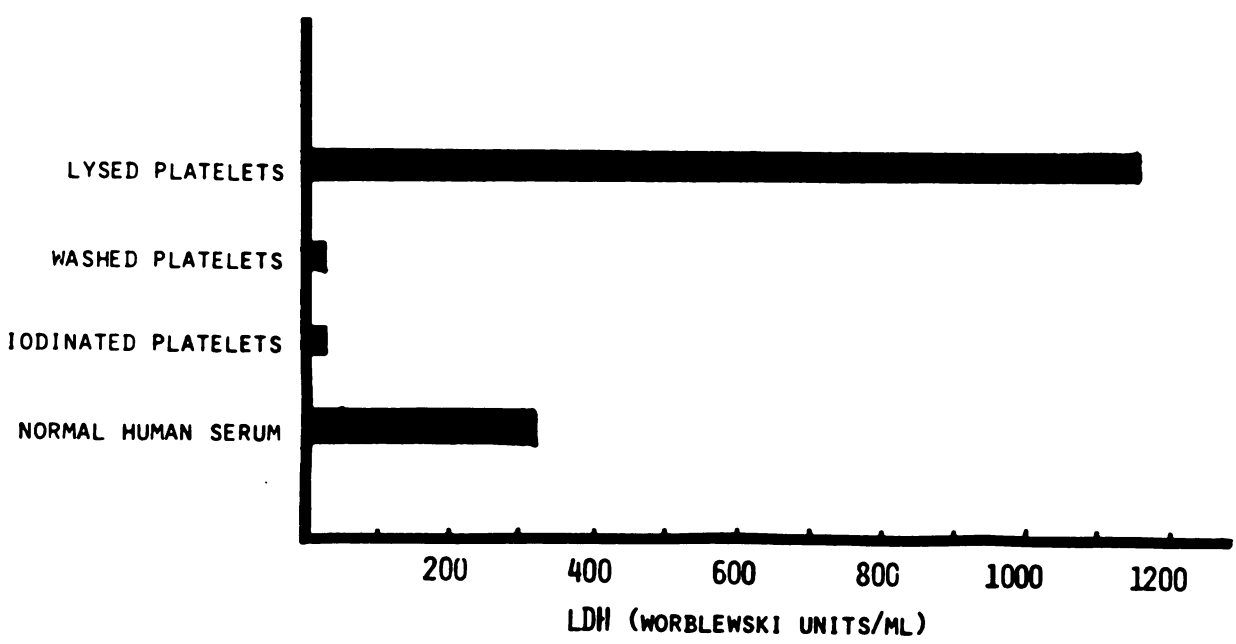


Figure 9. LDH activity of washed iodinated platelets.

DISCUSSION

It has been shown that a high degree of reproducibility in the determination of the molecular weight can be obtained in SDS-PAGE whether the proteins are run on the same or different gels (19). Different proteins have electrophoretic mobilities which are independent of the isoelectric point and the amino acid sequence of their polypeptide chains. Sodium dodecyl sulfate-PAGE yields not only excellent results for proteins which are globular in the native state, such as actin, but also for the highly helical, rod-shaped molecules, like myosin. With sufficient markers in the desired range it is possible to achieve an accuracy of about 10% in the determination of the molecular weight of an unknown protein falling in this range.

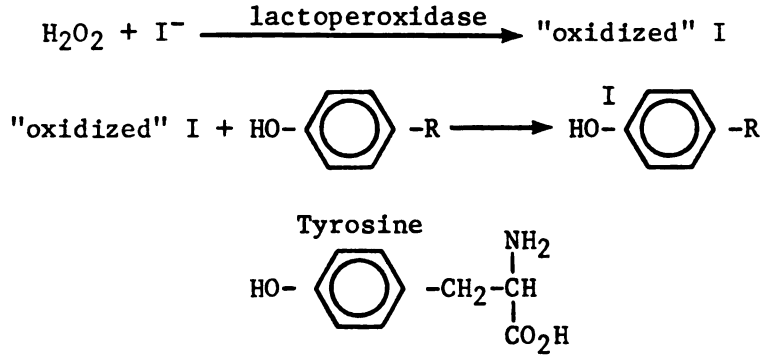
The molecular weight of actin, as reported in the literature, ranges from 37,000 to 44,000 daltons in non-muscle cells (34, 35) and 43,000 to 45,000 daltons in platelets (5, 32, 33). Platelet myosin heavy chain has been reported to have a molecular weight of 200,000 daltons and the 2 light chain molecular weights of 19,000 and 16,000 daltons (5). The reported molecular weights of actin and myosin subunits are comparable to the molecular weight of 41,000 daltons for actin, 196,000 daltons for myosin heavy chain, and 22,000 and 16,000 daltons for myosin light chains obtained in this study (Figure 2). Actin was the primary platelet protein identified in platelet homogenates. This agrees with reports that

actin is the most abundant platelet protein comprising 15% to 20% of the total platelet protein (4, 5).

It was suspected that the effect of EDTA on platelets might interfere with proper identification of membrane proteins. Booyse and Rafelson (15) found that by incubating platelets with EDTA, surface anti-actomyosin staining could be eliminated. Since EDTA had no effect on the immunochemical properties of isolated actomyosin, it was concluded that EDTA was releasing actomyosin from the cell surface. Actomyosin could be isolated from the incubation medium and characterized by its contractile protein and immunochemical properties. Curiously, all platelet lactoperoxidase iodination data to date has been performed in the presence of EDTA (20, 29, 30). Specific experiments were, therefore, performed to determine if EDTA altered the results of such studies by releasing surface components. Two proteins were altered by the presence of EDTA. A large 180,000 dalton protein was diminished in the presence of EDTA while a smaller 69,000 MW protein was slightly enhanced by the presence of EDTA. One assumes that the proteins altered with EDTA must have been located on the surface since EDTA was only exposed to intact whole platelets, yet they were not iodinated as indicated by autoradiography (Figure 6). A possible explanation for the lack of labeling of these 2 proteins is that these proteins contain insufficient tyrosine or that tyrosine residues are not present in the part of the protein exposed to lactoperoxidase iodination. Because of its effect on surface proteins EDTA was not used in subsequent radiolabeling procedures.

Lactoperoxidase catalyzes the reaction between oxidized iodide and a phenolic compound. Iodide is oxidized by the hydrogen peroxide (Figure 10).

Figure 10. Lactoperoxidase iodination.



The major amino acid iodinated is tyrosine, although histidine can also be iodinated but at a slower rate than tyrosine (21). Lactoperoxidase is an enzyme of 78,000 molecular weight which prevents it from entering an intact cell. The cellular integrity of the platelet during lactoperoxidase iodination is of utmost importance for the exclusive labeling of surface proteins. Phillips (20) showed no incorporation of iodine in control suspensions of platelets in the absence of lactoperoxidase. Examination of platelets by electron microscopy showed that his procedure did not induce any morphological alterations in the platelet (20).

Experiments were performed to demonstrate that no leakage of cytoplasmic components occurs during the iodination procedure. Marcus et al. (22) determined that 98% of the LDH in the platelet is not

associated with intracellular organelles and therefore free in the cytoplasm. If the platelet membrane is injured enough for lactoperoxidase to enter the cell then LDH should be able to leak out. Experiments designed to detect LDH leakage during iodination showed that the platelets contain adequate amounts of LDH and that iodination did not cause significant leakage of cytoplasmic LDH into the media. Further evidence that the platelets remained intact during iodination was the selective iodination of proteins; all proteins should have been iodinated if the platelets were not intact.

Using a modified Phillips' procedure in this study, in which EDTA was omitted, isotopic labeling results similar to those previously described were obtained. The 3 major surface glycoproteins were iodinated with the greatest labeling by glycoprotein III (28). A protein of 255,000 MW and one slightly larger than myosin heavy chain with a MW of 215,000 daltons were also labeled. Myosin heavy chain and both myosin light chains showed no isotopic labeling, but actin had slight radioactivity.

Comparison studies were performed before and after stimulation of platelets with ADP to see if platelet activation altered the composition of membrane proteins and to specifically determine if myosin appears on the membrane after activation. The concentration of ADP used (1×10^{-6} M) was intentionally very weak so that the platelets would not go through secondary aggregation which might cause possible injury to the membrane thus allowing lactoperoxidase to enter into the cell or cytoplasmic proteins to escape.

The lactoperoxidase iodination results indicate that myosin is not iodinated and therefore does not appear to be on the surface of

activated or non-activated platelets. This is in agreement with the results of Fugiwara and Pollard (18) using antimyosin rod immunofluorescent staining. Pollard et al. (17) described an ^{125}I lactoperoxidase surface-labeling experiment done with special emphasis on detecting any surface labeling of myosin in which no labeled myosin was detected. They suggest that a large portion of the myosin is not exposed on the platelet surface. Pollard also suggests that radioiodination results must be interpreted cautiously since the rod or tail portion of the myosin has few tyrosine residues. Portions of the tail could be exposed but still escape ^{125}I lactoperoxidase labeling.

The apparent ^{125}I lactoperoxidase surface labeling of actin seen in Figure 7 and its increase with ADP have not been appreciated in the previous radiolabeling studies of Phillips (20, 28) and Nachman et al. (30). The presence of actin on the surface is, however, supported in the literature. Perdue (11) using cultured chick embryo fibroblasts found "actin-like" filaments interacting with the plasma membrane. The filaments were called "actin-like" because they bound heavy meromyosin in the typical arrowhead-like pattern characteristic of actin. Electron micrographs and antibodies produced against the "actin-like" protein detected the protein on the cell surface, particularly at the ends of the cell where it makes contact with the dish and other cells. Perdue suggests that the presence of these external microfilaments at cell contact points may denote the function of these components in providing sites of cell attachment.

Bouvier et al. (9) using anti-actin antibodies (from the serum of patients with chronic aggressive hepatitis) showed fluorescent

staining of external actin in ADP activated platelets even after the platelets had recovered from the activation. Binding of anti-actin antibodies was also found in platelets which were pretreated with EDTA which inhibited ADP aggregation. No surface anti-actin antibody binding was observed in normal non-activated platelets however.

The radioactive labeling of actin in non-activated platelets observed in this study may indicate that platelets have been slightly stimulated during the washing and centrifugation used in the preparative steps. Alternately radioiodination may be a more sensitive tool for detecting actin in non-stimulated platelets than immunofluorescent studies. Three-fold increased in surface actin were detected in iodination studies using only weak concentrations of ADP. Bouvier et al. (9) report that weak concentrations of ADP gave only slight immunofluorescence. This further suggests that they were working at the threshold of the sensitivity of their techniques.

The presence of actin on the platelet surface is consistent with proposed mechanisms of platelet participation in clot retraction (17). During clot retraction activated platelets change from a discoid to a spherical shape, the body of the platelet is reduced in size, and numerous long filopodia form (17). Filopodia consist of bundles of parallel actin filaments surrounded by a closely fitting membrane (23). A large quantity of filopodia are seen in scanning electron micrographs of stimulated platelets. If platelet filopodia are similar to other microvilli, the tip of the filopodia will be an actin filament anchoring site equivalent to the Z line in striated muscle. Since muscle actin has been shown to cross-link with fibrin

in the presence of fibrin-stabilizing factor (24), actin exposed at the tip of the filaments could bind directly to fibrin. Platelet-fibrin interactions have frequently been noted (25) including associations seen in electron micrographs after clot retraction (17). The evidence for actin's presence on the surface of the platelet and its ability to cross-link with fibrin may help explain platelet-fibrin binding during clot retraction.

In summary, actin and myosin were identified in homogenates of human platelets by comparison with purified human platelet actin and myosin standards. Using ^{125}I lactoperoxidase iodination to label the external proteins of intact platelets, myosin was not labeled, whereas actin was. The radioactive labeling of actin increased when platelets were activated.

Although myosin was not labeled by lactoperoxidase iodination, it may still be present but not detectable since tyrosine residues may not be exposed. Bray (36) has stated that immunological evidence for the presence of surface myosin is difficult to defend against all objections and that some direct biochemical confirmation is needed. Myosin, in particular, is difficult to purify and few researchers have been able to prepare myosin that did not contain up to 5% contaminents so that the homogeneity of the antisera is always suspect. For this reason it was important to attempt identification of surface myosin by lactoperoxidase iodination to determine if this biochemical procedure could clarify some of the controversy concerning the presence of myosin on the platelet surface.

Factors that could have caused myosin labeling to be missed in previous lactoperoxidase iodinations (17, 20, 28, 30) were eliminated.

(1) The effect of EDTA on surface proteins was investigated because of the report by Booyse and Rafelson (15) that EDTA removed actomyosin from the platelet surface and the fact that EDTA was present in all previous lactoperoxidase iodinations. Our observation that EDTA appears to alter some surface proteins caused us to omit this chemical in all subsequent work.

(2) The possibility that myosin may have been hidden from the surface in resting platelets was investigated by stimulating the cell with ADP.

(3) The variability in the radiolabeling patterns of sliced gels was reduced by:

   (a) freezing the gels, to reduce crushing, and thereby producing slices of a more consistent shape and thickness;

   (b) decreasing the size of the slices from the usual 1.5 mm thickness to 1 mm thick slices, thus producing sharper peaks;

   (c) staining the labeled gels before slicing;

   (d) and, using autoradiography of whole gels to rule out the possibility of spurious gamma spectrophotometer counts on gel slices.

Controlling all of these variables in attempting to label surface myosin directly with the lactoperoxidase iodination technique has failed to detect myosin on the platelet surface.

CONCLUSION

Human platelet actin and myosin were found to have molecular weights of 41,000 and 196,000 daltons respectively. Washing of platelets with EDTA as opposed to Na_3 citrate did not appear to change the concentration of actin or myosin as determined by scans of SDS-polyacrylamide protein stained gels. There was, however, a change in 2 other platelet proteins of 180,000 and 96,000 molecular weights.

The platelet membrane was found to remain intact after lactoperoxidase iodination as determined by LDH analysis.

The ^{125}I lactoperoxidase labeling of the external proteins of resting and ADP stimulated platelets indicated no external labeling of myosin. Actin appears to be labeled in both activated and non-activated platelets with a three-fold increase in labeling after activation.

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VITA

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