SURFACE CHEMICAL STUDIES OF HUMAN PLATELETS

by

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ABSTRACT

The purpose of this project is to investigate the surface properties of platelet discocytes, echinocytes and spherocytes. Normal "non-sticky" discoid shaped platelets (discocytes) can be transformed by ADP into irregularly shaped echinocytes which are "sticky" and aggregate easily in media containing Ca^{++} and fibrinogen. A model is examined here in which an echinocyte attains its "sticky" properties by evagination of a surface-connected canalicular system. Platelets also evaginate this canalicular system upon hypotonic shock, in which case the platelets swell up to form spherocytes. By comparing the properties of the different geometric forms of platelets insight into the nature of "stickiness" was sought. The surface areas of the discocyte and spherocyte measured microscopically were found to be 16.4 and 36.7×10^{-8} cm^2 respectively while that of the echinocyte was estimated to be 23.7×10^{-8} cm^2 using surface chemical analysis. Electron microscopic examination showed that the canalicular system may not be totally evaginated in the echinocyte. Although it was found that the spherocyte could still be agglutinated passively by ristocetin it had completely lost its ability to aggregate. Microelectrophoretic studies revealed 8 and 6 fold increases in the density of Ca^{++} and Mg^{++} binding sites respectively on the echinocyte surface relative to the discocyte. The spherocyte on the other hand had lost most of its Ca^{++} binding sites. Electrokinetic analysis of live, fixed and neuraminidase or alkaline phosphatase treated platelets showed major differences in charge as well as amino, sialic acid and phosphate group densities among the discocyte, echinocyte and spherocyte. The evaginated canalicular membrane surfaces of the latter two were also different. SDS-PAGE of platelets radiolabelled

via lactoperoxidase iodination, periodate-borohydride tritiation or neuraminidase/galactose oxidase-borohydride tritiation failed to show any difference in the gel patterns between the three platelet forms. No new glycoprotein species appeared during the transformations. The presence of fibrinogen interferes in a concentration related manner with lactoperoxidase iodination of GP-III on the echinocyte surface. An overall picture is presented here showing differences between the surface properties of platelet discocytes, echinocytes and spherocytes. The accumulated evidence suggests that changes in the whole platelet surface occur during activation and the model of a cloistered "sticky" membrane may be an oversimplification.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
Beta-Tg	Beta-thromboglobulin
CPM	Counts per minute
DTT	Dithiothreitol
EPM	Electrophoretic mobility
GP	Glycoprotein
JBA	Jequirity bean agglutinin
LDH	Lactate dehydrogenase
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
PAGE	Polyacrylamide gel electrophoresis
PRP	Platelet rich plasma
RBC	Red blood cell
RCA_{60}	Castor bean agglutinin, MW=60,000
RCA_{120}	Castor bean agglutinin, MW=120,000
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
WGA	Wheat germ agglutinin

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SURFACE CHEMICAL STUDIES OF HUMAN PLATELETS

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CHAPTER 1

GENERAL INTRODUCTION

Platelets are circulating formed elements of mammalian blood that are essential for normal hemostasis. Their functions can generally be divided into two groups. The first is hemostatic, i.e. the physical occlusion of damaged blood vessels by masses of aggregated platelets. The second function is thromboplastic, i.e. the participation of the chemical constituents of the platelets in the blood coagulation mechanism.

When the blood vessel wall is transected, platelets come into contact with the basement membrane as well as collagen in the subendothelial tissues around the cut margin. They then rapidly undergo shape change and degranulation. Platelets are normally discoid in shape (a form referred to as a discocyte) but during the shape change reaction they become irregular in shape with pseudopods extending out from a more or less spherical central body (echinocyte). Adhesion of the platelet to the injured vessel wall is first initiated with the pseudopods and then the platelets spread out over the exposed subendothelial surfaces (Mohammad and Mason, 1981; Frojmovic and Milton, 1982). During this process platelet degranulation occurs and substances released such as adenosine diphosphate (ADP) and serotonin help stimulate other platelets to undergo shape change and adhere to the first layer of platelets. This "second wave" of adherent platelets also release their granules and causes more platelets to adhere and accumulate around the injured site until a hemostatic platelet plug is formed, stopping further loss of blood through the injured vessel (Zucker, 1972). By 30 sec after injury the hemostatic plug will be well formed and can grow to several times the diameter of the blood vessel. In the central part of the plug, activated platelets that have lost most of their granules form a cytoplasmic mass in which individual platelets are no longer distinguishable (Wester et al., 1977).

During platelet activation factor 3 becomes available for its thromboplastic function. Platelet factor 3 is a phospholipid associated with the platelet membrane which is not detected until activation occurs. Platelet factor 3 forms a complex with factor IXa, factor VIII and calcium to activate factor X, following which it forms a complex with factor Xa, factor V and calcium to convert prothrombin into thrombin (Zwaal, 1978). Other substances released from platelet granules during activation such as platelet factor 4 (anti-heparin activity), platelet fibrinogen and factor VIII may also contribute to the coagulation cascade. The time course of platelet-induced clotting is such that fibrin is not observed in the hemostatic plug until about 3 min after the initial activating event. However by 30 min most of the platelets in the plug have disintegrated and have been replaced by a massive accumulation of fibrin (Wester et al., 1977).

The introduction of the aggregometer allowed platelet aggregation to be studied in vitro in detail (Born 1962; Michal and Born, 1971). An aggregometer is a simple photometric device that measures the turbidity of a stirred suspension of platelets. A beam of light shines through the platelet suspension and the amount of light passing through is measured. Normal discoid platelets have the maximum turbidity and the least amount of light passes through. During aggregation the platelets clump together into aggregates and the suspension clears optically, allowing more light to pass through. A measure of the rate of aggregation is therefore provided by the rate of increase in transmission of light through the platelet suspension.

Among the agents which can cause aggregation, the most well studied one is ADP. Upon exposure to ADP, the platelets undergo a shape change from discocyte to echinocyte. Providing the right conditions exist, the platelets

will aggregate. Right conditions include the presence of the divalent cations calcium or magnesium, and fibrinogen (Zucker, 1972). A certain amount of agitation is also required to bring sufficient numbers of platelets into contact with each other. The aggregometer has a built-in magnetic stirrer for this purpose. Shape change alone does not require any of these conditions.

The first event in the stimulation of platelets by ADP is the binding of this molecule onto the surface of the platelets (Born and Feinberg, 1975; Legrand and Caen, 1978). A yet-to-be understood sequence of events then triggers the discocyte-echinocyte transformation. At the same time, the surface of the platelet becomes "sticky." The development of platelet "stickiness" is a process which parallels shape change but not directly caused by the discocyte to echinocyte transformation itself (Zucker, 1972; Barnhart, 1978). The biochemical nature of "stickiness" is still uncertain and remains to be defined. It may involve the increased, saturable binding of fibrinogen onto the ADP-stimulated platelet surface in the presence of Ca^{++} or Mg^{++} (Mustard et al., 1978; Marguerie et al., 1979; Bennett and Vilaire, 1979; Peerschke et al., 1980). Surface properties associated with changes in electrophoretic mobility (Hampton and Mitchell, 1974; Seaman and Vassar, 1977; Stoltz, 1979) or in the amount of neuraminidase-susceptible sialic acid on the platelet surface (Motemed et al., 1976; Ku and Wu, 1977) have also been suggested as being involved. Throughout the following sections, these and other properties that may be involved in platelet "stickiness" will be explored.

After echinocyte formation and the appearance of "stickiness", the platelets begin a two stage aggregation process. First is primary or reversible aggregation in which the platelets adhere to each other to form aggregates. Some

internal reorganization in the platelets occurs: organelles move towards the central part of the echinocyte and the circumferential bundle of microtubules also shifts internally. The degree of change varies from echinocyte to echinocyte but no granules are released at this point (White, 1972). When the concentration of the aggregation agent is not high enough or the temperature is below 30°C, the platelets will eventually disaggregate and revert to the discoid shape (Zucker, 1972).

On the other hand, if the concentration of the aggregating agent is high enough and the temperature is ideal, then secondary or irreversible aggregation proceeds after primary aggregation (White, 1972). At locations in the aggregates where the platelets are tightly squeezed together, their organelles and microtubule bundles beome more centralized and finally contents of the platelet granules are released. The platelets in the aggregates subsequently begin to disintegrate (Rodman, 1971; White, 1972). the release reaction induced by agents such as thrombin, arachidonic acid and A23187 can be studied without aggregation by not stirring the platelet suspension to promote aggregation (Holmsen, 1977). Aggregation induced by ADP is usually not followed by release action (Holmsen, 1977).

Platelets have three kinds of granules, alpha granules, dense bodies and lysosomes (Skaer, 1981; Nurden et al., 1982). Dense bodies are few in number and electron dense. They contain large quantities of serotonin, inorganic calcium and phosphorus as well as ADP and ATP. The alpha granules are less electron opaque and contain numerous proteins including fibrinogen, fibronectin, thrombospondin, factor VIII-related antigen, factor V, low affinity and high affinity (affinity for heparin) platelet factor 4, beta-thromboglobulin, platelet growth factor, chemotactic factor and bactericidal

factor. Platelet lysosomes which are also not electron dense contain various proteinases, glycosidases, esterases and phosphatases. Serotonin is usually used as a monitor for dense granule release while beta-thromboglobulin and platelet factor 4 levels reflect alpha granule release.

Transformation from a discocyte to an echinocyte requires the platelets to increase their apparent surface areas to provide for the formation of the pseudopods. The shape change occurs almost instantaneously after exposure to an aggregating agent and is completed within seconds. The discocyte must therefore have a mechanism to provide for the increase during this short time period. The most likely mechanism seems to be the provision of excess surface area within the discocyte. The surface-connected canalicular system (open canalicular system) is a likely source. This open canalicular system is a labyrinth of membrane channels that are connected to the platelet surface with openings to the outside (White and Clawson, 1980). The evagination of this membrane system could therefore provide the additional surface membrane required for transformation (Morgenstern and Kho, 1977; Frojmovic and Milton, 1982). A comparison of the surface properties of platelet discocytes and echinocytes may therefore provide some information on the properties of "sticky" membranes and/or the open canalicular membrane system. The degree to which the two are related remains to be seen.

Another way to study the invaginated open canalicular system is to evaginate it by means of hypotopnic stress. By lowering the osmotic pressure of the suspending medium, large swollen spherical platelets (spherocytes) are creatred with their surface-connected canalicular system apparently evaginated (Milton and Frojmovic, 1979). Treatment of the discocyte with cocaine will also produce a spherical form of platelet (Behnke, 1979) but one which is much

smaller in size than the spherocyte. Hereafter these will be referred to as cocaine spheres to distinguish them from the spherocytes produced by hypotonic stress. The general term spheres will include both spherocytes and cocaine spheres.

The project described herein was aimed at comparing the surface features of the discocyte, echinocyte, spherocyte and cocaine sphere. Properties studied included elctrokinetic behaviour, surface sialic acid, phosphate and amino groups, calcium and magnesium binding and surface labelling. Aggregation with various agents was also studied.

CHAPTER 2

PLATELET AGGREGATION AND MORPHOLOGY

2.1 INTRODUCTION

Mild hypotonic shock to platelets has been used in the blood banking community to test for platelet viability during storage. Platelets are very resistant to hypotonic stress. When introduced into a hypotonic environment with osmolarity as low as 150 mOsm, the platelets will change into an echinoid shape but can slowly reverse back into the discoid form (Fantl, 1966; Handin et al., 1970; Kim and Baldini, 1974; Odink, 1976; Milton and Frojmovic, 1977a). Under more severe hypotonic stress platelets will turn into swollen spheres or spherocytes. Zucker-Franklin (1969) suspended platelets in distilled water to obtain spherocytes for microfibril studies. Milton and Frojmovic (1977b) found that by lowering the osmolarity to as much as 60 mOsm they could produce spherocytes stable for at least an hour. They also suggested that the osmotic spherocyte formation provides an opportunity for the study of the surface-connected canalicular system.

Treatment of platelets with cocaine results in a total loss of aggregability (Aledort and Niemetz, 1968; O'Brien, 1976) and produces a spherical form of platelet (Mannucci and Sharp, 1967; Behnke, 1970). Other properties of this cocaine-induced sphere have yet to be explored.

In this chapter, the morphology and dimensions of the normal platelet discocyte, the ADP transformed echinocyte, the hypotonically induced spherocyte and the cocaine-induced sphere will be described using the results from phase and electron microscopy.

As discussed earlier, aggregation of platelets by ADP requires Ca^{++} or Mg^{++} ions as well as fibrinogen. Aggregation studies to find the optimum ADP, Ca^{++}, Mg^{++} and fibrinogen concentrations required are reported in this chapter as preparation for later experiments.

Aggregation induced by a variety of other agents was also examined, mainly to test the aggregability of the spherocyte. Of these, ristocetin is of particular interest because it passively agglutinates rather than pharmacologically inducing platelet aggregation, and a plasma co-factor (von Willebrand's factor) is also required (Phillips, 1980). Another group of agents, the lectins, were also of special interest. It will be shown here that removal of surface sialic acid with neuraminidase can modify the platelet's response to different lectins.

2.2 MATERIALS AND METHODS

2.2.1 Collection of Blood

Blood was drawn from healthy human volunteers using plastic syringes by venipuncture and anticoagulated with 0.38% sodium citrate. Volunteers were mostly from within the Pathology Department but in a few cases arrangements were made through the local Red Cross. Red Cross blood was collected in citrate-phosphate-dextrose (Masouredis, 1972). Plastic ware was used in all the handling of blood and platelets. Samples remained at room temperature at all times.

2.2.2 Preparation of Platelets

Within one hour after the collection of blood, platelet-rich plasma (PRP) was prepared by centrifugation at 120xg for 15 min (Day et al., 1976). Platelets were then isolated from the PRP by centrifugation at 1200xg for 15 min. They were washed twice in a calcium and magnesium free Tyrode's solution made up of 136.75 mM sodium chloride, 2.68 mM potassium chloride, 11.90 mM sodium bicarbonate, 0.36 mM sodium dihydrogen phosphate and 5.55 mM glucose. The pH was 7.4 unless otherwise indicated. To prevent platelet loss during the isolation and washing procedures, the PRP and Tyrode's solution were first acidified with citric acid to pH 6.5 according to the method of Zucker and Grant (1978). After wshing, the platelets were resuspended in pH 7.4 Tyrode's solution. It was found that washing with the acidified Tyrode's solution was essential. If platelets are centrifuged in a pH 7.4 solution they will aggregate. Fibrinogen as well as calcium and magnesium ions were added separately as required on an individual experiment basis.

2.2.3 Preparation of Echinoctyes and Spherocytes

Echinocytes were prepared by the addition of $2x10^{-5}$M final concentration of ADP to suspensions of platelets at room temperature. The suspensions were then gently mixed by inverting the test tube twice. Vigorous mixing or shaking was avoided.

Spherocytes were prepared by the addition of water to isotonic suspensions of platelets (in PRP or Tyrode's solution) in a ratio of three parts water to one part suspension (Milton and Frojmovic, 1979). Water was added slowly through the period of about a minute and vigorous mixing was also avoided. The osmolality of the final suspension was therefore lowered to about 75 mOsmol.

2.2.4 Cocaine Induced Spheres

Platelet suspensions were incubated for half an hour with cocaine at 10 mM final concentration (Behnke, 1970) to produce the cocaine spheres. Sphering occurred at both room temperature and 37°C.

2.2.5 Enumeration of Platelets

Platelets were counted using a hemocytometer under phase contrast microscopy.

2.2.6 Fixation of Platelets

Platelets in various forms were fixed at room temperature in 0.35% glutaraldehyde in Tyrode's solution. After one hour the platelets were spun down and resuspended with fresh glutaraldehyde solution for overnight fixation at 4°C. Fixation with glutaraldehyde was found to be very rapid (Vassar et al., 1972).

2.2.7 Measurement of Platelet Sizes

The morphology of fresh and fixed platelets was examined under phase contrast microscopy. Photomicrographs were taken with a Carl Zeiss Photo-

microscope II using a 40x objective and overall magnification of 800x. A grid of 0.1μ was also photographed in the same manner. To measure the sizes of the platelets, the negatives from the photomicrographs were projected onto a piece of paper using a photographic enlarger. Diameters measured from the outside edges and the inside edges of the diffraction ring of each platelet were averaged as described by Frojmovic and Panjwani (1976). They were compared to that of the grid traced out in the same way. Dimensions for the spherical forms of platelets were calculated from their measured radii (r) using simple geometric formulae for spheres:

$$\text{Area} = 4\pi r^2$$

and $$\text{Volume} = 4/3\pi r^3$$

Dimensions for the discocytes were calculated according to Frojmovic and Panjwani (1976) using a model of an oblate spheroid:

$$\text{Area} = (\pi/2)d^2 + (\pi/4)t^2\left\{(1+r)/(1-r)\right\} \log R^{-1}$$

and $$\text{Volume} = (\pi/6)d^2t$$

where d = diameter

t = thickness

and $R = t/d$

2.2.8 Electron microscopy

Glutaraldehyde fixed platelets were postfixed with 2% osmium tetroxide in 0.1M cacodylate buffer (pH 7.35) for 1 hour. After dehydration in a graded series of ethanol solutions the platelets were embedded in Epon 812. Thin sections were cut with an ultramicrotome equipped with a diamond knife and contrast of the sections enhanced by staining with uranyl acetate and lead citrate. Platelet samples from three healthy volunteers were viewed with a Phillips EM 300 electron microscope and photographs taken.

2.2.9 Tests for the Integrity of Platelet Forms

2.2.9.1 Lactate Dehydrogenase (LDH) Leakage

LDH assays were performed according to the kinetic method of Kachmar (1970). This method is based on the reverse reaction of pyruvate (substrate) to lactate and monitored as a decrease in absorbance at 340 nm when NADH is oxidized to NAD.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{NAD} + \text{lactate}$$

The unit of enzyme activity is the Wroblewski Unit which is the drop of absorbance per minute per volume of sample. Supernatants from discocyte, echinocyte and spherocyte suspensions were assayed for the leakage of the enzyme. Total LDH in platelets was assayed using supernates from frozen and thawed samples. More detailed time studies for spherocytes were also performed.

2.2.9.2 Beta-Thromboglobulin (beta-Tg) Release

Beta-thromboglobulin was assayed using an Amersham radioimmunoassay kit. Release of beta-Tg from echinocytes and spherocytes was monitored by comparing supernatant and platelet contents released by freeze-thawing. Some discocytes were also stimulated to release their granules by the addition of thrombin at 0.4 NIH units/ml.

2.2.9.3 ^{14}C-Serotonin Release

Platelet (discocyte) dense bodies were first loaded with ^{14}C-serotonin by the method of Clark and Harms (1978). About 25 μCi of ^{14}C-serotonin were incubated with each milliliter of PRP for one hour at room temperature. The platelets were then washed as before and resuspended in Tyrode's solution. After transformation to echinocytes or spherocytes ^{14}C-serotonin in the

supernatants and platelets were monitored using liquid scintillation counting. Samples were mixed with Atomlight scintillant fluid (New England Nuclear, Boston) in a ratio of 1 ml sample to 5 ml Atomlight and then counted with a Beckman LS-233 scintillation counter.

2.2.10 Platelet Aggregation

Platelet aggregation was studied with a Born-Michal Aggregometer (London, England). The velocity of aggregation was measured as the rate of decrease in the optical density of the platelet suspension (Born and Cross, 1963). This was done by drawing a line tangent to the steepest part of the slope of the aggregation tracing recorded by the chart recorder (Fig. 1.). The slope of this line was measured as the velocity of aggregation and had an arbitrary unit of inches (of chart paper) per minute. All aggregation experiments were done at $37^{o}C$ and at a concentration of about $2.5x10^{8}$ platelets/ml of Tyrode's solution.

A series of experiments was undertaken to determine the effects of different concentrations of ADP and fibrinogen as well as calcium and magnesium ions on platelet aggregation. In order to make inter-sample comparisons possible a normalization procedure similar to that used by Frojmovic (1973) was adopted. A concentration at which maximum aggregation velocity always occurred was referred to as producing a 100% velocity. Velocities at other concentrations studied were then expressed as percentages of this maximum velocity. In this way, percentage vs concentration curves from different samples could be combined.

ADP concentration studies were performed with platelets in Tyrode's solution containing 0.5 mg/ml of fibrinogen and 4 mM $CaCl_2$. Fibrinogen

concentration studies were done using platelets in Tyrode's solution containing 4 mM Ca^{++} and then $2x10^{-5}$M (final concentration) of ADP added as aggregating agent. Ca^{++} and Mg^{++} concentration studies were performed using platelets in Tyrode's solution containing 0.5 mg/ml of fibrinogen. ADP added was also $2x10^{-5}$M. Mn^{++} and Sr^{++} were also investigated for their abilities to support aggregation induced by ADP.

A panel of aggregating agents including ADP, thrombin, epinephrine, A23187, arachidonic acid and ristocetin was used to survey the aggregability of the spherocyte and cocaine sphere.

2.2.11 Lectin Studies

Lectins and ADP were used to study the effect of neuraminidase treatment on the aggregation of platelets. Platelets ($2.5x10^{8}$/ml) were incubated with 0.04 IU/ml of neuraminidase (Vibrio cholerae) for 90 min at 37^{o} in Tyrode's solution (see Chapter 3 for more information). The list of lectins used in these experiments includes wheat germ agglutinin (WGA), jeguirity bean agglutinin (JBA) and the two castor bean agglutinins (RCA_{60} and RCA_{120}). Sugars N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and D-galactose were used for blocking experiments.

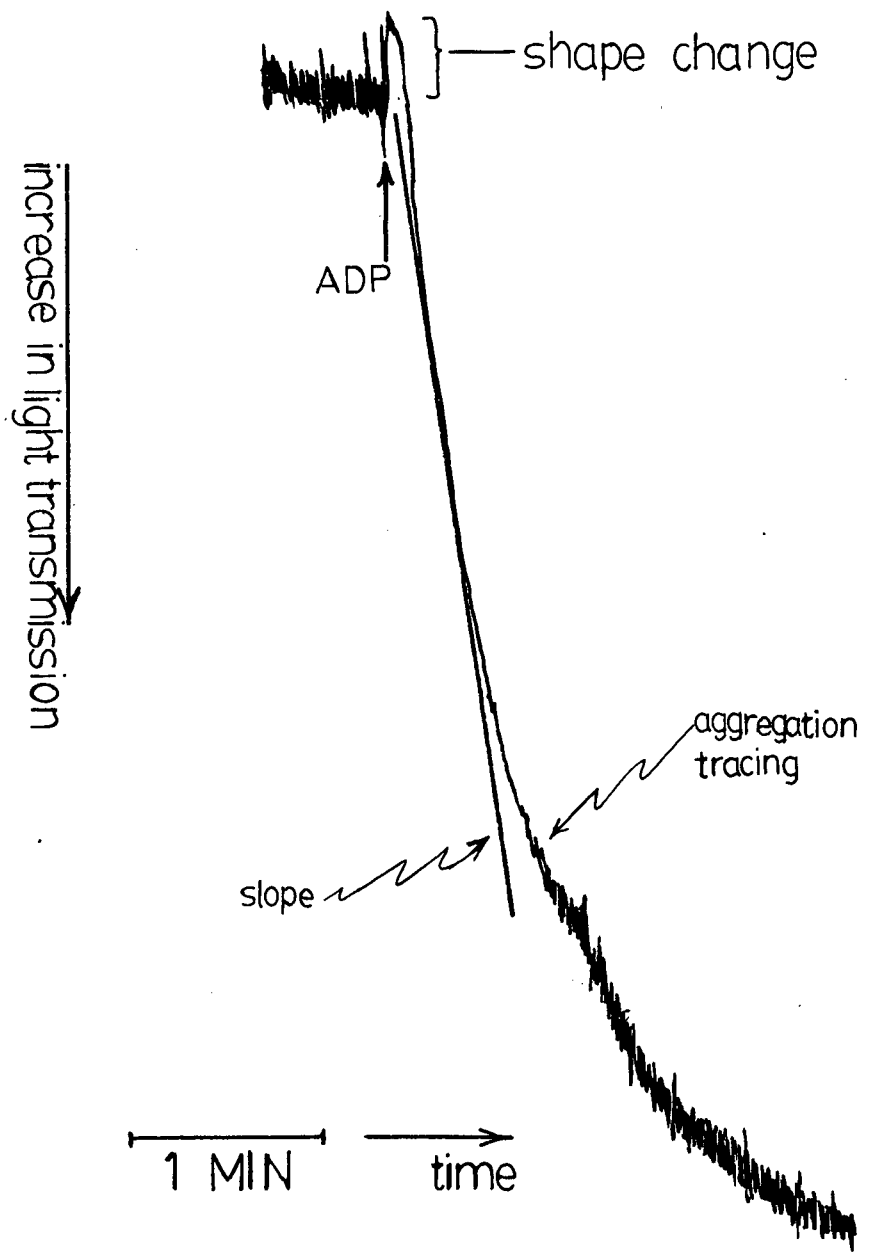
2.2.12 Statistical Methods

All statistical methods were according to Kalbfleisch (1974).

2.2.13 Materials

Fibrinogen was prepared and kindly supplied by Mr. Johan Janzen of our laboratory by precipitation with polyethylene glycol 6000 (Janzen, 1983). The fibrinogen was further purified by affinity chromatography using lysine-sepharose 4B and collagen-sepaharose 4B to remove plasminogen and fibronectin

Fig. 1. Example of a typical aggregation tracing. Aggregation velocity was found by drawing a line tangent to the steepest part of the tracing. The slope of this line was taken as the aggregation velocity. ADP is introduced into the platelet suspension as indicated by arrow. A rapid shape change phase occurred before aggregation.


shape change
ADP
increase in light transmission
aggregation tracing
slope
1 MIN
time

respectively. SDS-PAGE demonstrated no detectable contaminant and the preparation was considered to be 99% pure.

ADP, arachidonic acid, epinephrine, ristocetin sulfate, WGA, JBA, D-galactose, N-acetyl-D-glucosamine, N-acetyl-galactosamine, NADH and sodium phruvate were all from Sigma (St. Louis, Missouri).

The beta-thromboglobulin RIA kit and ^{14}C-serotonin were from Amersham (Arlington Heights, Illinois). The neuraminidase, both castor bean agglutinins and A23187 were from Calbiochem (La Jolla, California). Thrombin was from Park, Davis and Co. of Detroit, Michigan. Cocaine hydrochloride was purchased through the UBC Department of Family Practice pharmacy from Allen and Hanburys of Toronto, Ontario.

Arachidonic acid and A23187 were dissolved in ethanol. All others were dissolved in isotonic saline (pH 7.4) solution. Appropriate ethanol controls were included in all relevant experiments.

Calcium, magnesium, strontium and manganese were added as the chloride.

2.3 RESULTS

2.3.1 Platelet Dimensions and Morphology

Under phase microscopy, platelet discocytes could be seen to tumble through the field of view demonstrating their discoid shape. No differences were observed in the morphology of discocytes in platelet-rich plasma or in Tyrode's solution. Therefore the washing procedure proved to be quite satisfactory.

Addition of 2×10^{-5} M of ADP to the platelet suspension produced the echinocytes (Fig. 2). They appeared more "stationary" than the discocytes. Careful observations by focusing the microscope up and down showed long slender pseudopods which were extremely difficult to photograph. Echinocytes appeared as single platelets in suspension. Small aggregates would form if the suspension was shaken or stirred even in the absence of Ca^{++} and fibrinogen. Hypotonic shock at 75 mOsm produced the spherocytes which were much larger than the spheres induced by treatment with 10 mM of cocaine (Fig. 2).

Table 1 shows the measured diameters for the discocyte, spherocyte and cocain sphere. The discocyte has a mean diameter of 3.18×10^{-4} cm and a thickness of 0.89×10^{-4} cm. The thickness to diameter ratio is therefore 0.28. Its surface area and volume were calculated to be 16.48×10^{-8} cm^2 and 4.71×10^{-12} cm^3 respectively. Since the discocyte tumbles through the field of view, only platelet images considered as presenting the edge-on view were measured for thickness. Discocytes in PRP had similar dimensions. Table 1 also shows the spherocyte has a 123% and a 345% increase in surface area and volume respectively over the discocyte. The cocaine sphere is much smaller than the spherocyte and its increase in volume and area over the discocyte is not as large. No differences were found between fixed and non-fixed

Fig. 2. Phase photomicrographs of different forms of platelets.

(a) Discocytes (x1200 magnification*)

(b) Echinocytes (x1400 magnification)

(c) Spherocytes (x1000 magnification)

(d) Cocaine spheres (x1200 magnification)

*Magnification here and in all following figures depicts magnification of the platelets as they appear on the photographic prints.

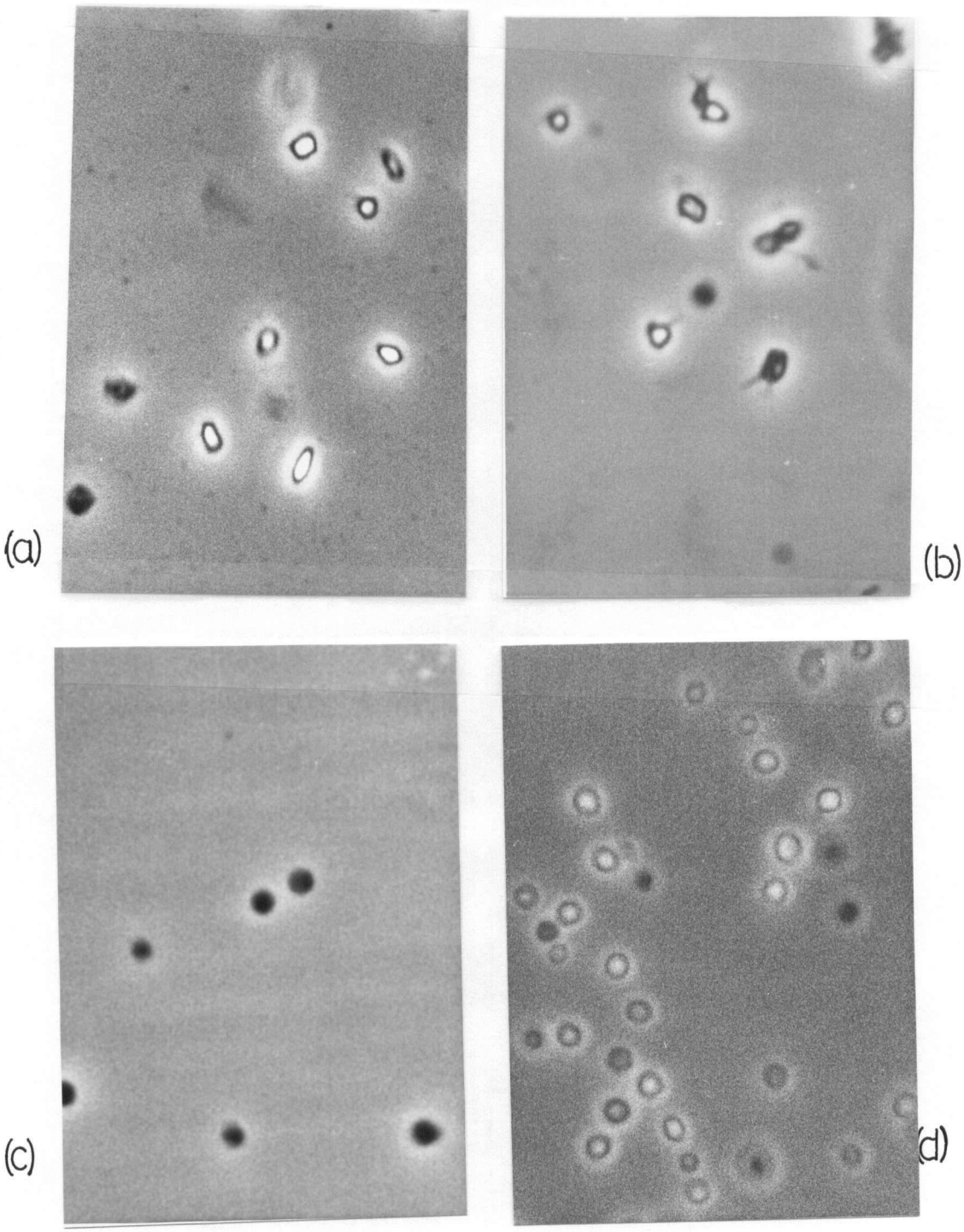

(a)
(b)
(c)
(d)

Table 1. Physical Dimensions of Washed Platelets*

	Diameter+ (cm)	Surface Area (cm^2)	Volume (cm^3)
Discocyte**	3.18×10^{-4} ($\pm 0.03 \times 10^{-4}$)++	16.48×10^{-8} ($\pm 0.35 \times 10^{-8}$)	4.71×10^{-12} ($\pm 0.14 \times 10^{-2}$)
Spherocyte	3.42×10^{-4} ($\pm 0.017 \times 10^{-4}$)	36.7×10^{-8} ($\pm 0.36 \times 10^{-8}$)	20.94×10^{-12} ($\pm 0.31 \times 10^{-12}$)
Cocaine spheres	2.90×10^{-4} ($\pm 0.025 \times 10^{-4}$)	26.38×10^{-8} ($\pm 0.45 \times 10^{-8}$)	12.77×10^{12} ($\pm 0.33 \times 10^{-12}$)

*Measured diameter of discoycte in PRP=3.17×10^{-4}cm ($\pm 0.035 \times 10^{-4}$)
+1000 platelets measured in each category
**Thickness for discocyte=0.89×10^{-4} cm ($\pm 0.06 \times 10^{-4}$ cm; n=130)
++Standard deviation

platelets. Addition of ADP ($2x10^{-5}M$) to the spherocytes and cocaine spheres produced no further change.

Transmission electronmicrographs of platelet discocytes, echinocytes, spherocytes and the cocaine spheres are shown in Figs. 3 to 6. At least 20 fields of view for each type of platelet were examined. All the platelets appeared to have retained their complement of granules. The discocytes (Fig. 3) ranged in appearance from discs (top view) to cigar shapes (edge-on view). The echinocytes (Fig. 4) were irregular in shape with long pseudopods. The spherocytes (Fig. 5) were large and swollen but the plasma membranes were intact. The spherocyte cytoplasm was less dense and had an "empty" appearance. There were large vacuoles apparent besides the storage granules. Because of the "empty" appearance of the cytoplasm the plasma membrane as well as the granules and vacuoles were highlighted better than in the discocytes and echinocytes. The cocaine spheres (Fig. 6) were round but smaller than the spherocyte.

The surface-connected canalicular system was obvious in the discocyte. Numerous openings to the outside could be seen. The canalicular system in the echinocytes was dilated. In the cocaine spheres dilation of the canalicular channels was even more obvious. No opening connecting the dilated channels of the echinocyte and cocaine spheres to the outside could be seen.

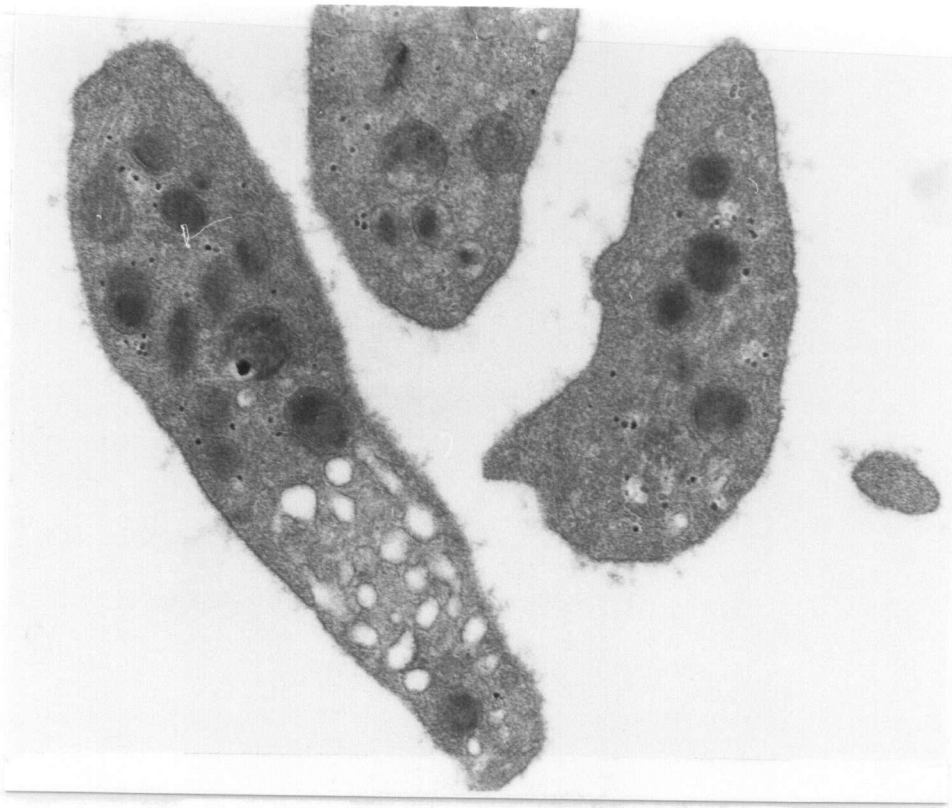
2.3.2 Platelet Integrity and Release of Contents

The supernatants of washed discocytes showed no sign (zero) of lactate dehydrogenase (LDH) activity. After the platelet suspension had been frozen and thawed once to damage the platelets, LDH activity was detected in the supernatant at 1.047 Wroblewski unit per ml per 10^6 platelets ($\pm$0.350 s.d.; n=3). This is considered as the total platelet LDH activity. After

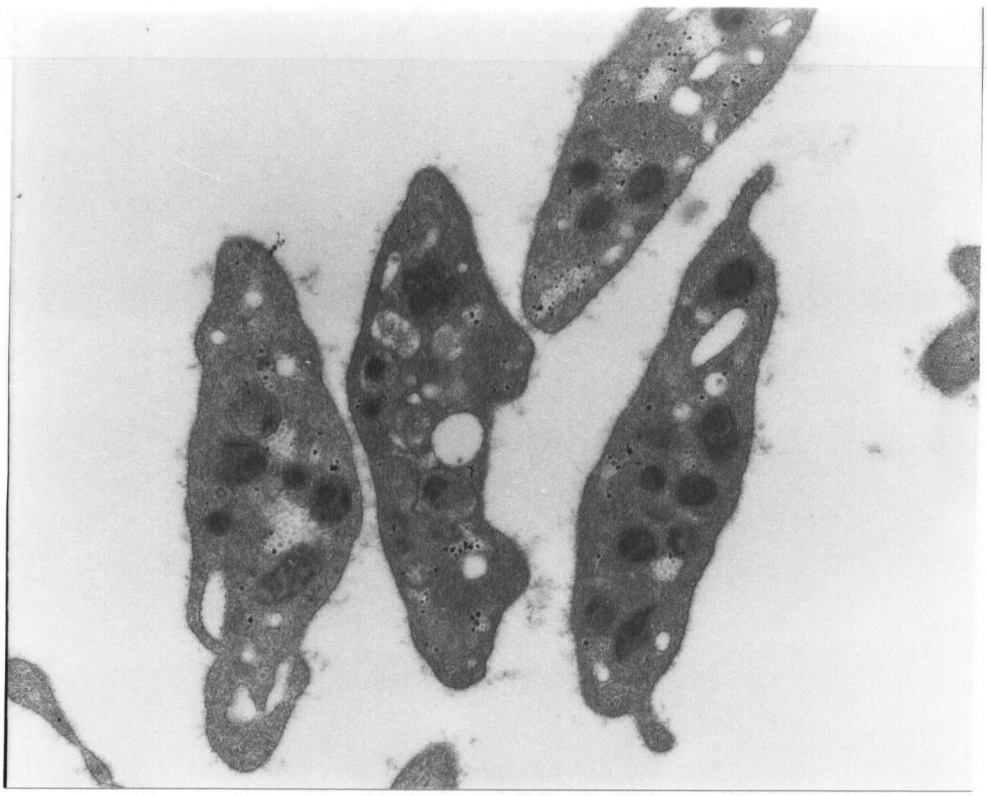
Fig. 3. Electron micrographs of platelet discocytes.

(a) x33600 magnification

(b) x22000 magnification



a



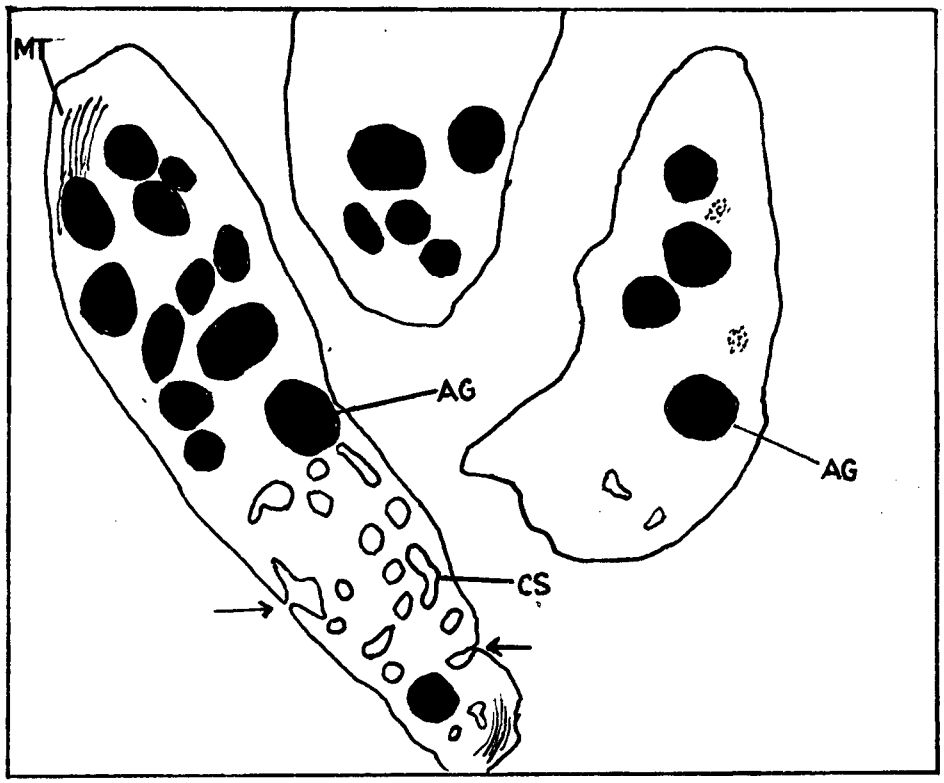
b

Fig. 3 (continued)

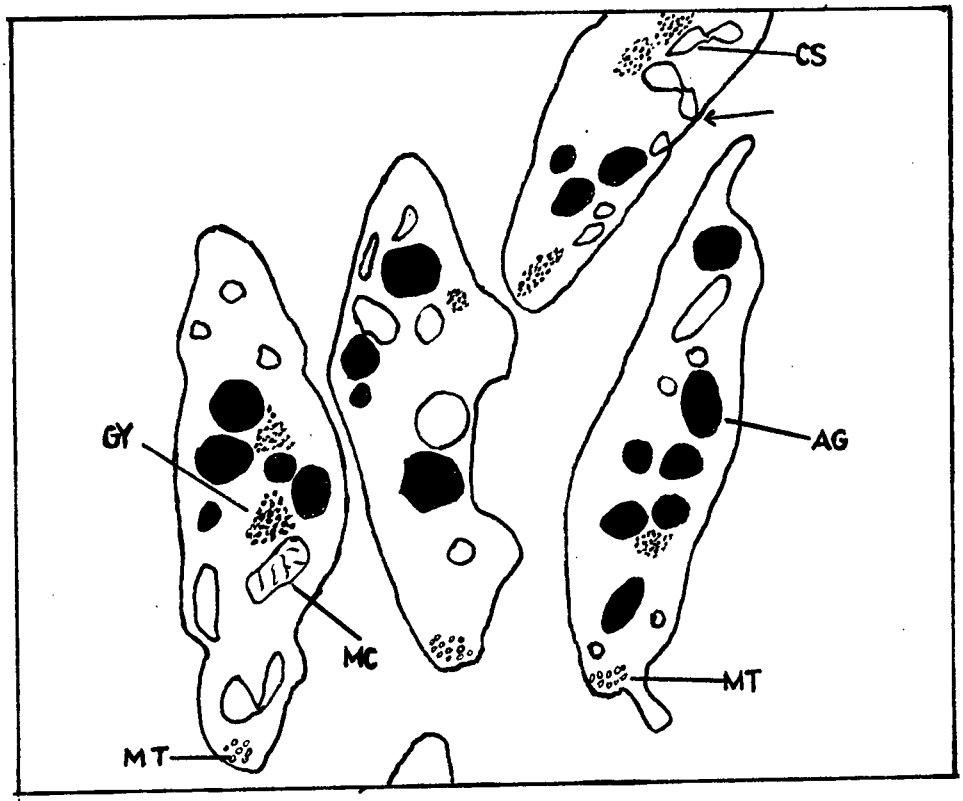
(c) schematic representation of (a)

(d) schematic representation of (b)

Two prominent features are the channels of the surface-connected canalicular system (CS) with openings on the platelet surface (arrows) and the alpha granules (AG). Microtubules (MT) from the circumplatelet bundles can be seen. Also visible are glycogen deposits (GY) as well as an occasional mitrochondrion (MC).



C

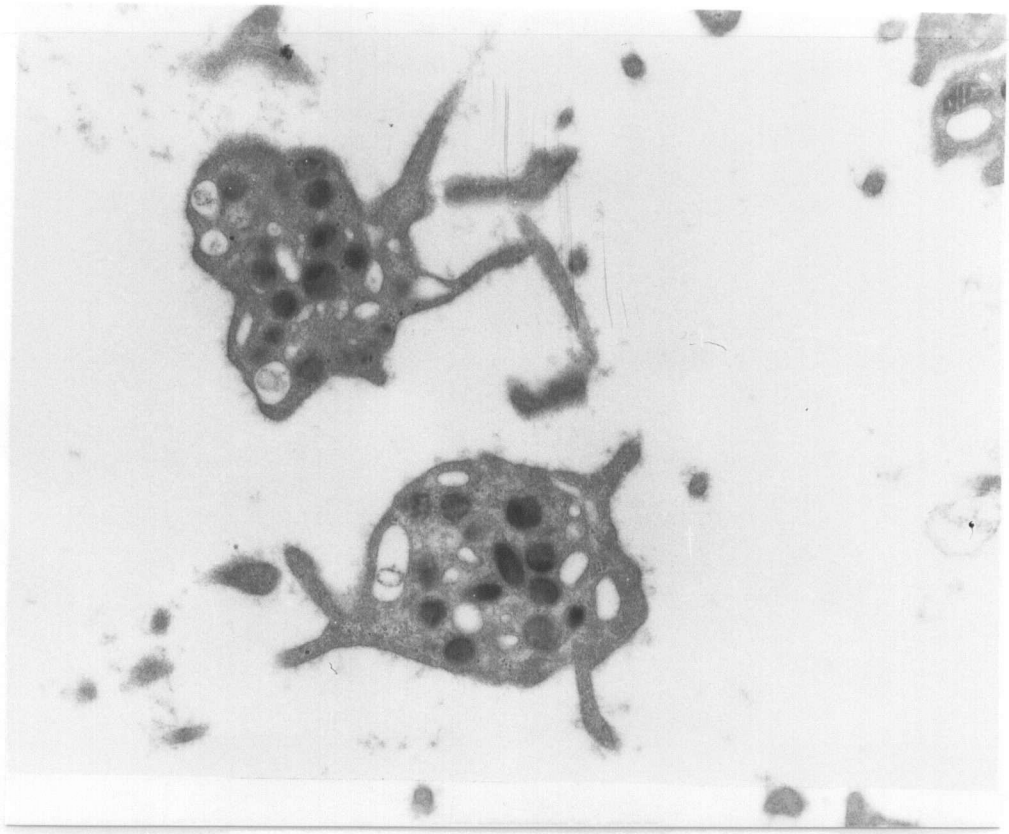


d

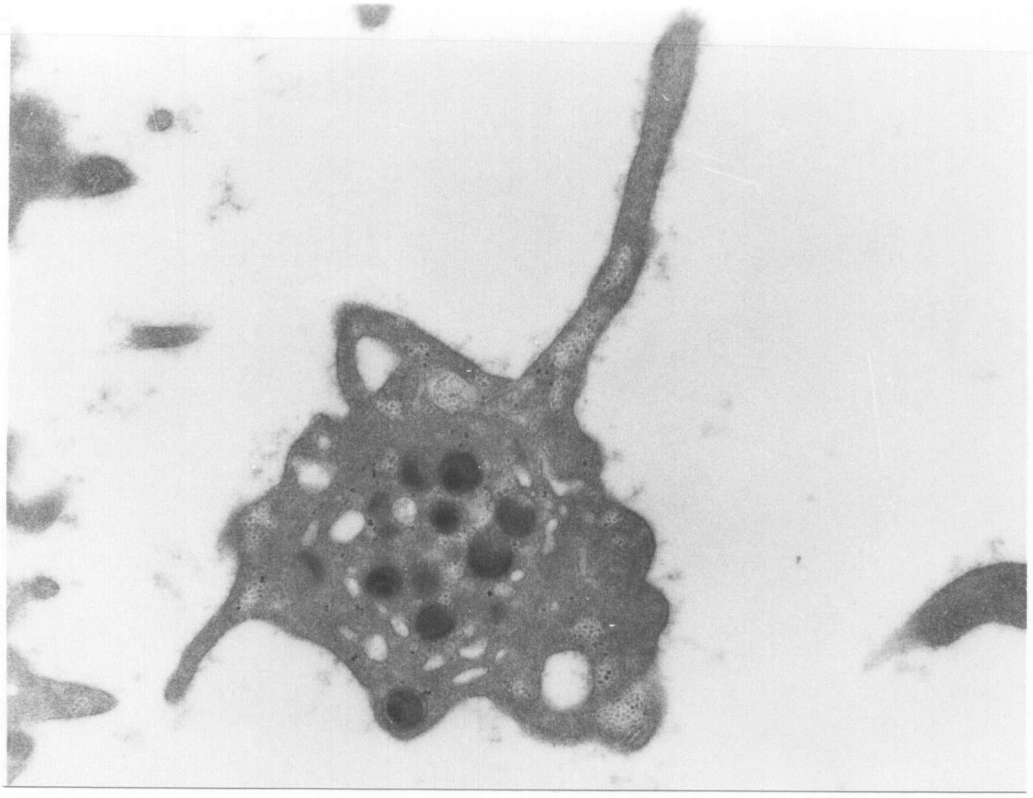
Fig. 4. Electron micrographs of platelet echinocytes.

(a) x16900 magnification

(b) x27600 magnification



a



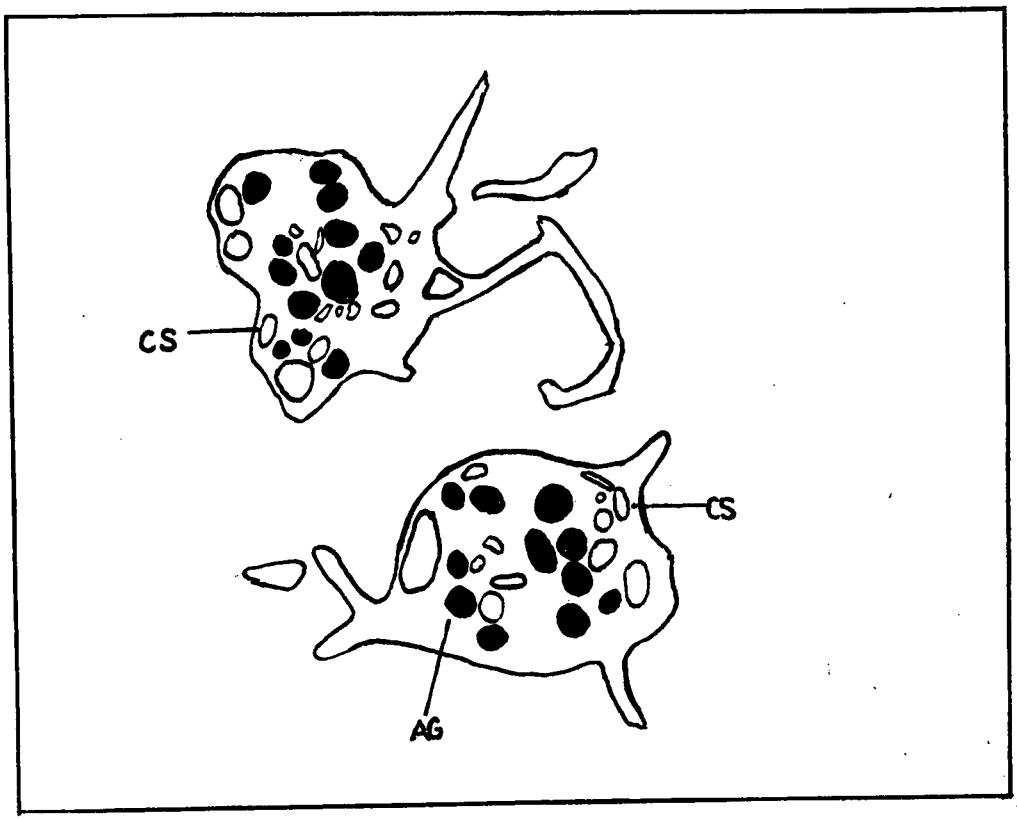
b

Fig. 4 (continued)

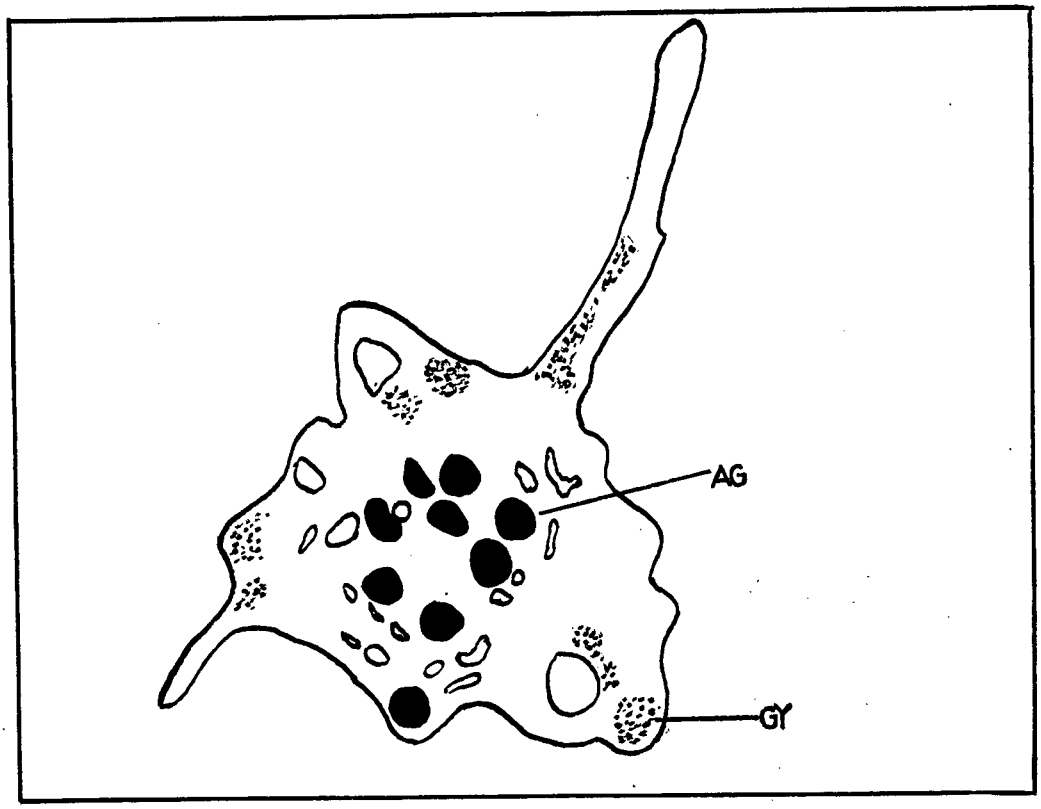
(c) schematic representation of (a)

(d) schematic representation of (d)

The echinocytes are irregular in shape with long pseudopods. The surface-connected canalicular system (CS) is dilated. The granules (AG) as well as the glycogen deposits (GY) can still be seen.



C

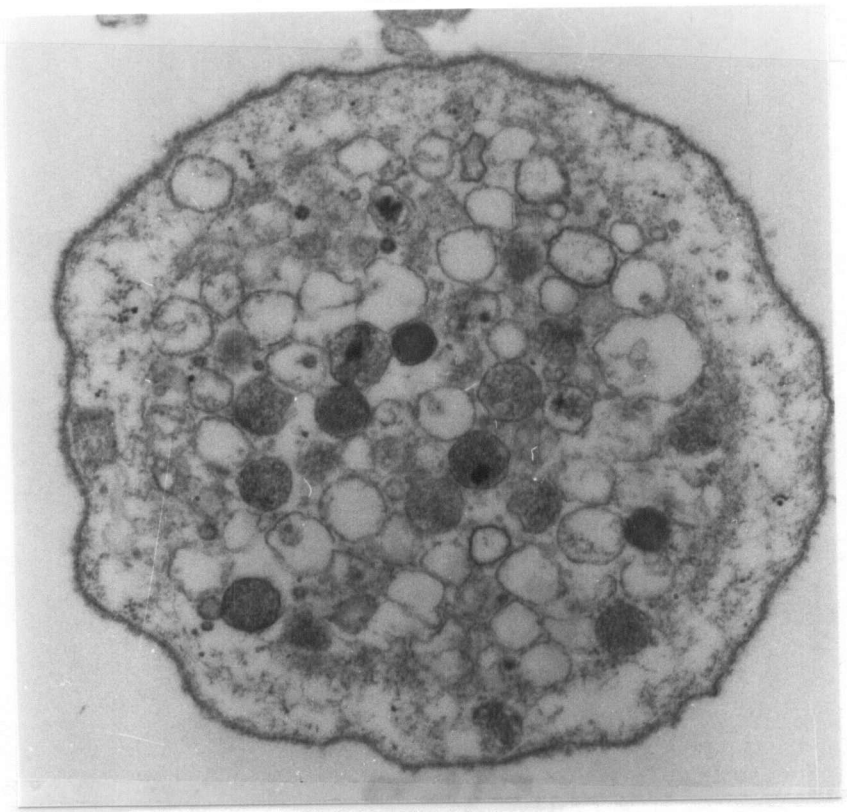


d

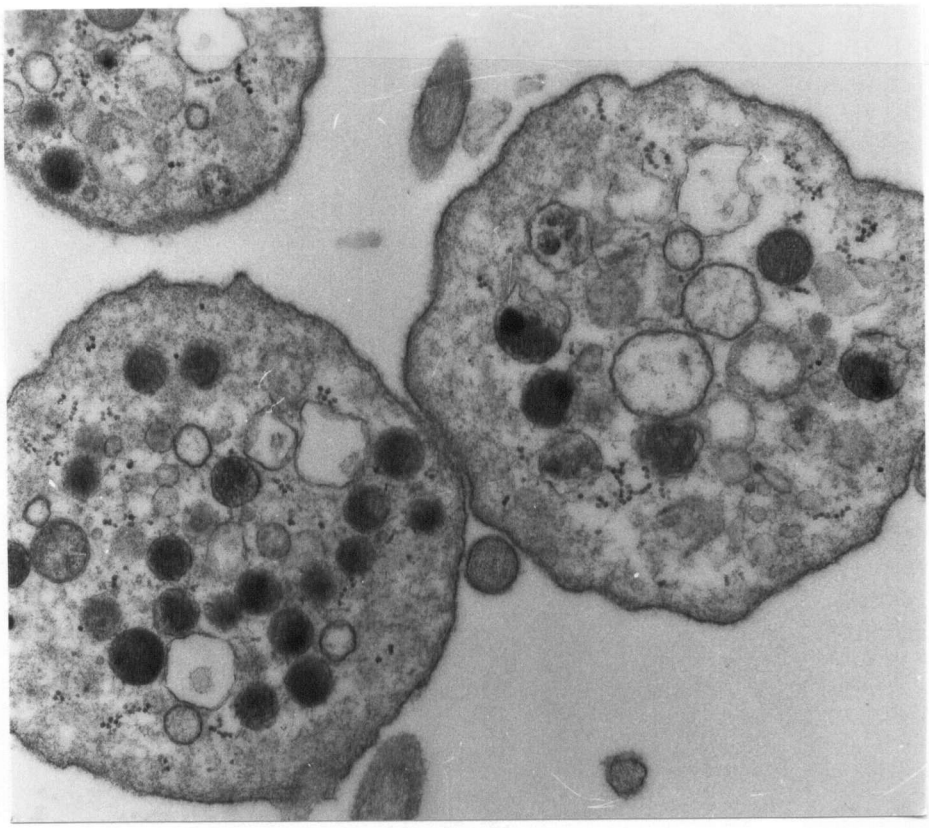
Fig. 5. Electron micrographs of platelet spherocytes.

(a) x29500 magnification

(b) x20800 magnification



a



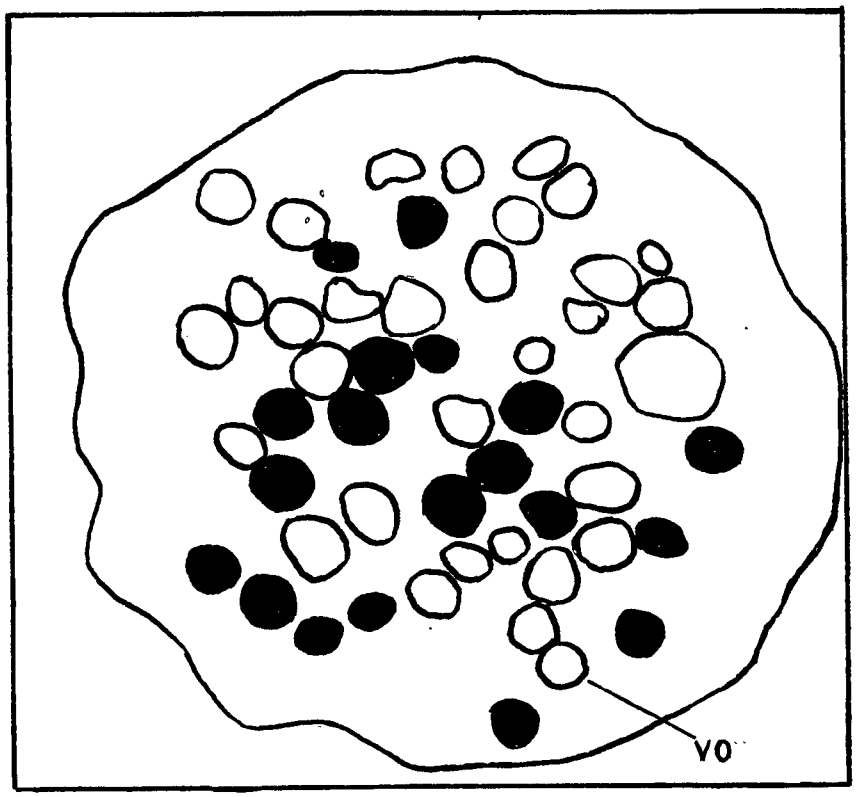
b

Fig. 5 (continued)

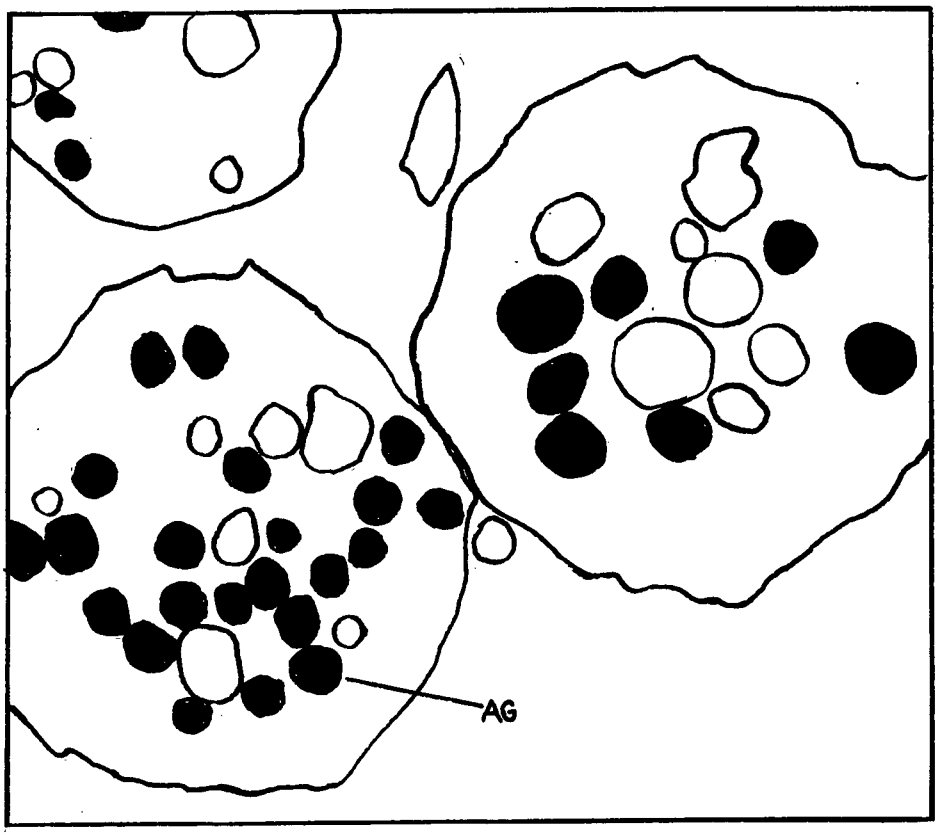
(c) schematic representation of (a)

(d) schematic representation of (b)

The platelets are swollen but the plasma membranes appear intact. There are swollen vacuoles (VO) as well as storage granules (AG).



C

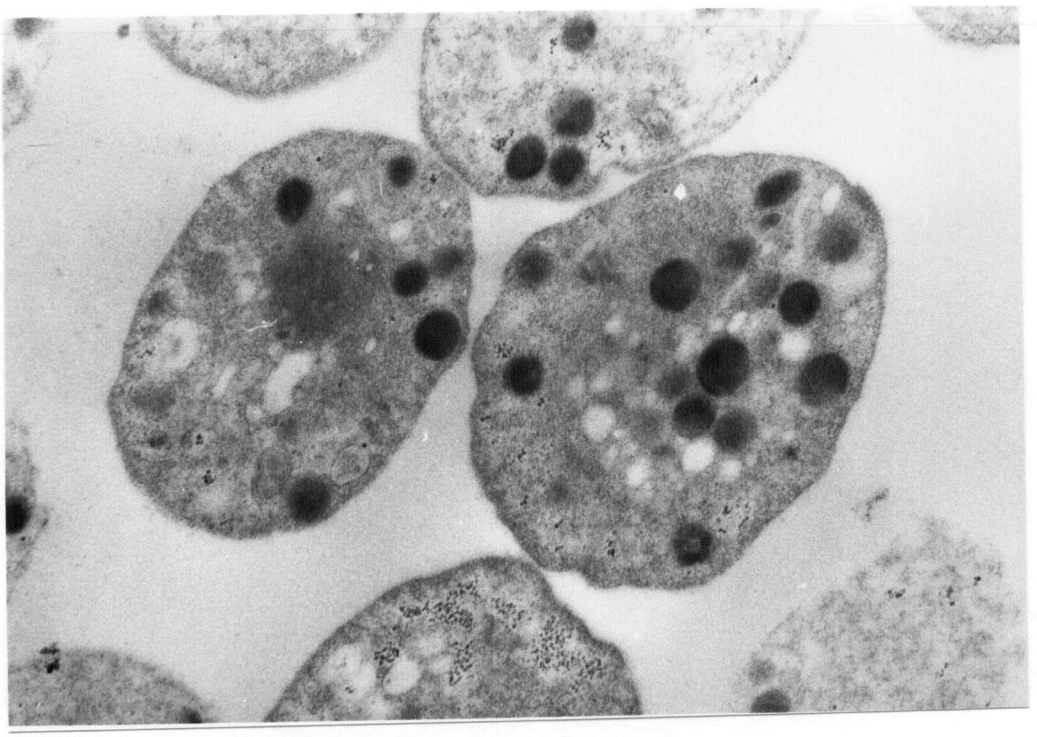


d

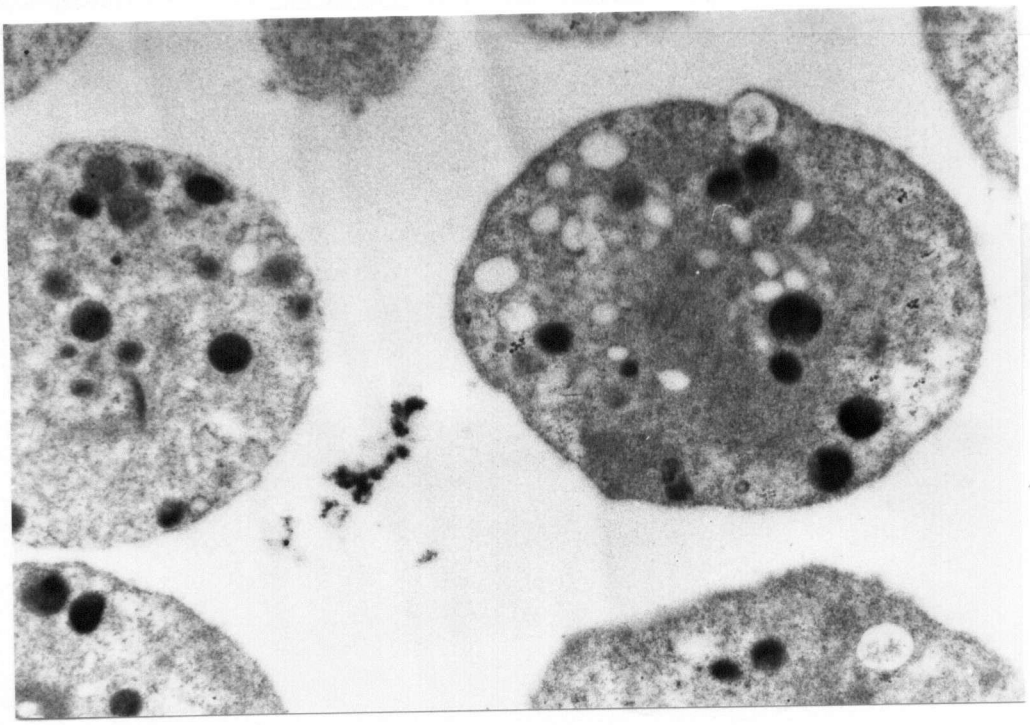
Fig. 6. Electron micrographs of the cocaine spheres.

(a) x18200 magnification

(b) x20800 magnification



a



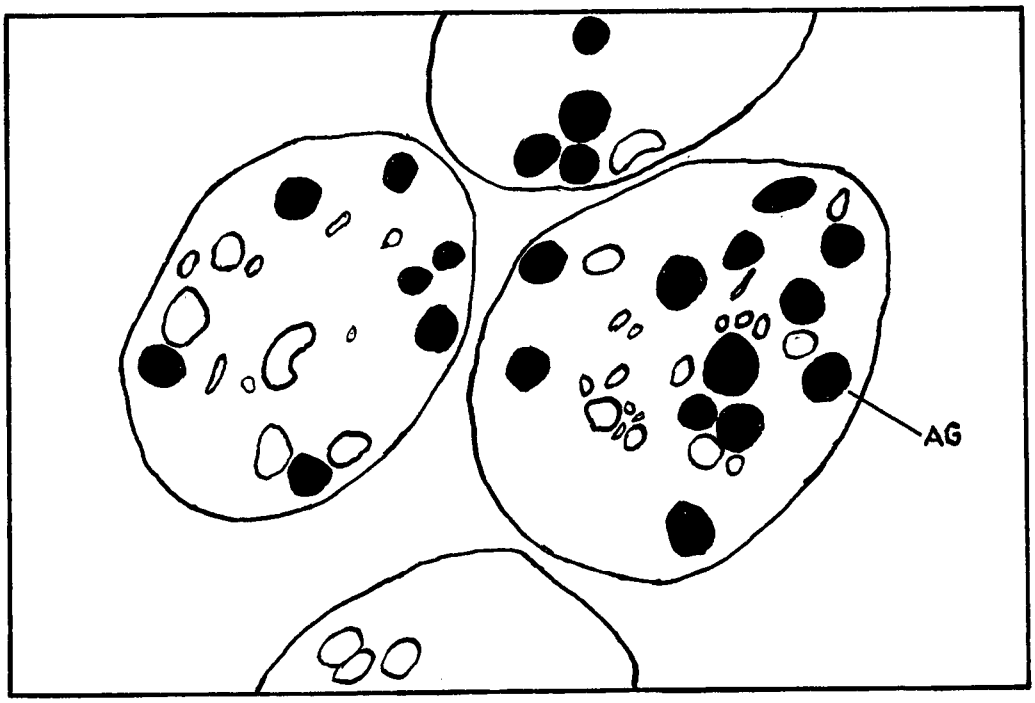
b

Fig. 6 (continued)

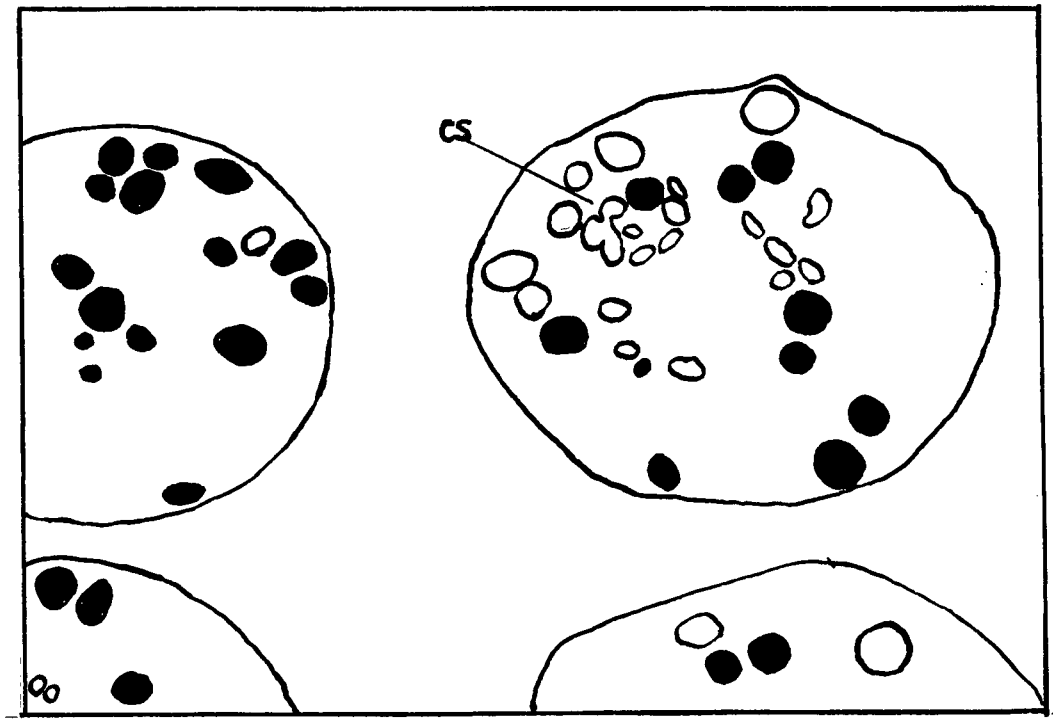
(c) schematic representation of (a)

(d) schematic representation of (d)

The platelets here are spherical but much smaller than the spherocytes. Storage granules (AG) are present and channels of the canalicular system (CS) can be seen.



C



d

transformation to echinocytes with ADP and to spheres with cocaine the supernatant remained negative. A more detailed timed study of LDH leakage from the spherocytes was done and shown in Fig. 7. After 90 min of hypotonic shock about 3.87% (±0.80%) of the total platelet LDH activity was found in the supernatant. The percentage increased to 10.19% (±1.70%) after 2½ hours and 25.51% (±1.23%) after 4 hours.

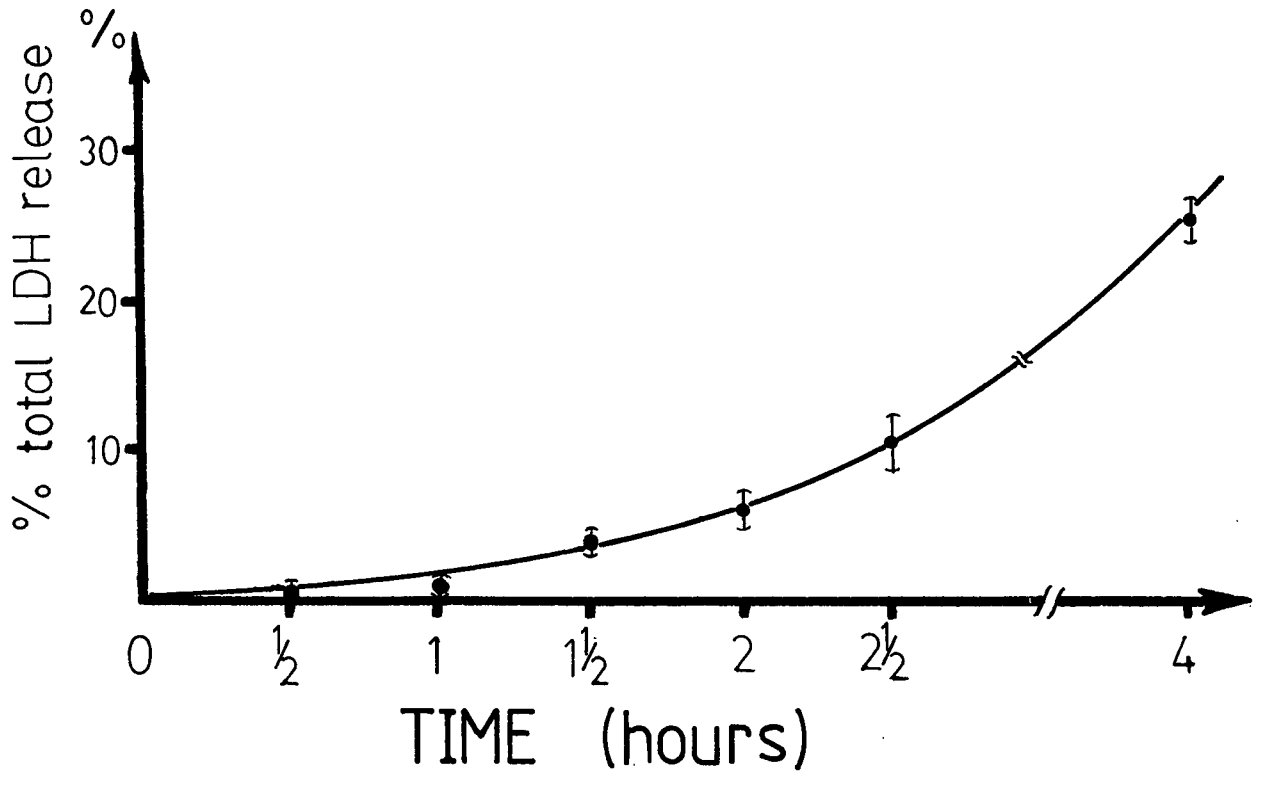
The platelet release experiments consisted of two parts, the ^{14}C-serotonin released from dense bodies and beta-thromboglobulin (beta-Tg) released from alpha-granules. It was found that practically no release of ^{14}C-serotonin had occurred after echinocyte and spherocyte transformations. Nearly all of the radioactivity (97.8% in echinocyte and 97.5% in spherocyte) could be recovered from the pellets after centrifugation.

The mean beta-Tg content of washed human discocytes from two determinations was 22.21 $\mu g/10^9$ platelets (22.23 and 22.19 $\mu g/10^9$ platelets). About 13% of this was found in the supernatant of the washed platelets as free beta-Tg. It was possible that some release of beta-Tg had occurred during the washing procedure. Additional releases of 4% during echinocyte transformation and 27% during spherocyte transformation were also found. If the discocytes were stimulated with 0.4 NIH units/ml of thrombin, 94% of their beta-Tg contents was released.

2.3.3 Platelet Aggregations

Fig. 8 demonstrates how aggregation velocity increased with ADP concentration. The curve was constructed from aggregation measurements of five different samples of washed platelets suspended in Tyrode's solution containing 0.5 mg/ml of fibrinogen and 4 mM Ca^{++}. Aggregation velocity was about maximum at 2×10^{-5}M ADP. Aggregations at this concentration were therefore

Fig. 7. LDH leakage from the spherocytes (as percentage of total platelet LDH activity) as a function of time of suspension in hypotonic Tyrode's solution. Values are means of three series of experiments; error bars indicate ± 1 standard deviation.


% total LDH release
%
30
20
10
0
½
1
1½
2
2½
4
TIME (hours)

considered as 100% in all samples for the normalization procedure mentioned before. This concentration was also used throughout this project for echinocyte transformation. An example of the actual aggregation tracings showing the effect of different ADP concentration is shown in Fig. 9.

The effect of fibrinogen concentration on aggregation velocity is shown in Fig. 10. In this case fibrinogen and 4 mM of Ca^{++} ions were added to the platelet suspensions before ADP ($2x10^{-5}M$). Maximum velocity achieved at 0.5 mg/ml of fibrinogen was designated as 100% according to the normalization procedure above. Fig. 10 is a composite of five different series of concentration experiments.

The curves showing the effect of divalent cation concentration on the aggregation velocity are shown in Fig. 11. Fibrinogen at 0.5 mg/ml together with either Ca^{++} or Mg^{++} ions were added to the platelet suspensions before ADP ($2x10^{-5}M$). A biphasic effect was observed in both the Ca^{++} and Mg^{++} curves with maximum aggregation at 4 mM of Ca^{++} ion. Velocity of this optimum concentration therefore serves as the 100% aggregation velocity for both the Ca^{++} and Mg^{++} series. In this way, it can be demonstrated that the ability of Mg^{++} ion to support aggregation is only about half that of Ca^{++} ion throughout the concentrations tested. Examples of individual aggregation tracings are shown in Fig. 12.

Two more divalent cations, strontium and manganese were tested for their abilities to support ADP induced platelet aggregation. In the presence of 0.5 mg/ml of fibrinogen, Sr^{++} ions at concentrations between 0-20 mM failed to support ADP ($2x10^{-5}M$) induced aggregation (Fig. 12). If added to a system already containing 4 mM of Ca^{++} ions, Sr^{++} would neither augment nor reduce the supportive role of Ca^{++} ions in ADP induced aggregation.

Fig. 8. The effect of ADP concentration on the aggregation velocity of washed platelets suspended in Tyrode's solution containing 0.5 mg/ml of fibrinogen and 4 mM Ca^{++}. Aggregation velocity at $2x10^{-5}$M ADP is taken as 100% and velocities at lower concentrations are expressed as percentages of it. Values are means of five series of experiments; error bars indicate $\pm$1 standard deviation.

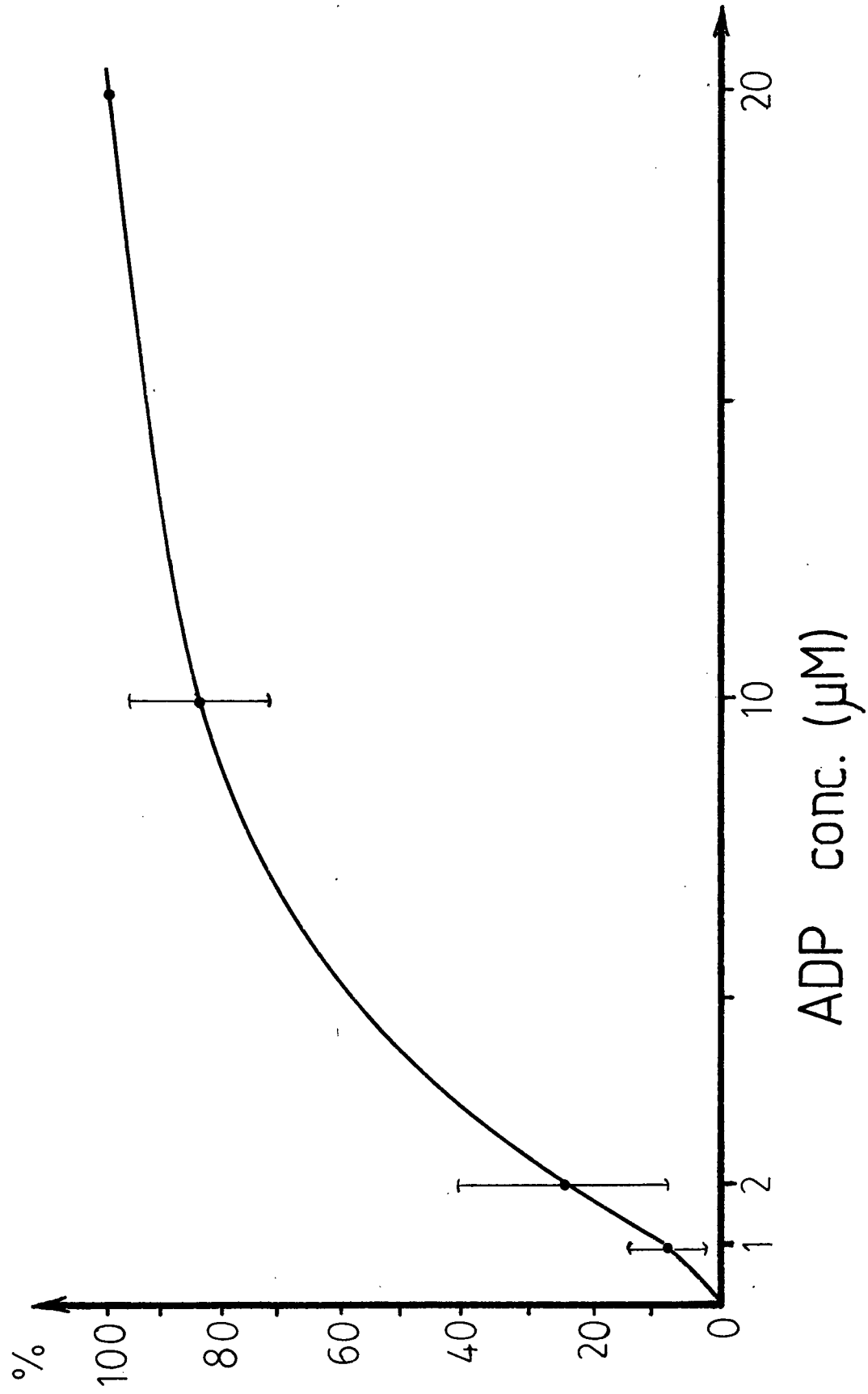

%
100
80
60
40
20
0
1
2
10
20
ADP conc. (µM)

Fig. 9. Examples of aggregation tracings showing the effect of ADP concentration on platelet aggregation velocity. ADP at (a) $2x10^{-5}M$; (b) $2x10^{-6}M$ or (c) $1x10^{-6}M$ was added at arrow.

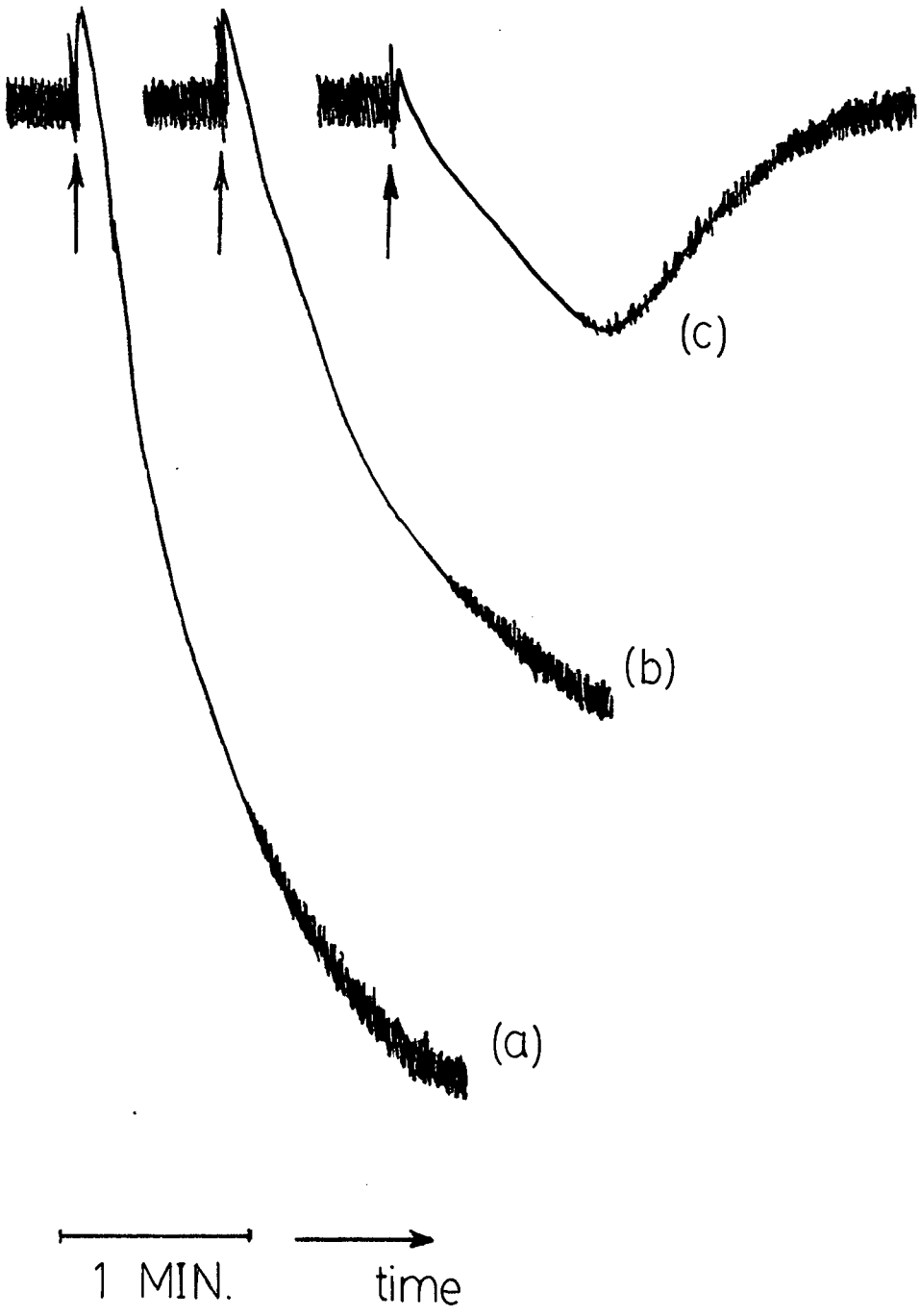

(c)
(b)
(a)
1 MIN.
time

Fig. 10. The effect of fibrinogen concentration on the aggregation velocity of platelets. Washed platelets were suspended in Tyrode's solution containing fibrinogen and 4 mM Ca^{++} ions. Aggregation was induced by $2x10^{-5}$M of ADP. Aggregation velocity at 0.5 mg/ml of fibrinogen was taken as 100% and velocities at lower concentrations were expressed as percentages of it. Values are from means of five series of experiments; error bars indicate $\pm$1 standard deviation.

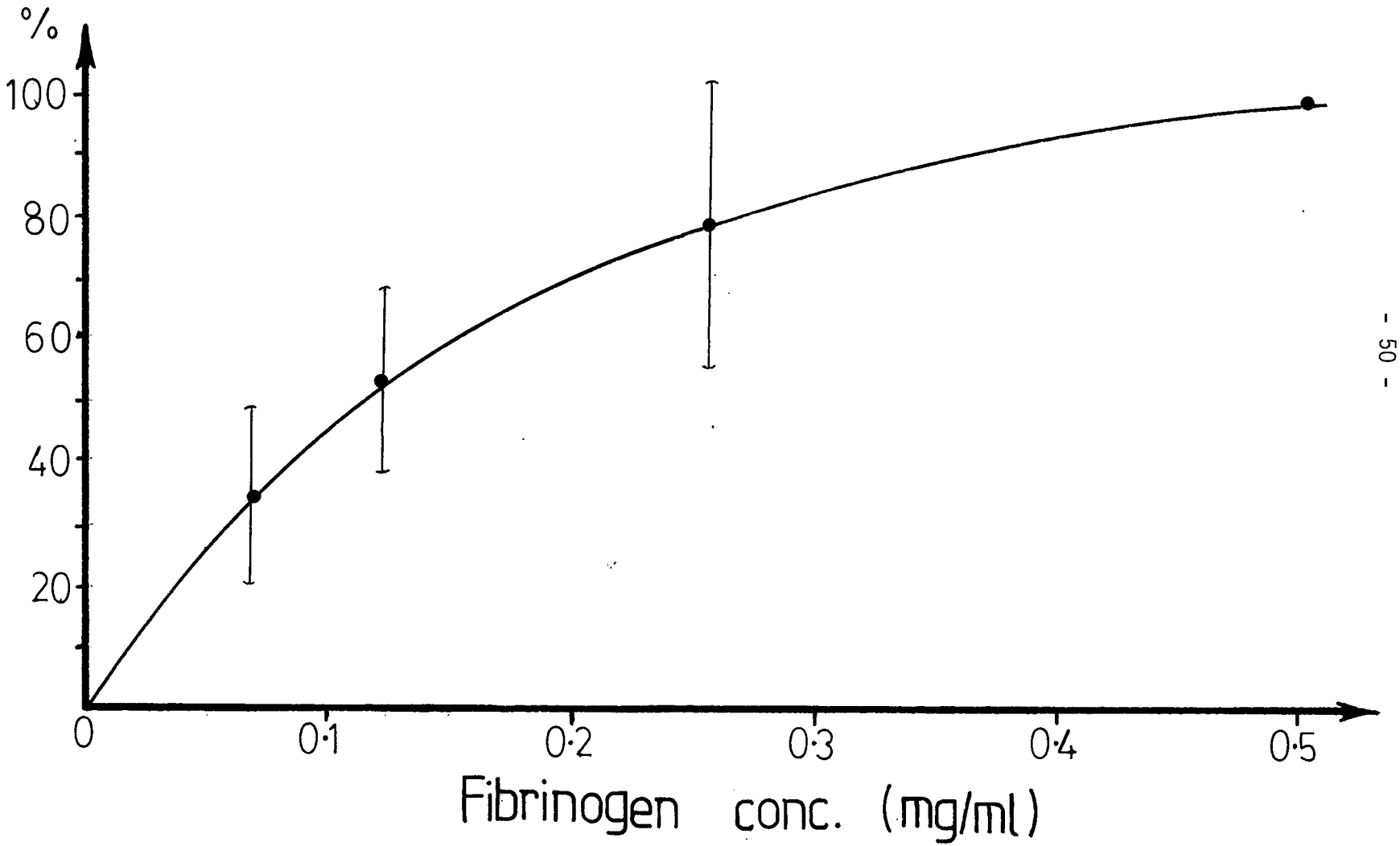

%
100
80
60
40
20
0
0·1
0·2
0·3
0·4
0·5
Fibrinogen conc. (mg/ml)

Fig. 11. The effect of divalent cation concentration on the aggregation velocity of platelets: Ca^{++} (-●-), Mg^{++} (-o-) and Sr^{++} (-Δ-). Washed platelets were suspended in Tyrode's solution containing 0.5 mg/ml fibrinogen and one of the cations. Aggregation was induced by $2x10^{-5}$M of ADP. Aggregation velocity at 4 mM Ca^{++} ions is taken as 100%. Values are means from five series of experiments; error bars indicate ± 1 standard deviation.

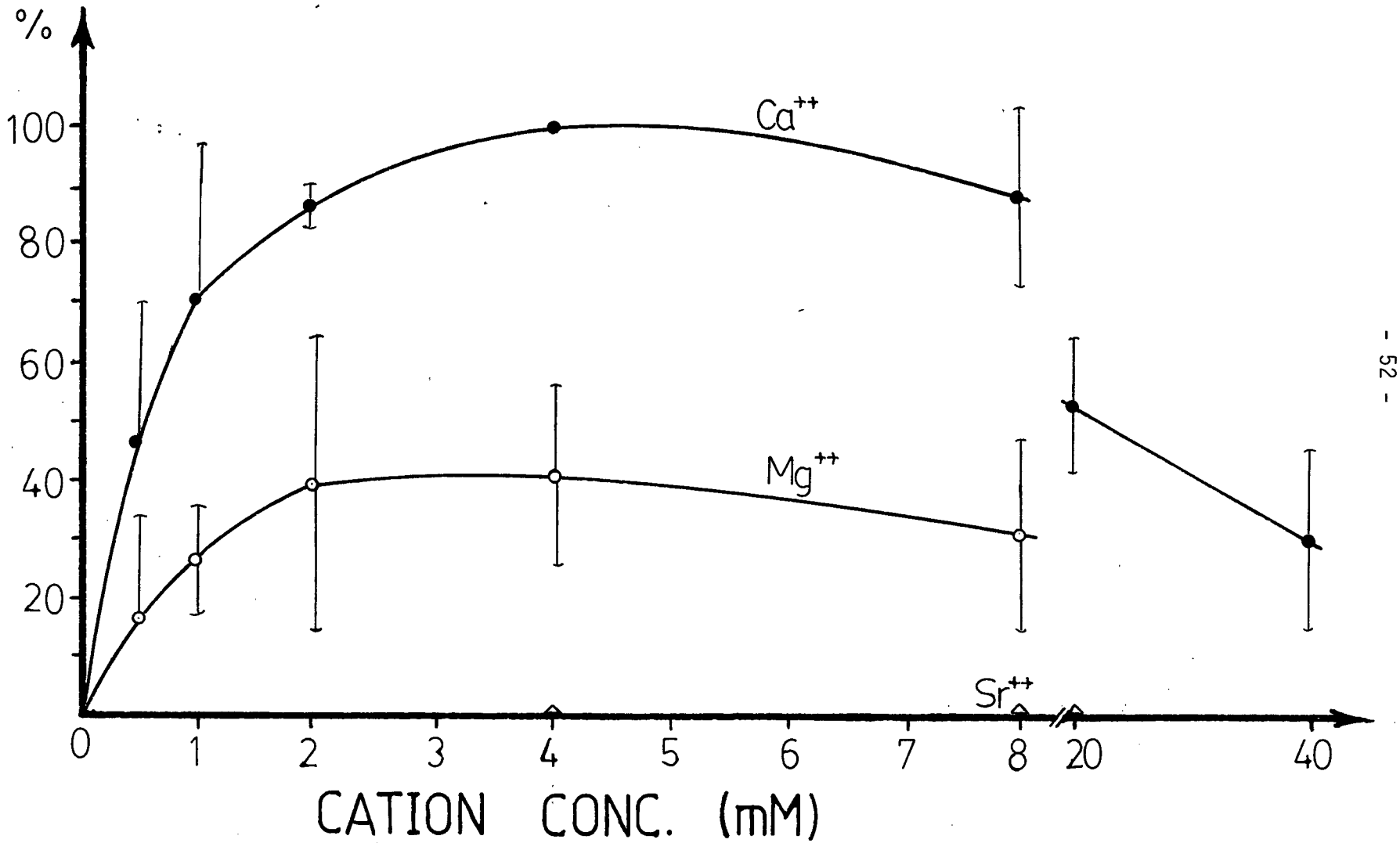
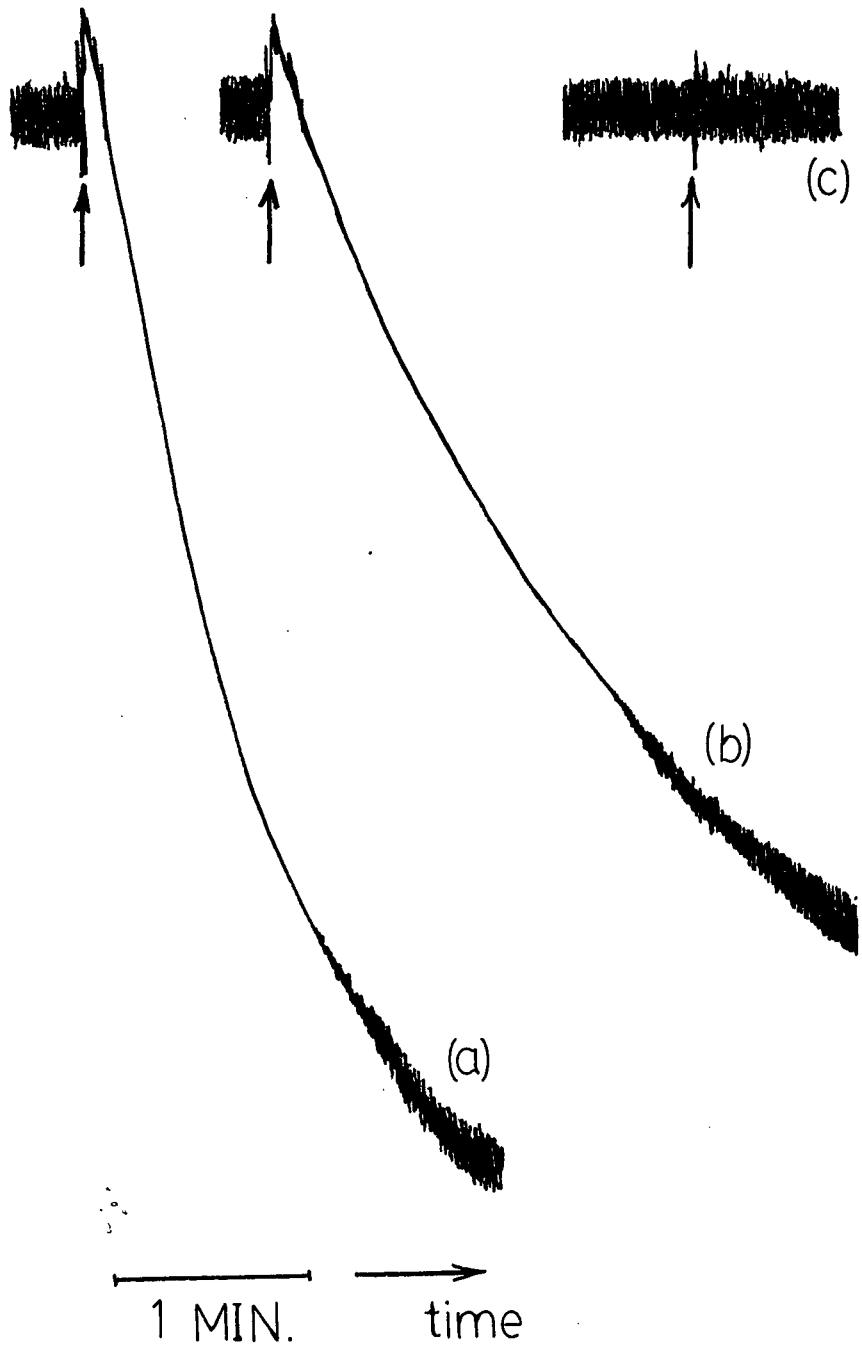

%
100
80
60
40
20
Ca++
Mg++
Sr++
0
1
2
3
4
5
6
7
8
20
40
CATION CONC. (mM)

Fig. 12. Examples of aggregation tracings showing the effects of different divalent cations. Platelets were suspended in Tyrode's solution containing 0.5 mg/ml of fibrinogen and 4 mM (a) Ca^{++} ions; (b) Mg^{++} ions or (c) Sr^{++} ions. ADP at $2x10^{-5}M$ was added as indicated by arrows.


(c)
(b)
(a)
1 MIN.
time

It was found that Mn^{++} ion was an inhibitor of platelet aggregation. Aggregation induced by $2x10^{-5}M$ of ADP in the presence of 0.5 mg/ml of fibrinogen and 4 mM Ca^{++} ions was greatly reduced by 2.5 mM and completely inhibited by 10 mM Mn^{++} ions (Fig. 13). Aggregation induced by 0.1 NIH unit/ml of thrombin or by $1x10^{-4}M$ of arachidonic acid was also fully inhibited by 10 mM of Mn^{++} ions.

A panel of aggregating agents including ADP ($2x10^{-5}M$ with 0.5 mg/ml of fibrinogen and 4 mM of Ca^{++} ions), epinephrine ($5x10^{-5}M$ with 0.5 mg/ml of fibrinogen and 4 mM of Ca^{++} ions), thrombin (0.1 NIH unit/ml), A23187 ($1x10^{-5}M$) and arachidonic acid ($1x10^{-3}M$) were tested for their abilities to aggregate spherocytes. They all failed to do so. These aggregating agents at the concentrations indicated, would strongly aggregate normal discoid platelets. The cocaine spheres also failed to aggregate upon stimulations with ADP, thrombin and arachidonic acid (same concentrations as above).

Although spherocytes have lost their ability to aggregate their reaction to ristocetin, an agglutinating agent, is quite different. When 1.5 mg/ml of ristocetin alone was added to either discocyte or spherocyte there was no response. If plasma was added to provide for the von Willebrand's factor then agglutination occurred in both spherocyte and discocyte samples. In fact, the agglutination of the spherocytes was twice the rate (velocity) of the discocytes (Fig. 14). Plasma was added at a ratio of 0.1 ml per ml of platelet suspension.

Under phase microscopy discocytes treated with neuraminidase still retained their discoid shape. Tested at $2x10^{-5}M$ of ADP (with 0.5 mg/ml of fibrinogen and 4 mM of Ca^{++} added) the aggregation rates were the same for the treated and non-treated discocytes (Fig. 15).

Fig. 13. The inhibitory effect of Mn^{++} on platelet aggregation. Washed platelets were suspended in Tyrode's solution containing 4 mM Ca^{++} and 0.5 mg/ml of fibrinogen. Mn^{++} at (a) 0 mM; (b) 2.5 mM or (c) 10 mM was then added. Aggregation was induced by adding $2x10^{-5}$M of ADP as indicated by arrows.

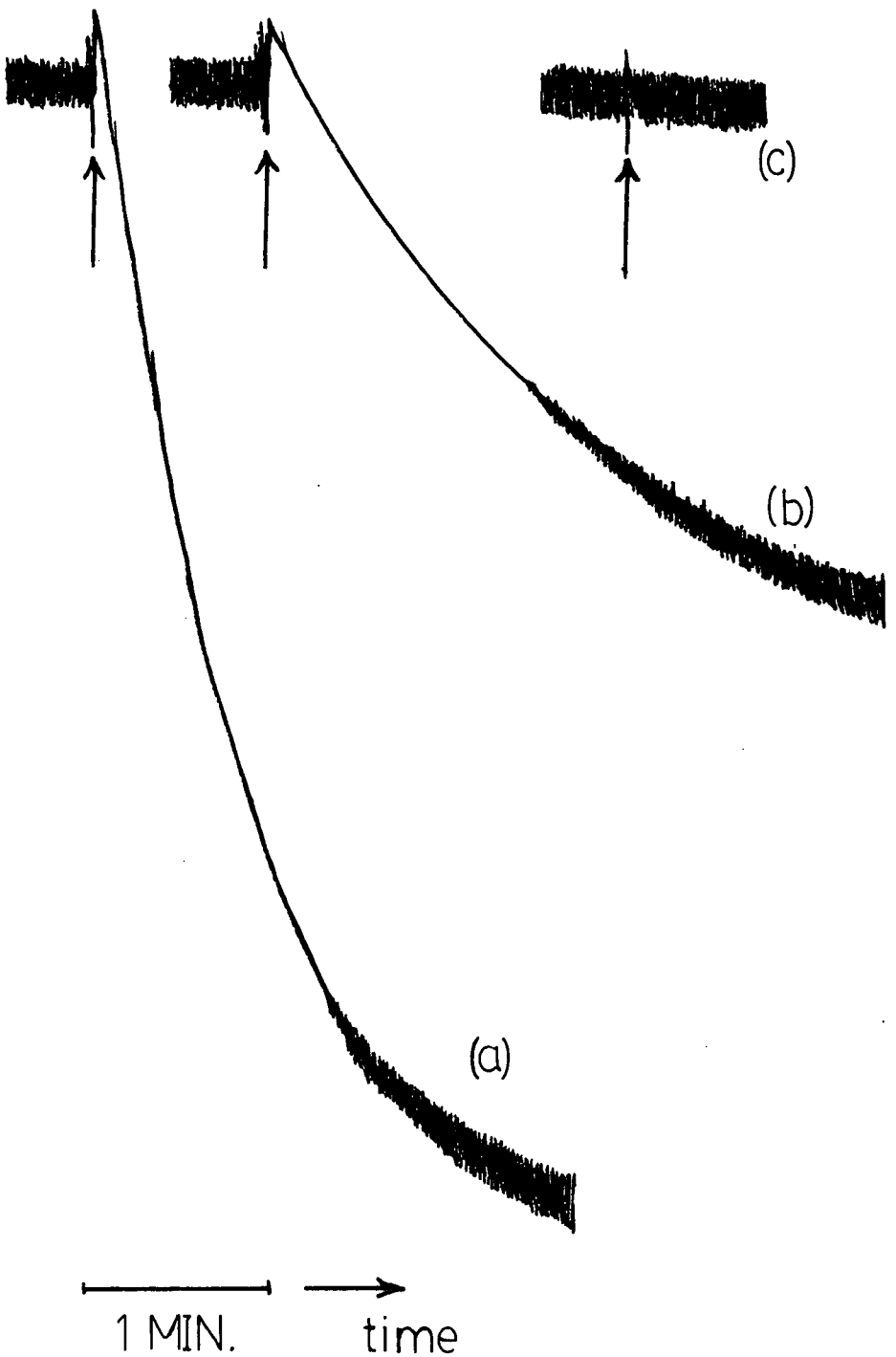

(c)
(b)
(a)
1 MIN.
time

Fig. 14. Agglutination of platelet (a) spherocytes and (b) discocytes by ristocetin. Discocytes were suspended in isotonic and spherocytes in hypotonic Tyrode's solution. Plasma was added to each sample at 0.1 ml of plasma per ml of platelet suspension, to provide von Willebrand's factor. Values are means from two series of experiments.

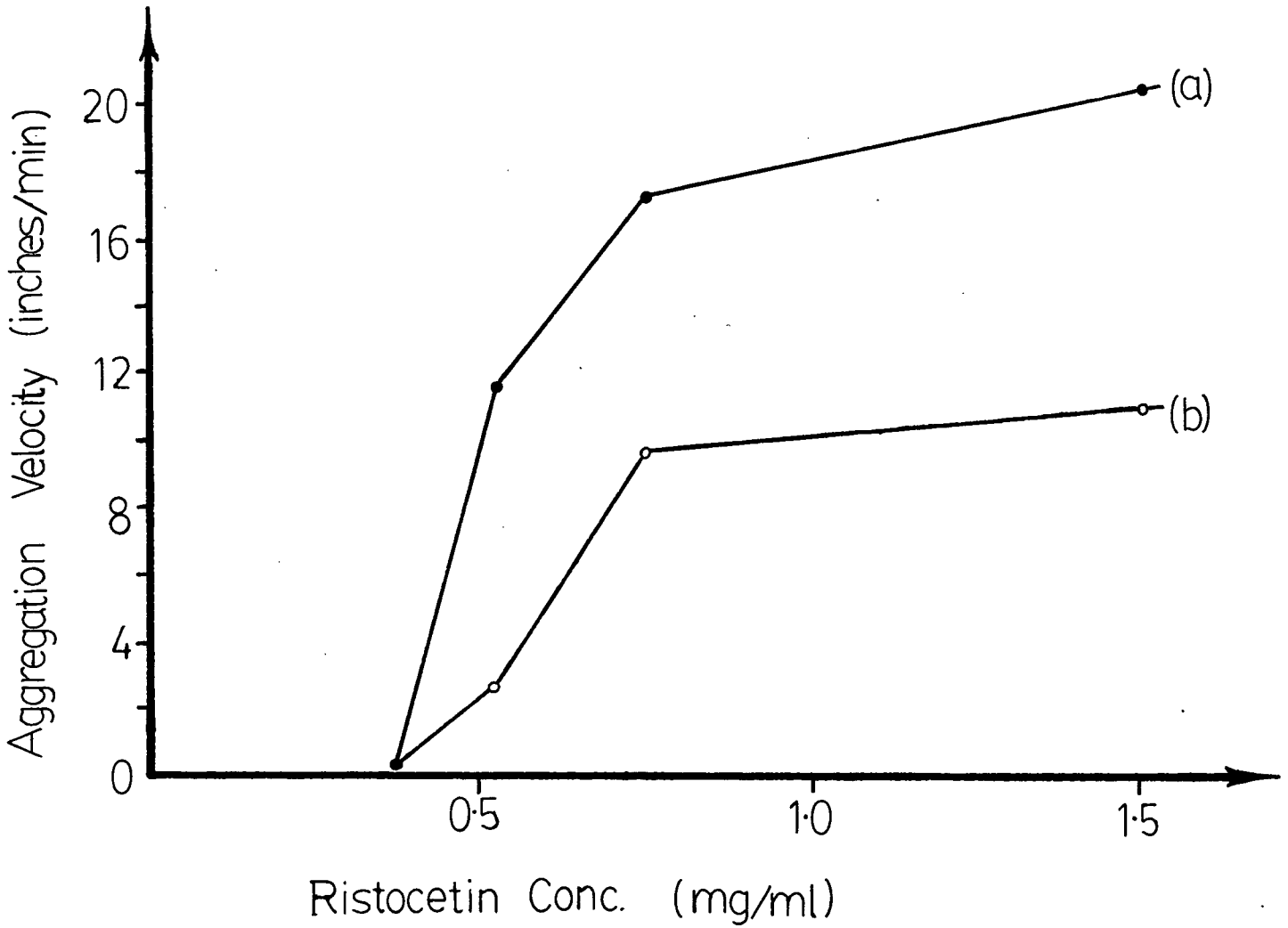

Aggregation Velocity (inches/min)
20
16
12
8
4
0
0·5
1·0
1·5
Ristocetin Conc. (mg/ml)
(a)
(b)

Fig. 15. Aggregation tracings from normal (left) and neuraminidase treated (right) platelets. Both were suspended in Tyrode's solution containing 0.5 mg/ml of fibrinogen and 4 mM Ca^{++} ions. Aggregation was induced by $2x10^{-5}$M of ADP as indicated by arrows.


1 MIN.
time

Wheat germ agglutinin (WGA) aggregated normal discocytes strongly. After neuraminidase treatment, the aggregation velocities dropped substantially at all the concentrations tested (Fig. 16). Typical aggregation tracings are shown in Fig. 17. Aggregation was inhibited by EDTA or EGTA (both at 2.25 mg/ml) as well as by N-acetyl-glucosamine (50 mM) but galactose and N-acetyl-galactosamine at this concentration were without effect.

Both the castor bean agglutinins (RCA_{60} and RCA_{120}) aggregated normal platelets only to a small extent but after neuraminidase treatment aggregation was greatly enhanced (Figs. 16 and 17). Aggregation was also inhibited by 2.25 mg/ml of EDTA or EGTA. Aggregation by RCA_{60} was inhibited by both N-acetyl-galactosamine (50 mM) and D-galactose (50 mM). Aggregation by RCA_{120} was inhibited only by D-galactose while N-acetyl-galactosamine had no effect. Neither were affected by N-acetyl-glucosamine.

Jeguirity bean agglutinin (JBA) did not aggregate non-treated platelets and after neuraminidase treatment the platelets were aggregated moderately (Fig. 16). The aggregation was inhibited by 2.25 mg/ml of EDTA or EGTA as well as 50 mM D-galactose. N-acetyl-galactosamine and N-acetyl-glucosamine were without effect.

Aggregation of glutaraldehyde fixed discocytes by WGA could not be demonstrated with the aggregometer but under the microscope numerous small aggregates of two or three platelets were seen.

Fig. 16. Aggregation responses of normal (-●-) and neuraminidase treated (-o-) platelets to the lectins.

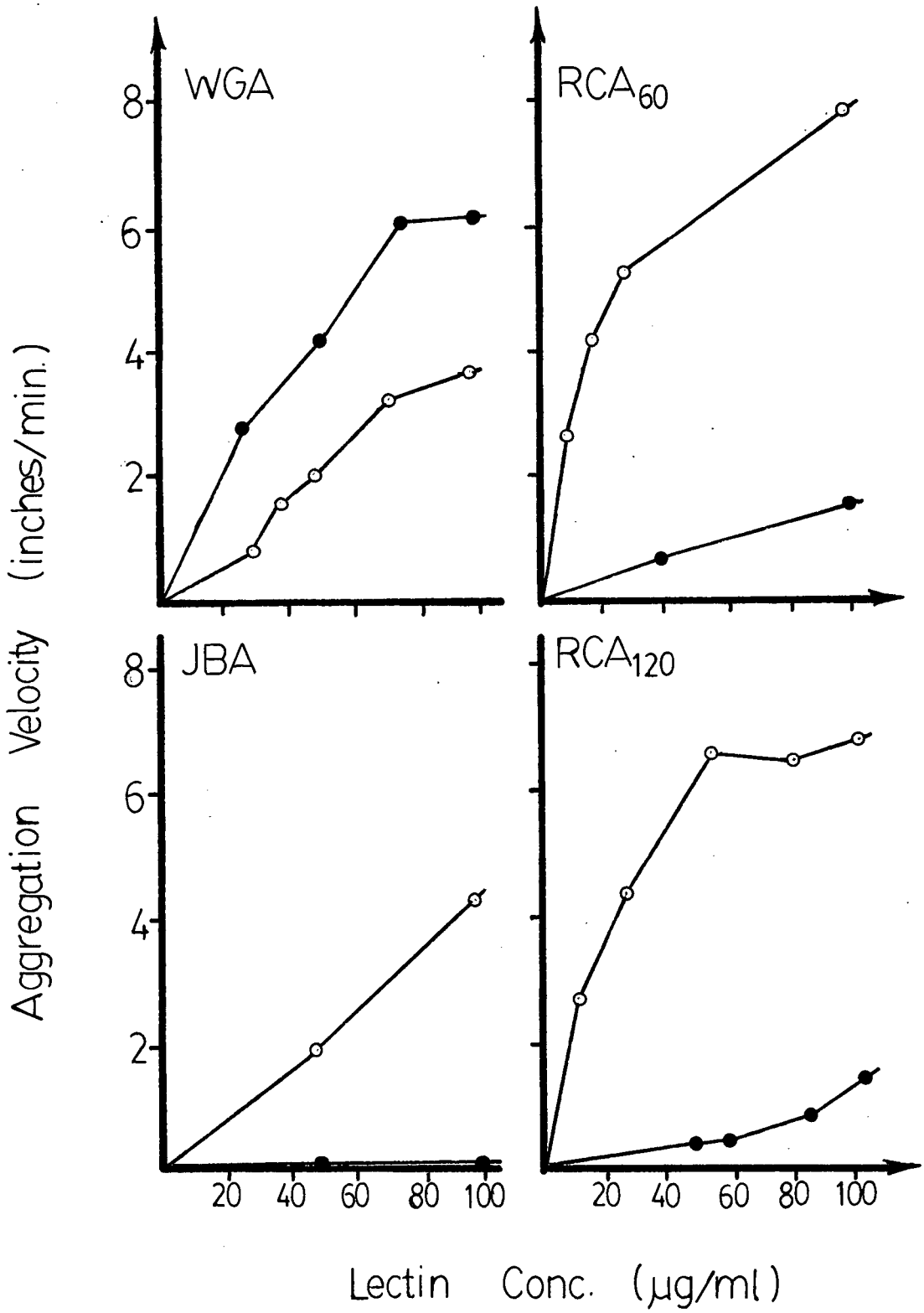

WGA
RCA60
JBA
RCA120
8
6
4
2
20
40
60
80
100
Aggregation Velocity (inches/min.)
Lectin Conc. (µg/ml)

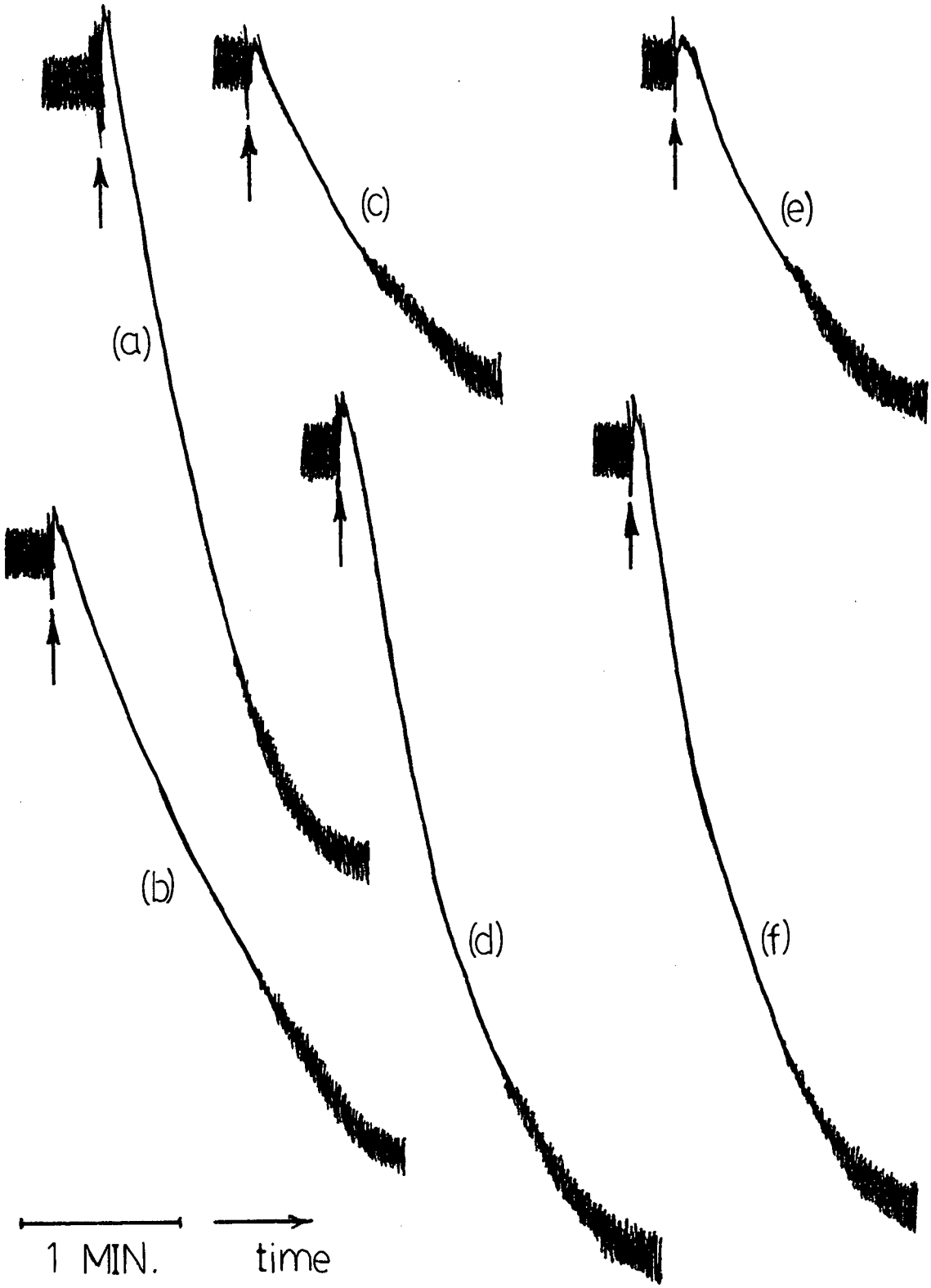
Fig. 17. Examples of platelet aggregation responses to lectins.

(i) Normal (a) and neuraminidase-treated (b) platelets aggregated by WGA

(ii) Normal (c) and neuraminidase-treated (d) platelets aggregated by RCA_{60}

(iii) Normal (e) and neuraminidase-treated (f) platelets aggregated by RCA_{120}

All lectins were added at 100 μg/ml as indicated by arrows.


(a)
(b)
(c)
(d)
(e)
(f)
1 MIN.
time

2.4 DISCUSSION

2.4.1 Platelet Dimension and Morphology

The mean diameter of the discocyte measured here 3.18×10^{-4}cm is similar to 3.2×10^{-4}cm reported by Milton and Frojmovic (1979). The R value of 0.28 for human discocyte measured at room temperature was close to that of 0.26 measured at 37°C by Frojmovic and Panjwani (1976). Although there was a small difference between the R values, the resulting difference in surface area (16.48×10^{-8} vs 16.42×10^{-8} cm^2) was minimal. Born et al (1978) reported R=0.25 for rabbit platelets. Using electronic particle counting Gear (1981) found the human platelet volume to be 4.94×10^{-12} cm^3 and Yamazaki and Motomiya (1980) found a similar value 4.66×10^{-12} cm^3. These two values are quite compatible with 4.71×10^{-12} cm^3 reported in this chapter.

Hypotonic stress at 75 mOsm produced spherocytes with a mean diameter of 3.42×10^{-4} cm. An identical value was found by Milton and Frojmovic (1979). Recently Boneu et al. (1982) found hypotonic treatment at 120 mOsm increased platelet diameter to 3.07×10^{-4} cm.

The surface area and volume of the echinocyte cannot be found from the geometrical means used here because of its irregular shape. However it will be shown in Chapter 3, from indirect calculations, that the surface area of the echinocyte is probably close to that of the cocaine sphere. Born et al (1978), using a high speed centrifugation radiolabel dilution technique found that rabbit platelets had a mean volume of about 5×10^{-12} cm^3. They found no change in platelet volume after echinocytic transformation.

The morphologies of the discocyte, echinocyte and the cocaine sphere under electron microscopy were not unlike those reported elsewhere (White, 1972;

Behnke, 1970). The surface-connected canalicular system was present in the discocyte, echinocyte and the cocaine spheres. The channels of the system were dilated in the echinocyte and severely dilated in the cocaine spheres. Presence of the canalicular system in the echinocyte suggested that evagination of the system was not total. This could be supported by the fact that the surface area of the spherocyte appears to be considerably larger than that of the echinocyte (Chapter 3). Our original hypothesis, that the increase in membrane surface area originates from the canalicular system does not dictate the degree of evagination of the canals. In fact White and Clawson (1980) suggested that the canalicular system within the cytoplasm is so tortuous and interwoven that complete evagination would result in the total destruction of the platelet. Platelets spreading on a surface can attain a surface area of $70 \times 10^{-8} cm^2$ (Frojmovic and Milton, 1982), double that of the spherocyte. This implies that even in the spherocyte evagination may not have been total. The large empty vacuoles in the spherocytes might be swollen canalicular channels. Platelet morphology and surface area related to surface chemistry will be discussed further in Chapter 3.

2.4.2 Platelet Integrity and Release of Contents

The enzyme LDH is a cytoplasmic enzyme (Gogstad *et al.*, 1981) and has been used extensively as a marker for cytoplasmic leakage from platelets (Milton and Frojmovic, 1979; Fratantoni and Poindexter, 1981; Ostermann *et al.*, 1982; Sturk *et al.*, 1982). The possibility that the transformation of platelets, especially during hypotonic shock could result in leakage or breaks in the plasma membrane is of considerable concern here. If even a small number of platelets burst, released material could coat the surfaces of the remaining intact ones and the resulting properties could be misinterpreted as platelet

surface changes during transformation. At 60 mOsm Milton and Frojmovic (1979) found that considerable enzyme leakage had occurred after only 10 min of hypotonic shock. In the present work it was found that LDH leakage was not measurable after 1 hour of stress at 75 mOsm (Fig. 7) and that the amount remained below 4% for 90 min, the time required to perform the H^3-borohydride labelling procedure in Chapter 4. Much shorter time periods (20-30 min) were needed to do cell electrophoresis (Chapter 3). Therefore within the time periods of the experiments the platelet spherocytes appeared to be intact by this criterion.

Beta-Tg is present in the alpha-granules and the sole source of plasma beta-Tg is from the platelets. Increased amounts of beta-Tg in the plasma indicate vascular events involving *in vivo* platelet activation (Kaplan and Owen, 1981). The radioimmunoassay kit developed by Amersham is intended for use with plasma but can easily be adopted to assay platelet beta-Tg with little change in the assay protocol. This kit has already been used by a number of groups for *in vitro* platelet experiments as an indicator for alpha-granule release (Ludlam and Cash, 1976; Bolton *et al.*, 1980; Gogstad *et al.*, 1981). Kaplan and Owen (1981) have reported that the mean platelet beta-Tg content is 17.7$\mu g/10^9$ platelets, a little lower than 22.2 $\mu g/10^9$ platelets found here. It seems that it is extremely easy to induce beta-Tg release. It was recommended by the manufacturer and others (Ludlam and Cash, 1976) that the blood should be collected in EDTA and handled at 4^oC. This was not possible in our circumstances and a considerable amount of beta-Tg was released during washing and hypotonic shock as a result. Examination of the platelets under electron microscopy, on the other hand, did not show the platelets to be grossly depleted of their granules. No release from the dense bodies was evident as monitored using ^{14}C-serotonin.

2.4.3 Platelet Aggregations

The effect of ADP concentration on platelet aggregation has been well studied. The results shown in Fig. 8 were not significantly different from those found by others (Mills et al., 1968; Tangen et al., 1971; Frojmovic, 1973; Stibbe and Holmsen, 1977; Marguerie et al., 1979). It is seen that $2x10^{-5}$M of ADP gives the maximum aggregation velocity. This concentration was therefore used throughout this project. In most of the experiments in Chapters 3 and 4 shape change (to echinocyte), and not aggregation, was desired. Fibrinogen and divalent cations were withheld from the platelets to avoid aggregation. Born (1970) has shown that the speed and extent of shape change also depend on ADP concentration. Michal and Born (1971) found that the velocity of aggregation was directly related to the velocity of shape change. In the present work it was also found, based on the magnitude of the initial decrease in light transmission preceding the increase due to aggregation, that higher ADP concentrations brought about larger shape change responses.

The effect of fibrinogen concentration on platelet aggregation induced by ADP has also been well studied and Fig. 10 is similar to concentration curves reported by others (McLean et al., 1964; Deykin et al., 1965; Niewiarowski et al., 1977; Marguerie et al., 1979; Harfenist et al., 1980). The fibrinogen concentration study here forms the basis for the fibrinogen adsorption experiments to be described in Chapter 4.

Fig. 11 shows the biphasic platelet aggregation response to increasing concentrations of Ca^{++} and Mg^{++} ions. Such biphasic responses were also observed by Born and Cross (1964) and Heptinstall (1976). It was found here that aggregation velocities increased with Ca^{++} or Mg^{++} ion concentration

until the optimum occurred at 4 mM. Further increases in concentration caused a decrease in aggregation velocity, the reason for which is not clear although it is likely associated with the high ionic strength of the suspending medium at the higher Ca^{++} or Mg^{++} concentrations. Born and Cross (1964) found the optimum Ca^{++} concentrations. Born and Cross (1964) found the optimum Ca^{++} concentrations to be 1.7 mM while Heptinstall (1976) found it at 2.5 mM. They also found, as shown here, that Mg^{++} ions were less effective in supporting ADP induced platelet aggregation.

The requirement of Ca^{++} or Mg^{++} ions for the binding of fibrinogen to ADP activated platelets has been discussed earlier. Marguerie et al. (1980) found that this requirement is also biphasic. The calcium or magnesium ion concentration optimal for fibrinogen binding is about 1 mM and binding decreases below or above this concentration. Marguerie et al. (1980) also found that Mg^{++} was less effective in fulfilling the requirement for fibrinogen binding than was Ca^{++}. At the optimum calcium ion concentration, about $3x10^4$ fibrinogen molecules bind to each platelet if magnesium is substituted. The calcium and magnesium ion concentration curves for platelet aggregation (Born and Cross, 1964; Heptinstall, 1976 and reported here) and fibrinogen binding (Marguerie, 1980) are strikingly similar, suggesting the presence of a close relationship between platelet aggregation and fibrinogen binding.

The contribution of strontium ions (Sr^{++}) to platelet aggregation is not as clear. It was found here that between 0 and 20 mM, Sr^{++} did not support ADP-induced aggregation. Lages et al. (1975) found that the presence of 0.2 mM of Sr^{++} had little or no effect on ADP and adrenalin induced platelet aggregation. On the other hand, Best et al. (1981) reported that addition of

Sr^{++} ions alone could result in platelet activation with thromboxane B_2 production and serotonin release.

Unlike magnesium, calcium and strontium, manganese is not a type IIA element. Its contribution to platelet aggregation is also uncertain. It has been proposed by Bosmann (1972) and Wu and Ku (1978) that sialyltransferase on the surface of platelets may be involved in the platelet aggregation process. The hypothesis is that the enzyme on one platelet interacts with the substrate sialic acid on the surface of another platelet forming a "bridge." This has never been substantiated. This enzyme has a requirement for Mn^{++} as co-factor. However, present results suggest that Mn^{++} is an inhibitor of platelet aggregation. It is therefore unlikely that sialyltransferase plays any essential part in platelet aggregation. The optimum concentration of Mn^{++} ions required by the enzyme is between 0.8 and 2 mM as reported by Wu and Ku (1978) and 20 to 60 mM according to Bosmann (1972). Manganese ions at a few millimolar concentration have an inhibitory effect on the movement of calcium ions across the sacroplasmic reticulum membranes in muscle fibres (Saida and Suzuki, 1981). Platelet activation by various aggregating agents involves the massive movement of compartmentalized calcium into the cytoplasm (Detwiler et al., 1978; Massini et al., 1978). Whether manganese inhibits platelet aggregation by interferring with calcium ion movements or not remains to be determined. The fact that Ca^{++}, Mg^{++}, Sr^{++} and Mn^{++} all have very different effects on ADP-induced aggregation at the same concentration strongly implies that specific binding of the divalent ions to, presumably, membrane sites is involved in their activity, since generalized electrostatic effects would depend only on the ionic strength, not on the chemical nature of the ions (Diamond and Wright, 1969).

After spherocyte transformation, the platelets were found to have lost their ability to aggregate. The most likely explanation is that hypotonic shock has caused platelet damage to the point that aggregation no longer is possible. The loss of aggregability in cocaine spheres is also expected (O'Brien, 1962; Aledort and Niemetz, 1968). In both types of spheres the loss of aggregability may be attributed to the total disruption of contractile and cytoskeletal elements (Zucker-Franklin, 1969; Nachmias et al., 1977; Nachmias et al., 1979). Membrane changes to be described in Chapter 3 may also be involved.

Ristocetin is not a pharmacological aggregating agent but is a direct agglutinating agent. That is, no physiological response from the platelet is required to induce agglutination. In fact, ristocetin has been shown to agglutinate formaldehyde fixed platelets (Allain et al., 1975). Agglutination requires the co-binding of ristocetin and a plasma factor (von Willebrand's factor) to the surface of platelets. The exact nature of the interaction is still uncertain and a variety of possible models have been reviewed elsewhere (Kirby, 1977; Solum and Peterka, 1977; Coller, 1978; Phillips, 1980). Ristocetin binds to the surface of platelets (Hashimoto and Suzuki, 1979) and produces a condition that favours the binding of von Willebrand's factor to the platelet surface (Hahsimoto and Suzuki, 1979; Kao et al., 1979; Schneider et al., 1979). The von Willebrand's factor and/or ristocetin then cross-bridge receptors on the surfaces of adjacent platelets, causing agglutination. The receptor for von Willebrand's factor was found to be associated with the surface glycoprotein designated Ib (Phillips, 1980). Patients with Bernard-Soulier syndrome lack this glycoprotein and their platelets fail to agglutinate upon exposure to ristocetin and the plasma factor. On the other hand persons

with von Willebrand's disease lack the factor in their plasma and their platelets will agglutinate only after external von Willebrand's factor is added.

Although spherocytes have lost their ability to aggregate they are still able to agglutinate in response to ristocetin and added plasma. Hypotonic shock has therefore not destroyed the receptors for either ristocetin or von Willebrand's factor. The reason why the rate of agglutination is greater for the spherocyte than the discocyte is uncertain. It may be related to the increase in surface area or platelet volume in the spherocyte, perhaps increasing the number of receptor sites per platelet for either type of molecule. The increase in volume might also lead to an increase in the number of platelet- platelet collisions in the spherocyte suspension, since a larger fraction of the suspension volume would be occupied by the spherocytes. The decrease in negative charge density on the surface of the spherocyte (Chapter 3) might also contribute to the increased aggregation rate. Ristocetin is a positively charged molecule and it has been proposed that its function in the agglutination is to neutralize some of the platelet surface negative charge (Phillips, 1980). If the surface charge density has already been lowered during spherocyte transformation then the action of ristocetin might be much enhanced. Finally, since the spherocytes were suspended in hypotonic Tyrode's solution while the discocytes were in isotonic Tyrode's solution, the low ionic strength might also play a role in facilitating agglutination of the spherocytes.

As expected, neuraminidase treatment of platelets did not alter their shape or their aggregability. Hovig (1965) as well as Bowles and Brunton (1982) found no alteration in platelet morphology under transmission and scanning electron microscopy after neuraminidase treatment. Greenberg et al.

(1975) found only a slight enhancement of ADP induced aggregation in treated platelets. The enhancement was most obvious at a low ($4.5x10^{-6}M$) ADP concentration with pratically no difference being observed at a higher ($9x10^{-5}M$) concentration. At $2x10^{-5}M$ no differences were found here.

Greenberg and Jamieson (1974) reported that human platelets can be aggregated by WGA and to a lesser extent by RCA. Ganguly and Fossett (1979) found that neuraminidase treatment of platelets reduced the extent of aggregation by WGA as well as the number of WGA binding sites on the platelet surface. Patscheke and Worner (1977) and Naim et al. (1982) observed that removal of terminal sialic acid residues from the platelet surface by neuraminidase actually enhanced the aggregation response to RCA. Similar phenomena were observed by us. The effects of lectin concentration on the aggregation of treated and control platelets was also studied here (Fig. 16).

WGA binding can be specifically inhibited by N-acetyl-glucosamine but in the absence of this sugar it can interact strongly with the terminal sialic acid residues of glycoproteins (Goldstein and Hayes, 1978; Bhavanandan and Katlic, 1979; Peters et al., 1979; Wright, 1980). In platelets it has been demonstrated by a number of workers that WGA binds to glycoprotein-Ib (GP-Ib) on the surface of the cell (Ganguly and Fossett, 1979; Marchesi and Chasis, 1979; McGregor et al., 1979; Naim et al., 1982). These experiments involved either the binding of radiolabelled WGA by separated glycoproteins in polyacrylamide gels or the isolation of solubilized GP-I using WGA-Sepharose affinity chromatography. Caution in interpreting these results was urged by Naim et al. (1982). Moreover Rock et al. (1980) found that Bernard-Soulier syndrome platelets which lack GP-Ib could still be aggregated by WGA. It therefore seems unlikely that GP-Ib is the only receptor on platelets. The

hypothesis is that WGA binds to terminal sialic acid residues of the glycoprotein(s) on the platelet surface and causes platelet aggregation via the usual cross-linking of adjacent surfaces by the multivalent lectin. Removal of sialic acid by neuraminidase therefore reduces the platelet's response to WGA (Ganguly and Fossett, 1979).

The removal of terminal sialic acid residues apparently exposes galactose or N-acetyl-D-galactosamine residues. This suggestion is based on the effect of RCA_{60}, RCA_{120} and JBA, all of which have a high affinity for galactose (Goldstein and Hayes, 1978) and all of which show enhanced activity with neuraminidase treated platelets (Patscheke et al., 1977). These galactose-binding lectins probably induce aggregation by the same mechanism(s) as does WGA. That galactose or N-acetyl-galactosamine residues can be exposed after neuraminidase removal of terminal sialic acid has been found in many systems (Steck and Dawson, 1974; Wright, 1980). The combined use of neuraminidase and galactose oxidase followed by reduction with tritiated $NaBH_4$ to label cell surface glycoproteins has become a standard procedure in recent years (Phillips, 1979). This labelling procedure as applied to platelet membrane studies will be discussed in Chapter 4.

Finally, a small degree of agglutination was observed when fixed platelets were exposed to WGA. Similar to our study Ganguly and Fossett (1980) observed agglutination of fixed platelets by WGA microscopically but were unable to demonstrate this with aggregometry. They therefore suggested that two mechanisms, passive agglutination and active aggregation, might be at work in this situation.

2.4.4 Summary of Chapter 2

The dimensions and morphology of the different forms of platelets were established. The most important point found was that most spherocytes formed via hypotonic shock remained intact for a reasonable period of time, although some release of beta-Tg occurred. The second half of Chapter 2 dealt with platelet aggregation in general, and it was found that the spherocytes had lost all ability to aggregate. They could only be agglutinated passively by ristocetin and von Willebrand's factor.

CHAPTER 3

MICROELECTROPHORESIS

3.1 INTRODUCTION

Microelectrophoresis or cell electrophoresis is a technique used to probe the electrokinetic properties of cell surfaces. An in depth theoretical discussion of microelectrophoresis and the electrokinetic behaviour of cells can be found in a review by Seaman, 1975. Briefly, the electrophoretic mobility (EPM) of a cell is measured by observing the velocity of its motion under the influence of the particular electric field applied. It is defined as the velocity per unit field strength and is usually expressed in terms of $cm^2.sec^{-1}.V^{-1}$. The EPM (μ) of a cell, assuming it behaves as a smooth particle bearing a uniform charge density, is directly related to its zeta potential (ζ), i.e. the electrostatic potential at the shear plane. The zeta potential is in turn related to the charge density (σ) apparently located at the plane of shear:

$$\mu = \left(\frac{\varepsilon \zeta}{4\pi\eta}\right) \qquad [1],$$

$$\zeta = \left(\frac{4\pi\sigma}{K\varepsilon}\right) \qquad [2],$$

where $K = \left(\frac{8\pi N e^2 I}{10^3 \varepsilon kT}\right)^{\frac{1}{2}}$

$1/K$ = double layer thickness $\approx 8\text{Å}$ at I=0.15

η = viscosity of suspending medium

N = Avogadro's number

I = ionic strength of suspending medium $= \frac{1}{2}\sum_i C_i Z_i$

c_i = molar concentration of i^{th} ionic species

z_i = valence of i^{th} ionic species

ε = dielectric constant

k = Boltzman's constant

T = absolute temperature

e = electronic charge unit (4.8×10^{-10} esu)

Equation [1] holds providing the smallest radius of curvature of the particle is much larger than the double layer thickness 1/K. Equation [2] is valid providing $z_i e\zeta \ll kT$. Both conditions are fulfilled by platelets suspended in physiological buffers. Combining [1] and [2] gives:

$$\sigma = \mu \eta K \qquad [3].$$

The charge density, σ, is expressed as esu/cm^2 but can be converted into the number of charges per cm^2 by dividing by e.

The surface of most cells contains both positive and negative charge groups but in general the net surface charge is a negative one at physiological pH. The charge groups on the surface of a cell attract ions of opposite charge (counter-ions) and repel charges of like sign (co-ions), the net effect being the formation of the diffuse double layer adjacent to the cell surface. In some instances it is found that a fraction of the counterions adsorb to the particle surface reducing the net surface charge density and forming what is known as a Stern layer. There is no evidence for the formation of a Stern layer on biological cells if only monovalent ions are present in the suspending medium, as no dependence of mobility on the chemical nature of the ions has been found (Heard and Seaman, 1960). When multivalent cations such as Ca^{++} or Mg^{++} are present, however, binding does occur and the mobility decreases associated with these events can be used to estimate the binding parameters, as will be discussed subsequently.

Equations [1] to [3] assume the surface of the particle is smooth and well defined, although it may be irregular in shape. However, the surfaces of biological cells are not smooth on the scale of double layer dimensions. The lipid bilayer anchors a diffuse layer of charged and neutral glycoproteins and glycolipids known as the glycocalyx which extends some distance out from the

plane of the lipid head groups. Recently, theories have been developed which explicitly model the effects of this layer on the electrophoretic mobility (Donath and Pastushenko, 1979; Wunderlich, 1982; Levine et al., 1983). The results provide a modification of [3] in the form of:

$$\sigma F = \mu \eta K \qquad [4].$$

The function F includes terms involving the thickness of the glycocalyx and the average size and volume concentration of the polymer segments in this region. None of these parameters are known for platelets, however. Equation [3] is therefore used to interpret electrophoretic mobilities in this work, the assumption being made throughout that the function F remains constant or changes in a consistent manner among the platelet forms examined as conditions are varied.

Like most other types of cells platelets have a negative net surface charge. The electrokinetic behaviour of platelets has been reviewed by Seaman and Brooks (1970), Mason and Shermer (1971) and Seaman (1976). The electrophoretic mobility of platelets was first investigated by Abramson in 1928. He suspected that the electrical charge on platelets could have something to do with platelet aggregation and thrombosis. However the lack of information about platelets at that time prevented him from further examining this point. More recently Seaman and Vassar (1966) found that addition of 1 μg/ml of ADP to platelets in PRP caused an 18% decrease in mobility. They suggested that the decrease in EPM might somehow be associated with aggregation induced by ADP. Hampton and Mitchell (1966) reported a biphasic change in the EPM of platelets (in PRP) induced by ADP. At extremely low concentrations the mobility increased, possibly due to the binding of ADP onto the platelet surface.

When ADP was above $5x10^{-2}$ g/ml (about $1x10^{-7}$M) the electrophoretic mobility ddecreased with increasing concentration. This biphasic phenomenon was also observed by Stoltz (1971) as well as Kosztolanyi et al. (1980) but several groups including Grottum (1968), Betts et al. (1968) and Seaman and Vassar (1966) could not confirm this effect. A drop in the surface negative charge would mean a drop in the mutual repulsive force between platelets. This might bring about a more favourite conidtion for platelet-platelet interaction and aggregation (Grotum, 1968 and Seaman, 1976). No morphological studies were undertaken by these authors to correlate between platelet shape change and the decrease in mobility. A French group, on the other hand, using $2x10^{-6}$M of ADP have reported that discocyte to echinocyte transformation in PRP results in a mobility increase (Boisseau et al., 1977).

Sialic acid is a major contributor to platelet surface negative charge. Jung et al. (1982) found a linear correlation between platelet surface sialic acid content and EPM. It has been estimated that about 41% of the net negative charge detected electrokinetically comes from terminal sialic acid residues (Seaman, 1976). Another 28% derives from phosphate groups. Neuraminidase removal of the terminal sialic acid groups has been found to result in a 40 to 60% drop in human platelet electrophoretic mobility (Madoff et al., 1964; Seaman and Vassar, 1966; Bray and Alexander, 1969; Stoltz and Nicolas, 1979). From the drop in mobility one can calculate the decrease in charge density and providing the surface area is known, convert that into the actual number of sialic acid molecules released (Seaman and Vassar, 1966). One must be aware that this number reflects only sialic acid released from near the effective plane of shear. Other sialic acid residues located at different distances from this plane contribute somewhat less to the electrokinetic properties of

the platelets and their release can be expected to have little effect on the mobility. The total number of sialic acid molecules liberated by the enzyme from the platelet membrane into the supernatant can be assayed chemically. The effective number of sialic acid molecules at the plane of shear, determined using equation [3], can be expressed as a fraction of the total platelet surface sialic acid removable by neuraminidase. This fraction is approximately 46% for human red blood cells for example (Cook et al., 1961). It will be shown here that this ratio can be very helpful in the determination of the echinocyte surface area.

The enzyme alkaline phosphatase has also been used to remove negatively charged phosphate groups from the surface of platelets (Mehrishi, 1979 and Stoltz et al., 1975). Decreases in mobility of 15 to 30% were reported. Similarly, the number of phosphate groups eliminated at the plane of shear can be calculated based on equation [3] and expressed as a fraction of the total amount of phosphate liberated from the platelet surface by the enzyme. Alkaline phosphatase has a specificity for monester orthophosphates, C-O-P (Fernley, 1971).

Fixation of platelets with acetaldehyde has been reported to bring about a 20% increase in negative mobility (Seaman and Vassar, 1966). Fixation of red blood cells with glutaraldehyde also results in an increase in RBC mobility (Vassar et al., 1972). The aldehydes block the positively charged amino groups eliminating their positive charge (Jentoft and Dearborn, 1979). As a result net negative charge increases. The number of negative charges increased, calculated from the rise in EPM, gives the apparent number of positive amino groups neutralized.

The electrophoretic mobilities of platelet discocytes compared to those of the platelet spheres as well as the electrokinetic properties of the different forms of platelets modified with neuraminidase, alkaline phosphatase and aldehydes are discussed in this chapter.

Electrophoretic mobility measurements have also been used to estimate the number of calcium ion binding sites on cells (Seaman et al., 1969). If red cells are suspended in solutions of constant ionic strength containing different concentrations of calcium ions, their mobilities will drop with increasing concentrations of calcium. By plotting the relationship between the decrease in apparent charge density ($\Delta\sigma$) and calcium concentration according to the equation:

$$\frac{1}{\Delta\sigma} = \frac{1}{2en} + \frac{1}{2enK} \cdot \frac{1}{[Ca^{++}] \cdot \exp(2e\zeta/kT)} \qquad [5],$$

where $K = \exp(\Delta G/kT)/55.6$

n = number of binding sites per cm^2

ΔG = chemical free energy of adsorption

$[Ca^{++}]$ = calcium concentration

ζ = zeta potential of platelet at that calcium concentration

the number of calcium ion binding sites as well as the chemical free energy of binding can be evaluated. If the binding obeys the above model, a plot of $1/\Delta\sigma$ versus the reciprocal of the concentration-zeta potential function will be a straight line. The intercept with the ordinate will give 1/(2en) and the slope will represent 1/(2enK). This operation can also be used to find the number of magnesium ion binding sites. Attempts had been made previously to determine the number of calcium ion binding sites on platelet surfaces by measuring the amount of $^{45}Ca^{++}$ adsorbed after an equilibrium incubation

(Peerschke et al., 1980; Taylor and Heptinstall, 1980; Brass and Shattil, 1982). However this technique suffers from the serious drawback that platelets also have an active process of calcium ion internalization or uptake and it becomes difficult to distinguish between surface associated and internalized $^{45}Ca^{++}$ (Peerschke et al., 1980). Moreover, there is no suitable magnesium isotope for this kind of study. The microelectrophoresis technique described here therefore offers a unique opportunity to investigate calcium and magnesium ion binding to platelet surfaces.

3.2 MATERIALS AND METHODS

3.2.1 Microelectrophoresis

The electrophorectic mobilities of platelets were measured in a cylindrical chamber essentially as described by Seaman and Heard (1961). The chamber was immersed in a waterbath at 25°C and measurements were made at 40V. Electrodes were of silver/silver chloride. Mobilities of between 10 to 20 platelets from each sample were usually measured with typical standard deviations of $\pm 0.07 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$. Unless indicated all the samples were measured in pH 7.4 isotonic Tyrode's solution except for the non-fixed spherocytes which were measured in the hypotonic Tyrode's described earlier (pH 7.4). Platelet samples were usually examined under phase microscopy before electrophoresis. The mobilities of fresh human red blood cells were measured as a control each day to ensure proper working conditions for the apparatus. The EPM of the human RBC at pH 7.4 in 0.15M NaCl is $-1.08 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ with a standard deviation of $\pm 0.05 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$. Measurements from the daily controls fell within one standard deviation of the quoted mean.

The pH-mobility profiles were constructed from measurements of platelets suspended in Tyrode's solutions having different pH values. Adjustments of the pH's were made with HCl or NaOH shortly before the platelets were introduced into the electrophoresis chamber.

The Ca^{++} and Mg^{++} ion binding experiments were done with platelets suspended in Tyrode's solutions containing different concentrations of calcium or magnesium chloride. However constant ionic strength and osmolality had to be maintained. With increasing Ca^{++} or Mg^{++} concentration, the ionic strength of the solution increases. Therefore the amount of sodium chloride In the solution has to decrease. At the same time, osmolality decreases as a result

because the ionic strength, I, varies as the square of ionic valence so to maintain I constant as Ca^{++} concentration is increased the tonicity of necessity drops. The addition of an appropriate amount of glucose is used to compensate. The resulting changes in viscosity have to be accounted for when calculating surface charge (Seaman et al., 1969). Viscosities were measured by a Cannon viscometer. Spherocytes were suspended in a solution with 1/4 isotonicity for all measurements.

3.2.2 Fixation of Platelets

Fixation of platelets with glutaraldehyde was the same as in Chapter 2. Formaldehyde fixation of platelets was done with 3.7% formaldehyde (final concentration) overnight at $4^{o}C$. Some samples of the formaldehyde fixed platelets were further treated with 5 mM sodium borohydride for 30 min at room temperature (Jentoft and Dearborn, 1979). They were then washed and resuspended in Tyrode's solution.

3.2.3 Platelet Surface Sialic Acid

Surface sialic acid from glutaraldehyde fixed platelets was removed with neuraminidase (Vibrio cholerae). Digestion time was 90 min at $37^{o}C$. This incubation period was determined by a time-release study. Platelet concentration was $2.5x10^{8}$/ml and neuraminidase 0.04 IU/ml. For optimum conditions (Ada et al., 1961) the pH of the Tyrode's solution was lowered to 6.5 and 0.1 mM of Ca^{++} was added. After digestion the platelets were spun down. The supernatants were used for the chemical assays of released sialic acid and the platelet pellets were resuspended in pH 7.4 Tyrode's solution for microelectrophoresis.

The charge density of control and enzyme treated platelets can be calculated from equation [3]. The number of sialic acid molecules removed per unit area at the plane of shear can be calculated by dividing the drop in charge density with the electron charge unit (e).

The procedure of Culling et al. (1977) was used to monitor the amount of sialic acid released into the supernatant. Sialic acid was first oxidized with sodium metaperiodate and then estimated colorimetrically with thiobarbituric acid reagent. N-acetyl-neuraminic acid was used as standard.

To find the total sialic acid content, fixed whole platelets were hydrolysed with 0.1N sulphuric acid at 80°C for 1 hour. Sialic acid released this way was assayed by the Warren (1959) procedure. To test for O-acetyl substitution at the C-4 position of sialic acid, fixed platelets were saponified with 0.1N potassium hydroxide at room temperature (Reid et al., 1975) before neuraminidase digest. Neuraminidase is not able to attack sialic acid residues bearing such a substitution, therefore more sialic acid will be released after saponification if C-4 is acetylated.

3.2.4 Platelet Surface Phosphate Groups

Alkaline phosphatase (calf intestine) was used to liberate terminal phosphate groups from the surface of glutaraldehyde fixed platelets. Incubation was at 37°C for 45 min using 0.1 mg/ml of the enzyme. Due to the presence of phosphate in Tyrode's solution, platelets were resuspended in pH 10 (optimum pH) Tris-buffered saline before enzyme treatment. After digestion the platelets were spun down, the supernatant was used for chemical assay of phosphate and the platelet pellet was resuspended in pH 7.4 Tyrode's solution for microelectrophoresis

The method of Chen et al. (1956) was used to assay phosphate ions liberated by the enzyme into the supernatant. Phosphate forms a complex with ammonium molybdate and color was developed by reduction of the complex with ascorbic acid.

3.2.5 Statistical Methods

Linear regressioin and other statistical comparisons were according to Kalbfleisch (1974). Further discussion can be found in the appendix.

3.2.6 Materials

Neuraminidase was from Calbiochem (La Jolla, California) and calf intestine alkaline phosphatase from Boehringer-Mannheim (Dorval, Quebec). Both enzymes were tested for the presence of proteolytic activity with azocoll (Rinderknecht et al., 1968). The enzymes were incubated with 3 mg/ml of azocoll (Calbiochem) overnight at 37^{o}C under the same conditions, such as pH, medium and concentration, as used in the platelet experiments. Trypsin was used as standard. No proteolytic activity was detected in either enzyme.

N-acetyl neuraminic acid and trypsin were from Sigma (St. Louis, Missouri). All other reagents were from Fisher (FairLawn, N.J.).

3.3 RESULTS

3.3.1 Platelet Electrophoretic Mobilities

Electrophoretic mobilities of the different forms of platelets are shown in Table 2. Table 3 shows the mobility values converted into number of charges per platelet and per unit area. Shape change from discocyte to echinocyte induced by $2x10^{-5}$M of ADP resulted in a 13% drop in mean mobility and mean electrokinetic charge density. Since the surface area of the echinocyte is not known at this point, one cannot calculate the actual number of charges per echinocyte from the data. We were not able to detect any change in the mobility of platelets stimulated with $1x10^{-7}$M ADP. The spherocyte had the lowest charge density although the mobility for the non-fixed spherocyte was relatively high. This is because non-fixed spherocytes were measured in low ionic strength hypotonic Tyrode's solution. Adding ADP to spherocytes did not change the mobility.

Formaldehyde fixation raised platelet mobilities by about 17%. Table 4 shows how these changes are translated into increases in net negative charge density. The apparent number of amino groups on the platelet surface can then be calculated. The surface densities of amino groups on the echinocyte and spherocyte were both lower than on the discocyte. Because of the increase in surface area the number of amino groups per spherocyte actually increased. Not knowing the surface area, the number of amino groups per echinocyte cannot be calculated. The mobilities of fixed platelets further treated with borohydride were the same as the untreated ones. Therefore there was no evidence for reversal of the fixation process.

The pH-mobility curves for fixed and non-fixed platelets are shown in Fig. 18 and Fig. 19 respectively. The plateau values for glutaraldehyde fixed

Table 2. The Electrophoretic Mobilities of Platelets in $x10^4 cm^2.sec^{-1}.V^{-1}$

	Non-fixed Platelets	Glutaraldehyde Fixed Platelets	Formaldehyde Fixed Platelets
Discocyte	-1.08 (±0.016)+	-1.41 (±0.020)	-1.26 (±0.012)
Echinocyte	-0.93 (±0.035)	-1.21 (±0.008)	-1.10 (±0.007)
Spherocyte	-1.55* (±0.016)	-1.00 (±0.010)	-0.90 (±0.007)
Cocaine sphere	-1.40 (±0.017)		

*Non-fixed spherocytes measured in hypotonic Tyrode's solution; all others measured in regular isotonic Tyrode's.

+Values in brackets represent standard error of the mean derived from mobility determinations of at least 10 different platelet samples in each category.

Table 3. Apparent Platelet Surface Charge

	Discocyte	Echinocyte	Spherocyte	Cocaine sphere
Electrophoretic Mobility ($x10^4 cm^2.sec^{-1}.V^{-1}$)	-1.082 ($\pm$0.016)	-0.933 ($\pm$0.035)	-1.552 ($\pm$0.016)	-1.405 ($\pm$0.017)
Negative Charge Density (esu/cm^2)	$3.74x10^3$ ($\pm 0.056x10^3$)	$3.23x10^3$ ($\pm 0.121x10^3$)	$2.60x10^3$ ($\pm 0.026x10^3$)	$4.87x10^3$ ($\pm 0.058x10^3$)
Number of Charge Groups per cm^2	$7.80x10^{12}$ ($\pm 0.12x10^{12}$)	$6.73x10^{12}$ ($\pm 0.25x10^{12}$)	$5.43x10^{12}$ ($\pm 0.05x10^{12}$)	$10.14x10^{12}$ ($\pm 0.12x10^{12}$)
Surface Area (cm^2)	$16.4x10^{-8}$	-	$36.7x10^{-8}$	$26.4x10^{-8}$
Number of Charge Groups per Platelet	$1.28x10^6$ ($\pm 0.019x10^6$)	-	$1.99x10^6$ ($\pm 0.020x10^6$)	$2.67x10^6$ ($\pm 0.032x10^6$)

Table 4. Platelet Surface Amino Groups Calculated from Charge Density Increases After Formaldehyde Fixation

	Discocyte	Echinocyte	Spherocyte
Charge density of non-fixed platelets (esu/cm^2)	3.74×10^3 ($\pm 0.056 \times 10^3$)	3.23×10^3 ($\pm 0.121 \times 10^3$)	2.60×10^3 ($\pm 0.026 \times 10^3$)
Charge density of formaldehyde-fixed platelets (esu/cm^2)	4.38×10^3 ($\pm 0.041 \times 10^3$)	3.80×10^3 ($\pm 0.024 \times 10^3$)	3.12×10^3 ($\pm 0.023 \times 10^3$)
Increase in charge density (esu/cm^2)	6.40×10^2 ($\pm 0.526 \times 10^2$)*	5.70×10^2 ($\pm 0.271 \times 10^2$)*	5.20×10^2 ($\pm 0.226 \times 10^2$)*
Amino groups per cm^2	1.33×10^{12} ($\pm 0.109 \times 10^{12}$)	1.19×10^{12} ($\pm 0.047 \times 10^{12}$)	1.08×10^{12} ($\pm 0.056 \times 10^{12}$)
Surface area (cm^2)	16.4×10^{-8}	-	36.7×10^{-8}
Amino groups per platelet	2.18×10^5 ($\pm 0.179 \times 10^5$)	-	3.97×10^5 ($\pm 0.172 \times 10^5$)

*Combined standard deviations from fixed and non-fixed platelet charge densities using the formula (Kalbfleisch, 1974):

$$S = \left(\frac{n_a - 1}{n_a + n_b - 2} \cdot s_a^2 + \frac{n_b - 1}{n_a + n_b - 2} \cdot s_b^2 \right)^{\frac{1}{2}}$$

where S is the combined standard deviation, s_a and s_b are standard deviations of the two individual components, and n_a and n_b are number of samples from the two individual components.

Fig. 18. The pH-electrophoretic mobility profiles of fixed platelet discocytes (-●-), echinocytes (-▲-) and spherocytes (-X-). Solid lines represent glutaraldehyde fixed platelets and dash lines represent formaldehyde fixed platelets. Values are from means of four series of experiments. Error bars represent one standard deviation.

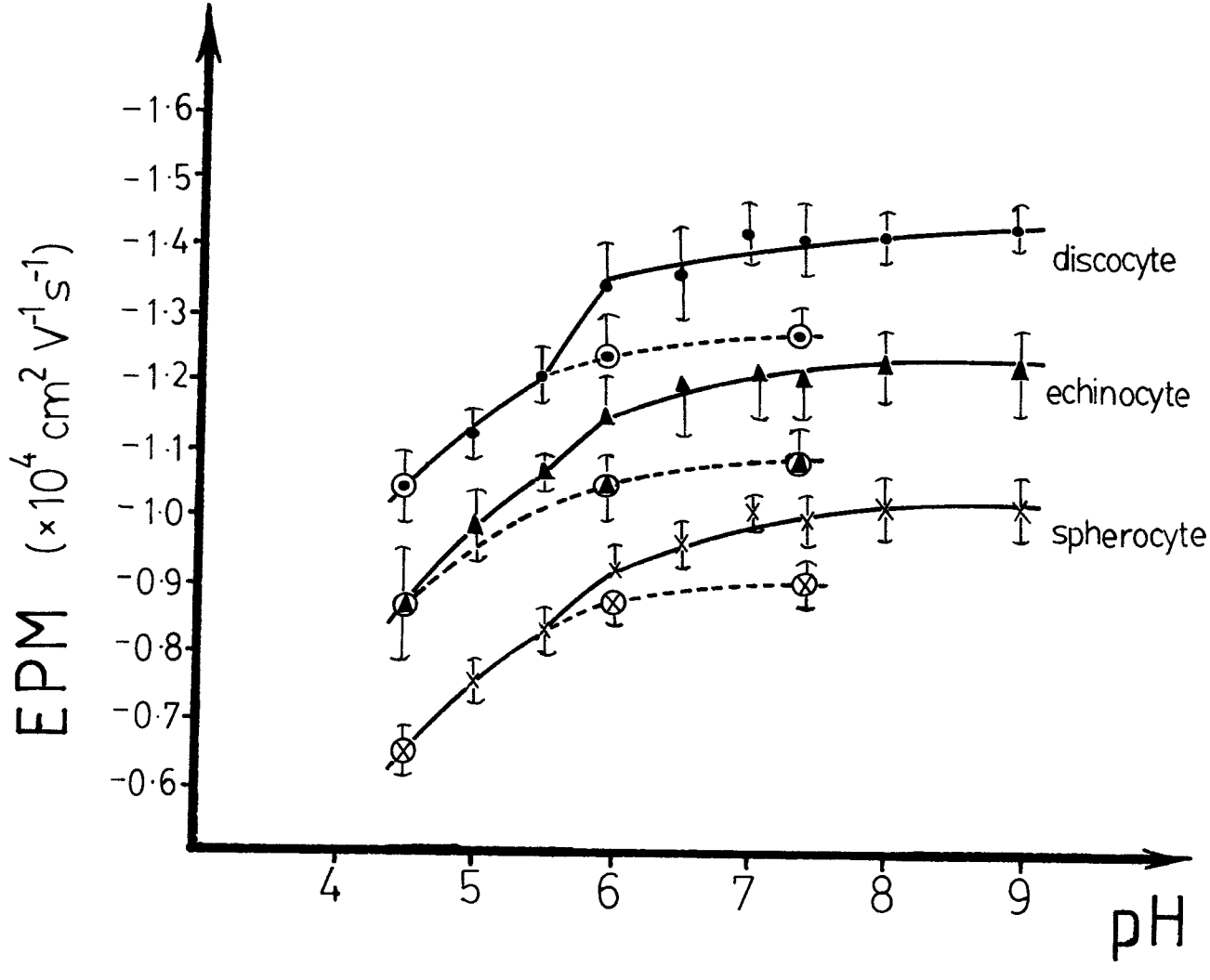
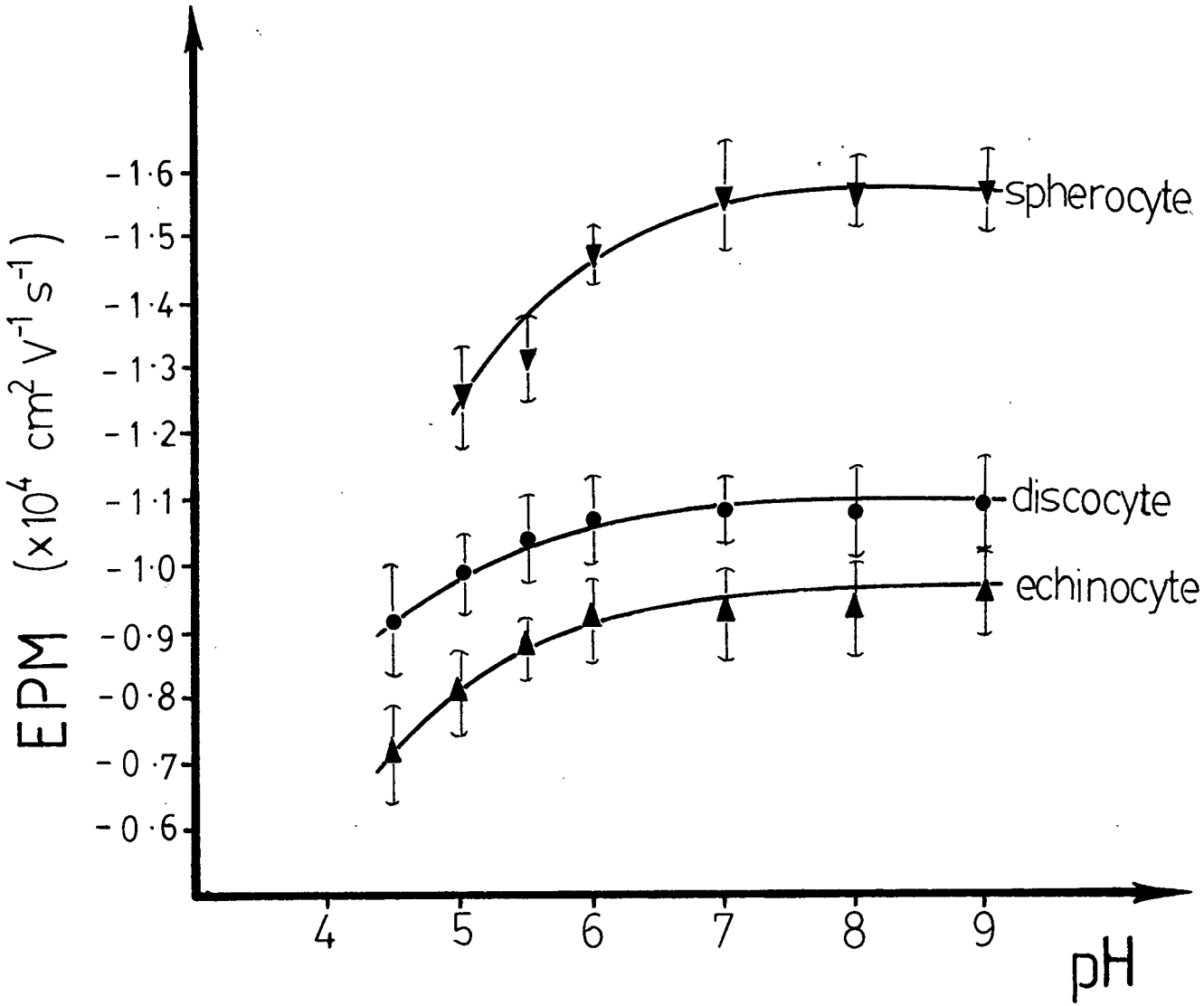

EPM (×10⁴ cm² V⁻¹s⁻¹)
−1·6
−1·5
−1·4
−1·3
−1·2
−1·1
−1·0
−0·9
−0·8
−0·7
−0·6
4
5
6
7
8
9
pH
discocyte
echinocyte
spherocyte

Fig. 19. The pH-electrophoretic mobility profiles of fresh platelet discocytes (-●-), echinocytes (-▲-) and spherocytes (-▼-). Values are means from three series of experiments. Error bars represent one standard deviation.


EPM (x10⁴ cm² V⁻¹ s⁻¹)
-1·6
-1·5
-1·4
-1·3
-1·2
-1·1
-1·0
-0·9
-0·8
-0·7
-0·6
4
5
6
7
8
9
pH
spherocyte
discocyte
echinocyte

platelets are about 10% higher than those of the formaldehyde fixed ones (Fig. 18 and Table 2). At low pH's two problems were encountered. The first problem was platelet agglutination which occurred at pH 3 or below. The second problem was irreversible changes in the mobility at low pH. For example, when the pH of the discocyte suspension was brought down to 4.0 from 7.4 the mobility went from -1.08 to $-0.75 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$. Upon returning to pH 7.4, the discocyte mobility went back only to $-0.98 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$. Therefore mobilities below pH 4.5 were not explored further.

3.3.2 Platelet Surface Sialic Acid

The neuraminidase digest-time curve is shown in Fig. 20. After 30 min. most of the neuraminidase susceptible sialic acid was released from the glutaraldehyde fixed discocytes. An arbitrary time of 90 min (3x30 min) was therefore used for further enzyme digestions.

The amount of sialic acid liberated from the platelet surfaces was determined in two ways: first, by chemically assaying the platelet suspending media and second, by calculation from the drop in platelet electrophoretic mobility. The chemical analysis data is shown in Table 5. No major differences were found in total sialic acid content between the discocyte, echinocyte and spherocyte, total sialic acid being sialic acid liberated by acid hydrolysis. It represents sialic acid from within as well as on the surface of the platelets. Neuraminidase, on the other hand, removed more sialic acid (17-18%) from the echinocyte and spherocyte than from the discocyte surface (Table 5). Neuraminidase digestion of saponified platelets failed to release any more sialic acid than from the non-saponified ones. No O-acetyl substitution was therefore evident.

Fig. 20. The time-release curve for the digestion of sialic acid from the surface of fixed platelet discocytes by neuraminidase. 2.5×10^8/ml of platelets were incubated with 0.04 IU/ml of enzyme. Values are means from three series of experiments. Error bars represent one standard deviation.

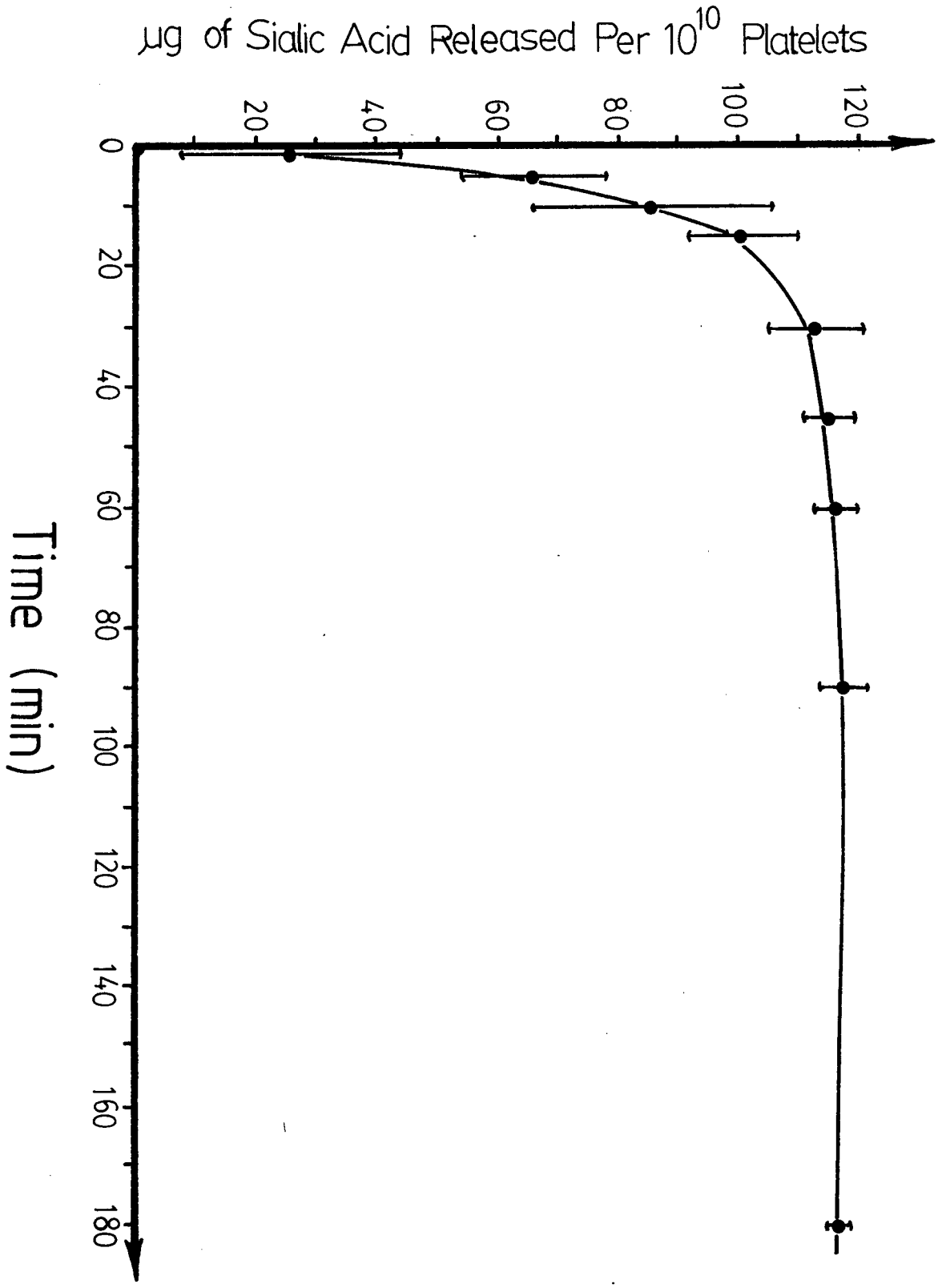

µg of Sialic Acid Released Per 10^{10} Platelets
20
40
60
80
100
120
Time (min)
0
20
40
60
80
100
120
140
160
180

Table 5. Platelet Surface Sialic Acid Determined Chemically

	Discocyte	Echinocyte	Spherocyte
Total sialic acid released by acid hydrolysis: (n=10)			
$\mu g/10^{10}$ platelet	205 ($\pm$6.0)	203 ($\pm$3.8)	204 ($\pm$4.9)
molecules/platelet	4.00×10^7 ($\pm 0.117 \times 10^7$)	3.96×10^7 ($\pm 0.074 \times 10^7$)	3.98×10^7 ($\pm 0.095 \times 10^7$)
Neuraminidase removable sialic acid: (n=18)			
$\mu g/10^{10}$ platelet	116 ($\pm$1.9)	137 ($\pm$3.3)	136 ($\pm$3.2)
molecules/platelet	2.26×10^7 ($\pm 0.037 \times 10^7$)	2.67×10^7 ($\pm 0.064 \times 10^7$)	2.65×10^7 ($\pm 0.062 \times 10^7$)
Percent of total sialic acid removed by neuraminidase	56.6%	67.5%	66.7%

Table 6 shows how the decrease in platelet electrophoretic mobility after neuraminidase treatment translates into molecules of sialic acid removed. By comparing the electrophoresis data to the chemical analysis data (Table 7), it is obvious that only 2.9% of the neuraminidase susceptible sialic acid on the surfaces of the discocyte and spherocyte contributed to the platelet electrokinetics. An opportunity is therefore presented to estimate the surface area of the echinocyte. Assuming the 2.9% ratio to be true for the echinocyte as well, then the number of sialic acid molecules removed from the plane of shear per echinocyte can be calculated (line (a) in Table 7). This is found to be 7.74×10^5 (2.67×10^7 x 0.029). Since the number of molecules removed per cm^2 at the plane of shear is known (3.52×10^{12}), the echinocyte surface area is calculated to be $22.0 \times 10^{-8} cm^2$ ($7.74 \times 10^5 \div 3.5 \times 10^{12}$).

3.3.3 Platelet Surface Phosphate Groups

The time-digest curve for alkaline phosphatase is shown in Fig. 21. It seems only 15 min is required to liberate the phosphate groups from the fixed discocytes. Therefore the phosphate experiments were carried out with 45 min (3x15 min) of incubation with the enzyme. The amounts of phosphate terminal groups released by alkaline phosphatase as determined chemically and electrophoretically are shown in Table 8 and Table 9 respectively. The percentage of phosphate groups at the plane of shear as determined electrophoretically is about 3.9% of the total amount determined chemically (Table 10). Again if one takes the percentage also to be true for the echinocyte, then its area can be back calculated as before. This calculation gave $25.3 \times 10^{-8} cm^2$, only slightly higher than the area found from the sialic acid calculations. An average of the two ($23.7 \times 10^{-8} cm^2$) is therefore taken.

Table 6. Platelet Surface Sialic Acid Determined from the Decrease in Electrophoretic Mobility after Neuraminidase Treatment

	Discocyte	Echinocyte	Spherocyte
EPM:glutaraldehyde fixed platelet ($x10^4 cm^2.sec^{-1}.V^{-1}$)	-1.41 ($\pm$0.020)	-1.21 ($\pm$0.008)	-1.00 ($\pm$0.100)
EPM:neuraminidase treated fixed platelet ($x10^4 cm^2.sec^{-1}.V^{-1}$)	-0.86 ($\pm$0.010)	-0.72 ($\pm$0.010)	-0.70 ($\pm$0.007)
EPM decrease ($x10^4 cm^2.sec^{-1}.V^{-1}$)	0.55 ($\pm$0.014)*	0.49 ($\pm$0.005)*	0.29 ($\pm$0.004)*
Decrease in charge density (esu/cm^2)	$1.91x10^3$ ($\pm 0.047x10^3$)	$1.69x10^3$ ($\pm 0.016x10^3$)	$1.02x10^3$ ($\pm 0.015x10^3$)
Sialic acid molecules removed per cm^2	$3.98x10^{12}$ ($\pm 0.098x10^{12}$)	$3.52x10^{12}$ ($\pm 0.033x10^{12}$)	$2.12x10^{12}$ ($\pm 0.031x10^{12}$)
Sialic acid molecules removed per platelet	$6.53x10^5$ ($\pm 0.161x10^5$)	-	$7.80x10^5$ ($\pm 0.114x10^5$)

Electrophoretic mobility (EPM) values are means from 10 series of paired experiments.

*Combined standard deviation (same as in Table 4)

Table 7. Ratio of Platelet Surface Sialic Acid Removed by Neuraminidase as Determined by Microelectrophoresis to That Determined Chemically

	Discocyte	Echinocyte	Spherocyte
Surface area (cm^2)	16.4×10^{-8}		36.7×10^{-8}
Electrophoresis data:			
Sialic acid molecules removed per cm^2	3.98×10^{12}	3.52×10^{12}	2.12×10^{12}
Sialic acid molecules removed per platelet (a)	6.53×10^{5}	-	7.80×10^{5}
Chemistry data:			
Sialic acid molecules removed per platelet (b)	2.26×10^{7}	2.67×10^{7}	2.65×10^{7}
Ratio (a)/(b)	0.0288	-	0.0294

Fig. 21. The time-release curve for the digestion of phosphate groups from the surface of fixed platelet discocytes by alkaline phosphatase. 2.5×10^8/ml of platelets were incubated with 0.01 mg/ml of the enzyme. Values are means from three series of experiments. Error bars represent one standard deviation.

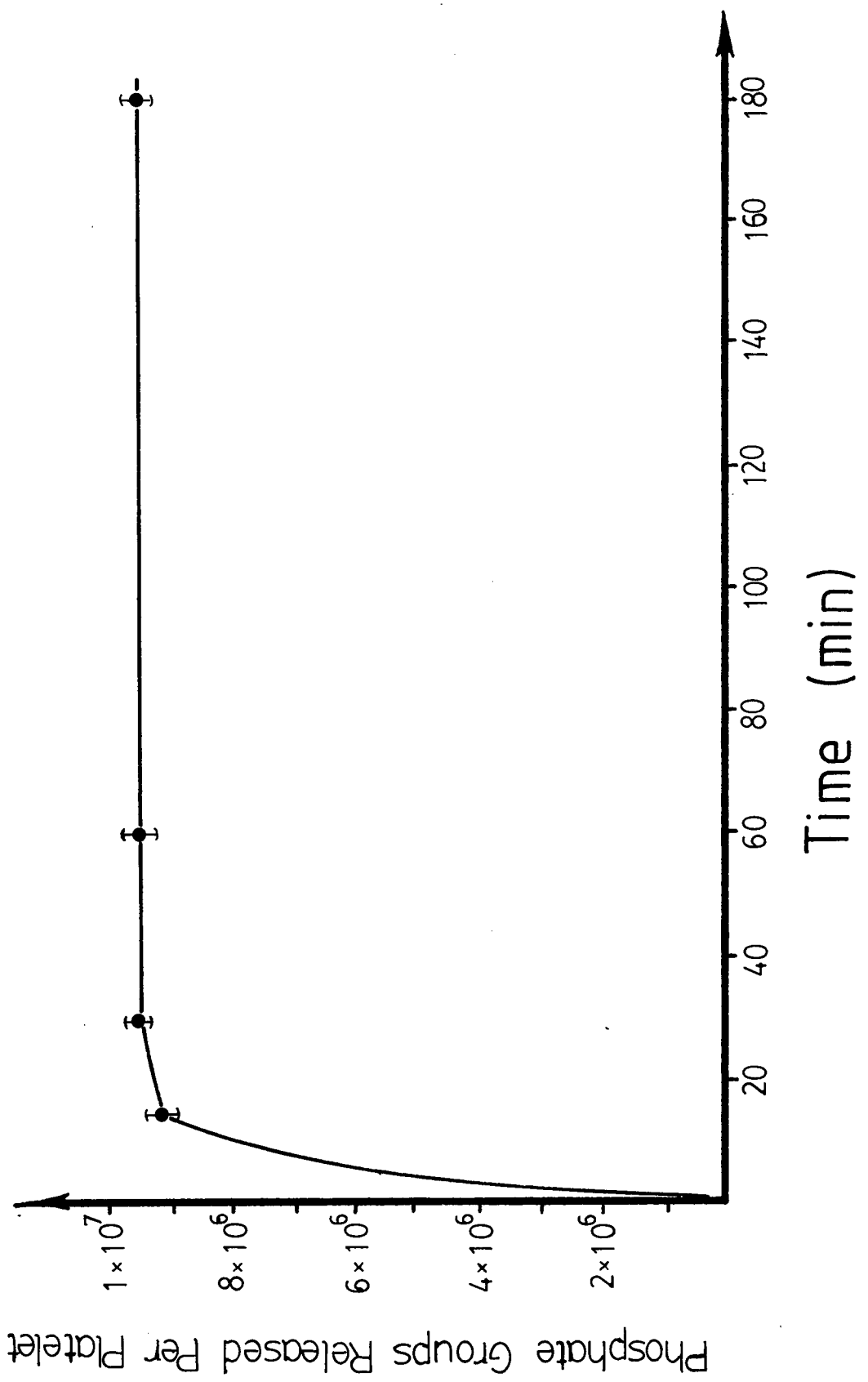

Phosphate Groups Released Per Platelet
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
20
40
60
80
100
120
140
160
180
Time (min)

Table 8. Platelet Surface Phosphate Groups Released After Alkaline Phosphatase Treatment Determined Chemically

	Discocyte	Echinocyte	Spherocyte
Phosphate groups/ platelet	9.44×10^6 ($\pm 0.199 \times 10^6$)	12.50×10^6 ($\pm 0.184 \times 10^6$)	18.51×10^6 ($\pm 0.130 \times 10^6$)

n=5

Table 9. Platelet Surface Phosphate Groups Determined from the Decrease in Electrophoretic Mobility after Alkaline Phosphatase Treatment

	Discocyte	Echinocyte	Spherocyte
EPM:glutaraldehyde fixed platelet ($x10^4 cm^2.sec^{-1}.V^{-1}$)	-1.41 ($\pm$0.020)	-1.21 ($\pm$0.008)	-1.00 ($\pm$0.010)
EPM:alk. phosphatase treated fixed platelet ($x10^4 cm^2.sec^{-1}.V^{-1}$)	-1.10 ($\pm$0.005)	-0.94 ($\pm$0.008)	-0.73 ($\pm$0.009)
EPM decrease ($x10^4 cm^2.sec^{-1}.V^{-1}$)	0.31 ($\pm$0.015)*	0.27 ($\pm$0.008)*	0.27 ($\pm$0.009)*
Decrease in charge density (esu/cm^2)	$1.09x10^3$ ($\pm 0.052x10^3$)	$0.94x10^3$ ($\pm 0.027x10^3$)	$0.94x10^3$ ($\pm 0.032x10^3$)
Phosphate groups removed per cm^2	$2.28x10^{12}$ ($\pm 0.108x10^{12}$)	$1.96x10^{12}$ ($\pm 0.056x10^{12}$)	$1.96x10^{12}$ ($\pm 0.067x10^{12}$)
Phosphate groups removed per platelet	$3.74x10^5$ ($\pm 0.177x10^5$)	-	$7.19x10^5$ ($\pm 0.245x10^5$)

Electrophoretic mobility (EPM) values are means from 10 series of paired experiments.

*Combined standard deviation (same as in Table 4)

Table 10. Ratio of Platelet Surface Phosphate Groups Removed by Alkaline Phosphatase as Determined by Microelectrophoresis to Those Determined Chemically

	Discocyte	Echinocyte	Spherocyte
Surface area (cm^2)	16.4×10^{-8}		36.7×10^{-8}
Electrophoresis data:			
Phosphate groups removed per cm^2	2.28×10^{12}	1.96×10^{12}	1.06×10^{12}
Phosphate groups removed per platelet (a)	3.74×10^{5}	-	7.19×10^{5}
Chemistry data:			
Phosphate groups removed per platelet (b)	9.44×10^{6}	12.50×10^{6}	18.51×10^{6}
Ratio (a)/(b)	0.0396	-	0.0389

3.3.4 Cocaine Spheres

Non-fixed cocaine spheres were treated with neuraminidase and alkaline phosphatase. The amounts of sialic acid and phosphate groups liberated as determined chemically from the supernatant and determined from the reductions in electrophoretic mobility after enzyme treatment are shown in Table 11. Since the electrokinetic properties of the cocaine sphere are much different from the other forms of platelets (Table 3), no attempt was made to compare the fixed cocaine spheres to the other forms of fixed platelet.

3.3.5 Calcium and Magnesium Ion Binding

Fig. 22 shows how the electrophoretic mobility of platelets decreases with increasing divalent ion concentration. Fig. 23 is a plot of the double reciprocal relationship between the drop in negative charge density and divalent cation concentration according to the formula given in the Introduction. The number of binding sites calculated from the intercepts in Fig. 23 are shown in Table 12 for calcium ions and Table 13 for magnesium ions. There are twelve times more Ca^{++} and eight times more Mg^{++} binding sites on the echinocyte than on the discocyte. On the other hand, the amount of Ca^{++} binding to the spherocyte is 61% lower than to the discocyte. Statistical comparisons of the binding density and free energy of Ca^{++} and Mg^{++} adsorption to the discocyte, echinocyte and spherocyte are summarized in Table 14. It is evident that the binding densities and free energies of Ca^{++} and Mg^{++} to the echinocyte are significantly different from those to the discocyte. The binding of Ca^{++} and of Mg^{++} to the discocyte is not significantly different and the same applies for the echinocyte.

Table 11. Sialic Acid and Phosphate Groups Released by Enzymes from Cocaine Sphere* Determined Electrophoretically and Chemically

	Control	Neuraminidase treatment	Aklaline Phosphatase treatment
Electrophoresis data:			
EPM ($10^4 cm^2.sec^{-1}.V^{-1}$) n=8	-1.40 ($\pm$0.017)	-0.67 ($\pm$0.012)	-1.00 ($\pm$0.018)
Charge density (esu/cm^2)	4.87×10^3 ($\pm 0.058 \times 10^3$)	2.34×10^3 ($\pm 0.041 \times 10^3$)	3.48×10^3 ($\pm 0.062 \times 10^3$)
Number of charges per cm^2	10.14×10^{12} ($\pm 0.121 \times 10^{12}$)	4.88×10^{12} ($\pm 0.085 \times 10^{12}$)	7.25×10^{12} ($\pm 0.129 \times 10^{12}$)
Number of charges per platelet	2.68×10^6 ($\pm 0.032 \times 10^6$)	1.29×10^6 ($\pm 0.022 \times 10^6$)	1.91×10^6 ($\pm 0.03 \times 10^6$)
Drop in number of charges per platelet (a)		1.39×10^6	0.76×10^6
Chemistry data:			
Sialic acid molecules released per platelet (b) (n=4)		2.69×10^7 ($\pm 0.031 \times 10^7$)	
Phosphate groups released per platelet (b) (n=4)			1.27×10^7 ($\pm 0.023 \times 10^7$)
Ratio (a)/(b)		0.0515	0.0601

*Area=$26.4 \times 10^{-8} cm^2$

Fig. 22. Influence of calcium and magnesium ion concentration on the electrophoretic mobility of the platelet discocyte, echinocyte, and spherocyte. Each data point represent a mean of 100 mobility determinations. Error bar represent one standard deviation.

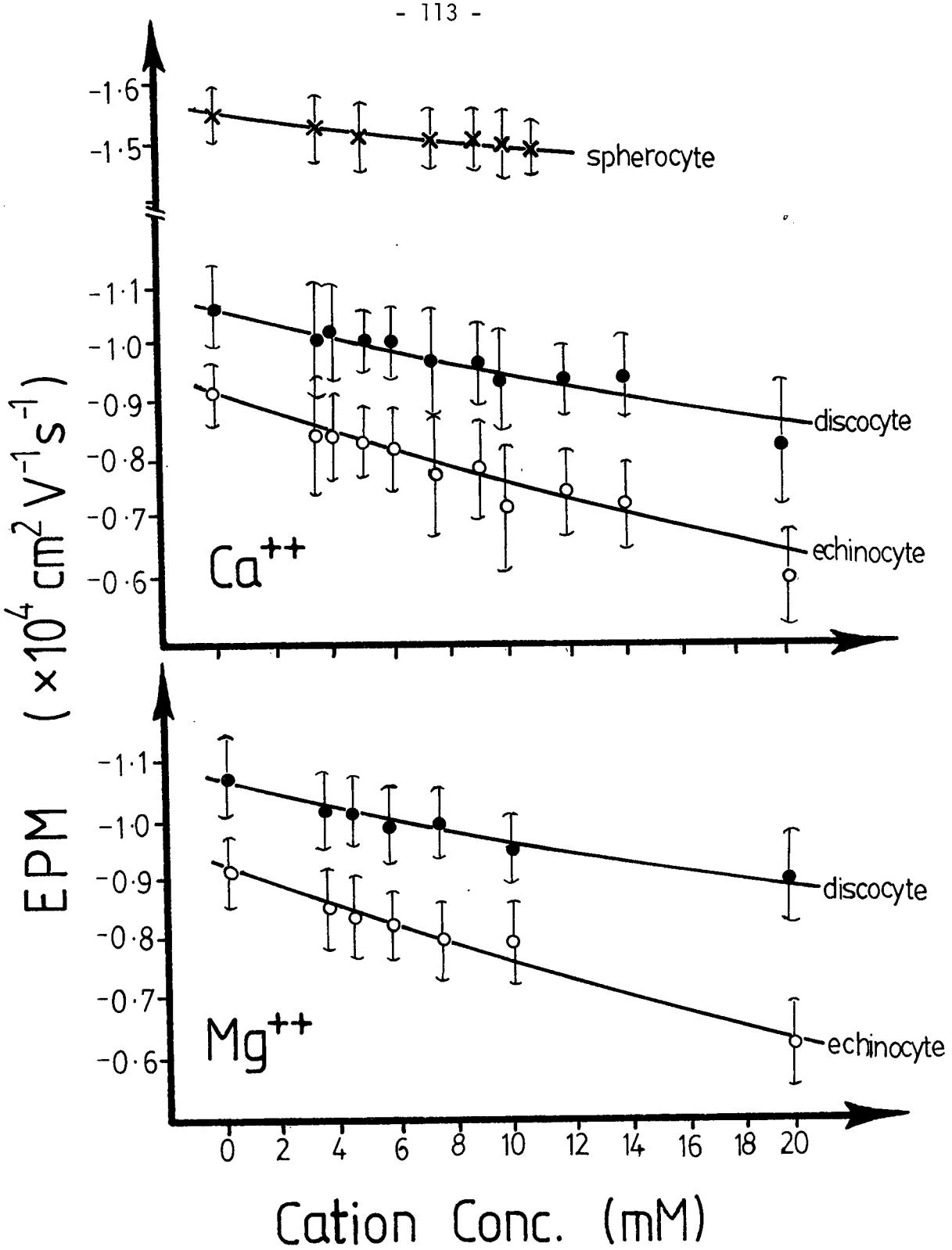

-1·6
-1·5
spherocyte
-1·1
-1·0
-0·9
-0·8
-0·7
-0·6
discocyte
echinocyte
Ca++
EPM (x10^4 cm^2 V^-1 s^-1)
-1·1
-1·0
-0·9
-0·8
-0·7
-0·6
discocyte
echinocyte
Mg++
0 2 4 6 8 10 12 14 16 18 20
Cation Conc. (mM)

Fig. 23. The double reciprocal plots to find the densities of calcium and magnesium ion binding sites on the discocyte and echinocyte. Error bars represent one standard deviation. [cat.]=concentration of either Ca^{++} or Mg^{++}

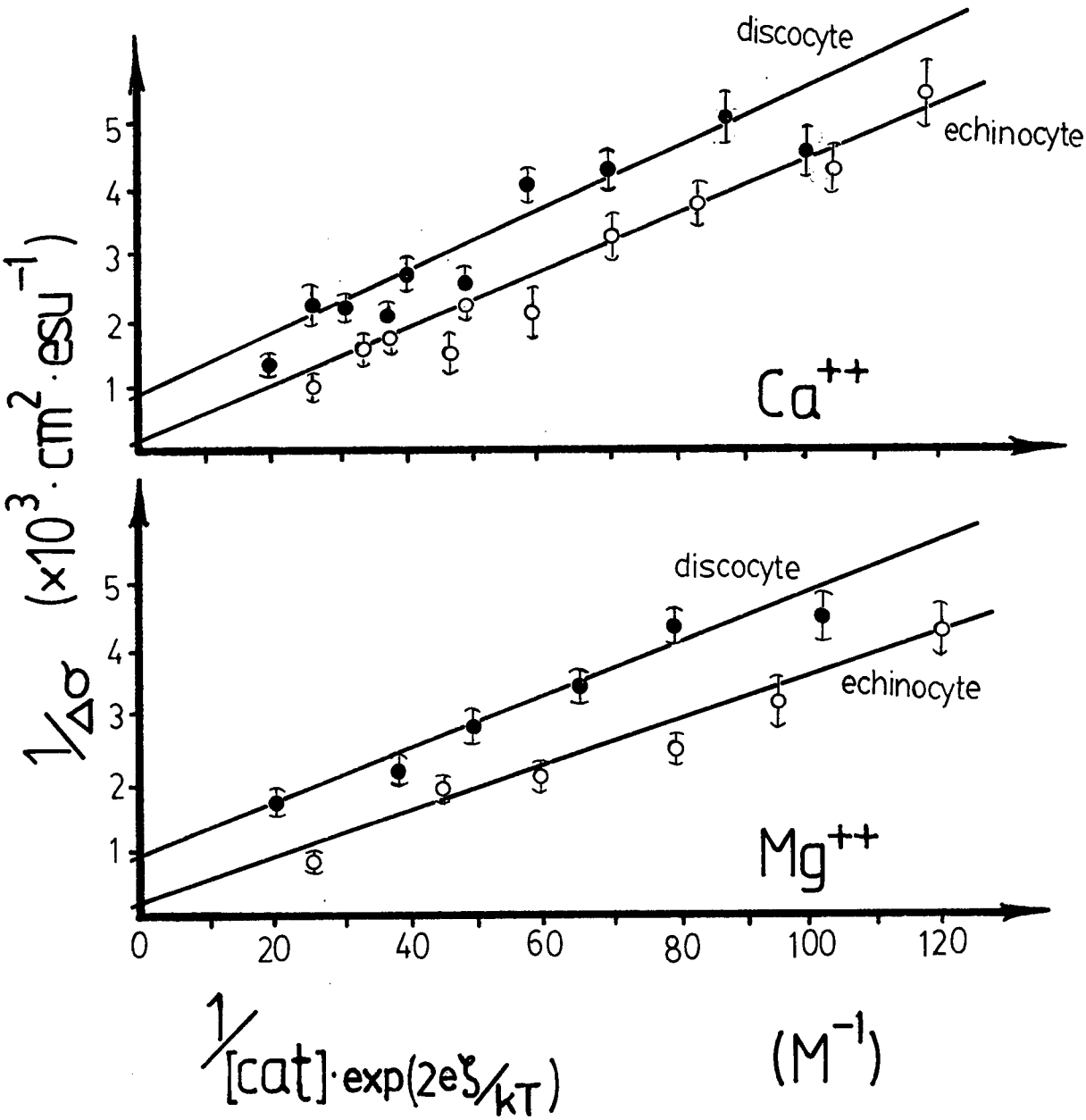

discocyte
echinocyte
Ca++
discocyte
echinocyte
Mg++
1/Δσ (x10^3 · cm^2 · esu^-1)
1/[cat]·exp(2eζ/kT)
(M^-1)
0
20
40
60
80
100
120
1
2
3
4
5

Fig. 23 (continued). The double reciprocal plot to find the density of calcium ion binding sites on the spherocyte. Error bars represent one standard deviation. [cat.]=concentration of Ca^{++}.

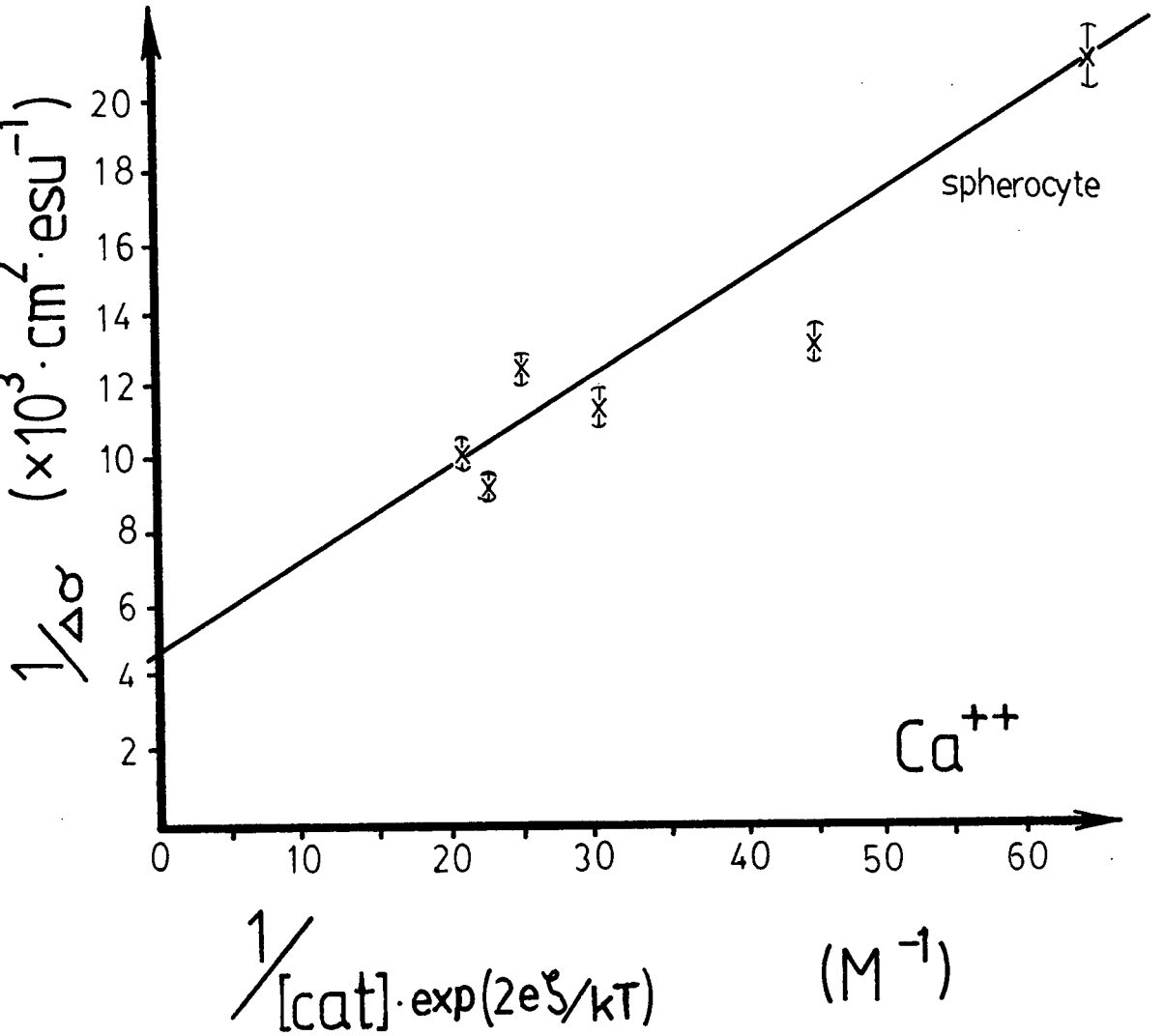

$1/\Delta\sigma$ $(\times 10^3 \cdot cm^2 \cdot esu^{-1})$
20
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
spherocyte
Ca^{++}
$1/[cat] \cdot \exp(2e\zeta/kT)$
(M^{-1})

Table 12. Calcium Ion Binding Sites

	Discocyte	Echinocyte	Spherocyte
Intercept on ordinate $(esu/cm^2)^{-1}$	8.15×10^{-4} ($\pm 6.8 \times 10^{-4}$)	9.50×10^{-5} ($\pm 2.07 \times 10^{-4}$)	4.69×10^{-3} ($\pm 1.5 \times 10^{-3}$)
Slope	4.54×10^{-5} ($\pm 8.45 \times 10^{-6}$)	4.03×10^{-5} ($\pm 2.24 \times 10^{-6}$)	2.43×10^{-4} ($\pm 4.06 \times 10^{-5}$)
Correlation coefficient	0.88	0.98	0.95
Binding sites:			
Density (cm^{-2})	1.28×10^{12} ($\pm 1.07 \times 10^{12}$)	1.09×10^{13} ($\pm 2.39 \times 10^{13}$)	2.22×10^{11} ($\pm 7.17 \times 10^{10}$)
Per platelet	2.10×10^{5}	2.58×10^{6}*	8.16×10^{4}
$-\Delta G$ (Kcal/mole)	4.09 (± 0.11)	2.89 (± 0.03)	4.13 (± 0.10)

*Calculated using the surface area of $23.7 \times 10^{-8} cm^2$.

Table 13. Magnesium Ion Binding Sites

	Discocyte	Echinocyte
Intercept on ordinate $(esu/cm^2)^{-1}$	9.66×10^{-4} ($\pm 3.49 \times 10^{-4}$)	1.58×10^{-4} ($\pm 2.55 \times 10^{-4}$)
Slope	4.03×10^{-5} ($\pm 5.32 \times 10^{-6}$)	3.57×10^{-5} ($\pm 3.38 \times 10^{-6}$)
Correlation coefficient	0.97	0.98
Binding sites:		
Density (cm^{-2})	1.08×10^{12} ($\pm 3.89 \times 10^{11}$)	6.60×10^{12} ($\pm 1.07 \times 10^{13}$)
Per platelet	1.77×10^{5}	1.56×10^{6}*
$-\Delta G$ (Kcal/mole)	4.26 (± 0.08)	3.26 (± 0.06)

*Calculated using the surface area of $23.7 \times 10^{-8} cm^2$.

Table 14. Statistical Comparison of Ca^{++} and Mg^{++} Binding to the Discocyte, Echinocyte and Spherocyte (Sig.=significant differences; not=no significant difference)

	Binding site density	Free energy (G)
Ca^{++}, discocyte vs echinocyte	sig., 0.025 p 0.05	sig., 0.0005 p 0.001
Ca^{++}, discocyte vs spherocyte	sig., p 0.005	sig., 0.01 p 0.025
Ca^{++}, echinocyte vs spherocyte	sig., p 0.005	sig., 0.005 p 0.01
Mg^{++}, discocyte vs echinocyte	sig., 0.005 p 0.01	sig., p 0.0005
discocyte, Ca^{++} vs Mg^{++}	not, 0.50 p 0.75	not., 0.50 p 0.75
echinocyte, Ca^{++} vs Mg^{++}	not, 0.50 p 0.75	not., 0.50 p 0.75

Statistical analysis using Analysis of Variance Tables and F-distribution according to Kalbfleisch (1974). See also the appendix.

3.4 DISCUSSION

3.4.1 Platelet Electrophoretic Mobilities

The electrophoretic mobility of the human discocyte in Tyrode's solution was found to be $-1.08 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ (non-fixed). This is similar to the mobility of the fresh human RBC. A review of the literature shows a variety of values for human platelet mobility. They are -0.635 (Coller and Zarrabi, 1981), -0.71 (Boisseau et al., 1971), -0.806 (Kosztolanyi et al., 1980), -1.07 (Hampton and Mitchell, 1974) and $-1.17 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ (Seaman and Vassar, 1966) for platelets measured in platelet-rich plasma and -0.84 (Mehrishi, 1970), -0.85 (Seaman and Vassar, 1966), -0.86 (Kosztolanyi et al., 1980), -1.08 (Bray and Alexander, 1969), -1.09 (Bosmann, 1972), -1.1 (Ross and Ebert, 1959), -1.14 (Madoff et al., 1964), -1.25 (Kirschmann et al., 1959), -1.4 (Yamazaki et al., 1980) and $-1.58 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ (Shimizu et al., 1979) for washed platelets measured in simple buffered saline-type media. Our result of $-1.08 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ for washed platelets is similar to the ones obtained by Bray and Alexander and Bosmann. The great range of mobility values probably reflects differences in the electrophoretic systems involved as well as the conditions of the platelet samples (this point is to be discussed further below).

An electrophoretic mobility of $-1.08 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ translates into a charge density of 3.74×10^3 esu/cm^2 or 1.28×10^6 net negative charge groups per discocyte (Table 3). The number of net negative charge groups is the actual (total) number of negative charge groups minus the positive charge groups.

After stimulation by 2×10^{-5}M ADP, there was a 13.7% drop in mobility from -1.08 to $-0.93 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$. The net negative charge density

decreased correspondingly from 3.74×10^3 to 3.23×10^3 esu/cm^2 (Table 3). As discussed earlier, Seaman and Vassar (1966) reported an 18% drop in human platelet electrophoretic mobility after addition of 1 μg/ml (2.3×10^{-6}M) of ADP. The decrease was concentration dependent and the reduction was up to 40% at 100 μg/ml (2.3×10^{-4}M) of ADP. However, they failed to confirm the biphasic mobility behaviour reported by Hampton and Mitchell (1966), Stoltz (1970) and Kosztolanyi et al. (1980). These workers reported that at low ADP concentrations, the platelet mobility increased up to a concentration of 5×10^{-2} μg/ml (1×10^{-7}M) after which the mobility decreased with concentration. However the maximum mobility increase at 1×10^{-7}M was only about 8% in all three reports. Confirming Seaman (1976) we were able to observe only the decrease in electrophoretic mobility. At 1×10^{-7}M ADP concentration we were not able to detect any change in platelet mobility. Takano and Suzuki (1981) working on rabbit platelets recorded only decreases in mobility with increasing ADP concentration. Finally, Boisseau et al. (1977) reported an increase in platelet mobility of 10% at 2×10^{-6}M ADP concentration.

Interestingly, platelet electrophoretic mobilities were all measured in platelet-rich plasma in the above papers. This work is the first to report a change in the mobility of washed platelets induced by ADP. Seaman and Vassar (1966) failed to find any change in the mobility of washed platelets in saline after exposure to ADP. The mobility -0.85 m.sec^{-1}.V^{-1}.cm for their washed platelets was very low, in fact close to the mobility of our echinocyte. They washed their platelets simply by centrifuging them down in saline. The difficulties encountered in washing platelets have already been discussed in Chapter 2. By repeating of the Seaman and Vassar washing procedure by us produced platelet aggregates of various sizes. Therefore it is possible that the above

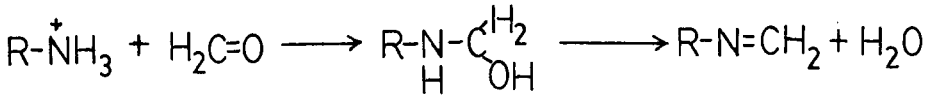
authors were actually measuring the mobility of small platelet aggregates or echinocytes. This could well be the reason why their washed platelets would not respond to ADP. The great variety of published platelet mobilities mentioned above may also in part be caused by different platelet handling procedures. Parallel microscopic monitoring of platelet morphology similar to that done here is therefore considered essential. Handling and transformation (to echinocyte and spherocyte) protocols were set up and standardized with the help of electron microscopy and all platelet samples were examined under phase microscopy before electrophoresis.

The spherocyte had an electrophoretic mobility of $-1.55 \times 10^{-4} cm^2 . sec^{-1} . V^{-1}$ measured in hypotonic Tyrode's solution. The charge density was 2.60×10^3 esu/cm^2 (Table 3). Presumably due to the increase in surface area, the number of charge groups per spherocyte increased by 55%.

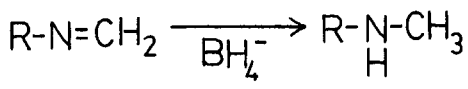
The hypotonic Tyrode's solution has an osmolality of 75 mOsmol and an ionic strength of 0.0379. The relationship between mobility and ionic strength is an inverse one (see equation in Introduction). Kirschmann et al. (1959) have examined the effect of low ionic strength on the electrokinetic properties of human platelets. As ionic strength was decreased the mobility increased. They found that the change in mobility was totally reversible. If platelets were suspended in 0.015 ionic strength medium and then resuspended back in an iso-ionic strength medium, the mobility returned to normal. We made similar observations. Discocytes suspended in a 0.04 ionic strength isotonic Tyrode's solution had a mobility of about $-2.08 \times 10^{-4} cm^2 . sec^{-1} . V^{-1}$ and after resuspension in regular Tyrode's solution the mobility was returned back to $-1.08 \times 10^{-4} cm^2 . sec^{-1} . V^{-1}$. No shape change was observed in the discocyte due to change in ionic strength.

The pH-mobility curves are shown in Fig. 18 and 19. It was hoped that information concerning platelet surface ionogenic groups could be obtained by constructing these curves. However, as a result of the problems encountered at low pH already discussed, the curves cannot be extrapolated below pH 4.5 and values such as pKa for the platelet surfaces cannot be deduced. The mobilities of glutaraldehyde fixed platelets were about 10% higher than the mobilities of formaldehyde fixed platelets. This phenomenon has also been found in red blood cells (Vassar et al., 1972) and lymphocytes (Vassar et al., 1973). The reason for the apparent additional charge remains unclear. Because of its fast action (Vassar et al., 1972) glutaraldehyde remained the fixative of choice for microscopic and enzyme digestion studies. Since the 10% increase occurred similarly to the discocyte, echinocyte and spherocyte no correction is required for the enzyme digestion studies. Acetaldehyde fixation tends to agglutinate platelets during fixation.

To study the amino groups on platelet surfaces formaldehyde fixation was chosen. The reaction of formaldehyde with cellular components to induce fixation is complex but presumably formaldehyde interacts with amino groups in the following way:



The positive charge is thus eliminated (Jentoft and Dearborn, 1979). To test the possibility that reversal of the fixation process could have occurred the platelets were further treated with borohydride (Jentoft and Dearborn, 1979):



A stable neutral species is then obtained. If there were significant reversal of the fixation process, the borohydride treated fixed platelets ought to have had a greater increase in mobility than the non-treated ones. We found no differences, indicating no reversal of fixation had occurred.

The value obtained here for the number of amino groups per platelet was 2.18×10^5 (Table 4) which compares well with those of Stoltz and Nicolas (1979), 3×10^5, and Mehrishi (1970), 2.2 to 2.5×10^5. The amino group surface density on the echinocyte is 10.5% lower and on the spherocyte 18.8% lower than on the discocyte.

3.4.2 Platelet Surface Sialic Acid

By acid hydrolysis one can find the total sialic acid content in the platelets. Mild hydrolysis with H_2SO_4 released sialic acid from the surface as well as from within the fixed platelets. The released sialic acid was then assayed. The total sialic acid content in the discocyte was found to be 205 $\mu g/10^{10}$ platelets or 3.99×10^7 molecules/platelet. The discocyte, echinocyte and spherocyte all had similar total sialic acid contents (Table 5) showing that during the transformations no sialic acid is lost. Both Motamed *et al.* (1976) and Martin *et al.* (1982) found 207 μg of sialic acid per 10^{10} platelets by acid hydrolysis. Ku and Wu (1977) found 185 and Stoltz and Nicolas (1979) found about 197 $\mu g/10^{10}$ platelets.

Neuraminidase (*Vibrio cholerae*) removed 56.6% of the total sialic acid from the discocyte. Higher percentages, 67.5% and 66.7% of total were removed from the echinocyte and spherocyte respectively (Table 5). In other words, neuraminidase susceptible sialic acid goes from 116 $\mu g/10^{10}$ discocytes to 137 $\mu g/10^{10}$ echinocytes and 136 $\mu g/10^{10}$ spherocytes. Ku and Wu (1977)

found that 48% of the total platelet sialic acid is released by neuraminidase and after ADP ($5x10^{-6}$M) stimulation the percentage increased to 65%. Motamed et al. (1976) found 40% for the unstimulated platelets and about 49% for ADP stimulated ones. Peerschke et al. (1978), on the other hand, found no difference between the amount of sialic acid removed by the enzyme from the surface of unstimulated and ADP stimulated platelets. No explanation was offered by them for the discrepancy.

Neuraminidase (Vibrio cholerae) has a molecular weight of 90,000 (Gottschalk and Bhargava, 1971; Behring, 1979). From sedimentation and diffusion coefficients it has an estimated radius of 32Å (Pye and Curtain, 1961). It is the belief of most workers that it will not gain access to the interior of the platelet and its enzymatic action is strictly on the platelet surface (Stoltz and Nicholas, 1979; Packham et al., 1980; Coller and Zarrabi, 1981). It is our hypothesis that an enzyme of this size will not be able to penetrate into the surface-connected canalicular system. Electron microscopic studies by Behnke (1968) showed that horseradish peroxidase, which has a molecular weight of 40,000 (Worthington, 1972) did stain the inside surface of some of the canalicular system. White (1970) made a more detailed study of the system by using two electron dense tracers of different sizes. Lanthanum nitrate (forming 25Å diameter particles) penetrates easily into the surface-connected canalicular system. On the other hand, ruthenium red (forming 50Å diameter particles) stains just the outside of platelets and only penetrates "superficially" into the canalicular system. After ADP stimulation, ruthenium red readily penetrates into the canalicular system demonstrating an apparent opening up process. White (1980) further showed that the openings of the

the channels at the discocyte surface are rather constrictive (see Fig. 3) and may not be as simple as once thought.

Our results show quantitative differences between the discocyte and the echinocyte as well as between the discocyte and the spherocyte. The additional sialic acid susceptible to neuraminidase most probably comes from evaginated or newly exposed membrane surfaces. Given White's results and the increase in surface area believed to occur upon echinocyte transformation (Frojmovic and Milton, 1982) it seems unlikely that neuraminidase penetrates into the discocyte canalicular system. The same argument applies to the enzyme alkaline phosphatase whose molecular weight is considerably larger at 140,000 (Frenette, 1980).

Electrophoretic measurements of fixed platelet discocytes showed a 39% drop in mobility after neuraminidase treatment (Table 6). Madoff et al. (1964) found a 42% decrease while Seaman and Vassar (1966) and Bosmann (1972) found 53% reductions. The decrease in mobility was 40% for the echinocyte and only 29% for the spherocyte (Table 6). This reflects the much lower sialic acid charge density on the spherocyte.

Only 2.9% of the neuraminidase susceptible sialic acid on the platelet surface is apparently responsible for the electrokinetic charge properties of the platelet (both discocyte and spherocyte). This figure derives from the 30 fold difference between the amount of sialic acid assayed chemically in the enzyme digest supernatant and that calculated from electrophoretic mobility measurements. Stoltz and Nicolas (1979) found a figure of 3.2%. The percentage is higher in polymorphonuclear leukocytes (10%) and considerably higher still for red blood cells (46%) (Cook et al., 1961 and Vassar et al., 1969). If we assume the percentage for the echinocyte to be also 2.9%, then

the surface area of the echinocyte can be back calculated from the chemistry data as described above (Table 7), giving the value of $22.0 \times 10^{-8} cm^2$.

3.4.3 Platelet Surface Phosphate Groups

Alkaline phosphatase removed 9.44×10^6 phosphate groups from each fixed discocyte (Table 8). This value is double that of 4.2×10^6 found by Bik *et al.* (1982). The enzyme liberates 32% and 96% more phosphate from the surfaces of the echinocyte and spherocyte respectively.

The electrophoretic mobility of the discocyte decreased by 22% after enzyme digestion corresponding to a removal of 3.74×10^5 phosphate groups from the discocyte plane of shear (Table 9). Mehrishi (1970) found a drop in mobility of between 30 and 37%, corresponding to a removal of 5×10^5 groups per platelet. Stoltz (1975) estimated platelet surface phosphate groups to be between 3 and 5×10^5 per platelet from microelectrophoresis experiments.

Again, by comparing electrophoresis data with chemistry data (Table 10) it was found that only 3.9% of the phosphate groups removed are apparently responsible for the electrokinetic charge contribution of the discocyte and spherocyte. If it is assumed that the same percentage applies to the echinocyte its surface area can again be calculated. The value based on phosphatase digestion data was $25.3 \times 10^{-8} cm^2$, quite close to $22.0 \times 10^{-8} cm^2$ obtained from the sialic acid calculations. An average of $23.7 \times 10^{-8} cm^2$ was therefore taken to be the estimate of the echinocyte surface area accessible to these two enzymes.

The established area of $23.7 \times 10^{-8} cm^2$ is larger than that of the discocyte but much smaller than that of the spherocyte (Table 1). Presuming that the newly-exposed membrane originates from the surface-connected canalicular

system, it is apparent that it could not be totally evaginated in the echinocyte. This conclusion is consistent with the morphological evidence for a partially intact canalicular system presented in Chapter 2.

In a recent review, Frojmovic and Milton (1982) made an estimate of the surface area of an echinocyte based on microscopic observations. By assuming an echinocyte to have a central spherical body of 13 μm^2 with thin cylindrical pseudopods extending out of the central body, they arrived at an area of between 17.7 and $22.4 \times 10^{-8} cm^2$. Their pseudopods had a mean radius of 0.075 μm and total length of between 10 and 20 μm. The closeness of this estimation to the one we obtained via surface chemical means is remarkable.

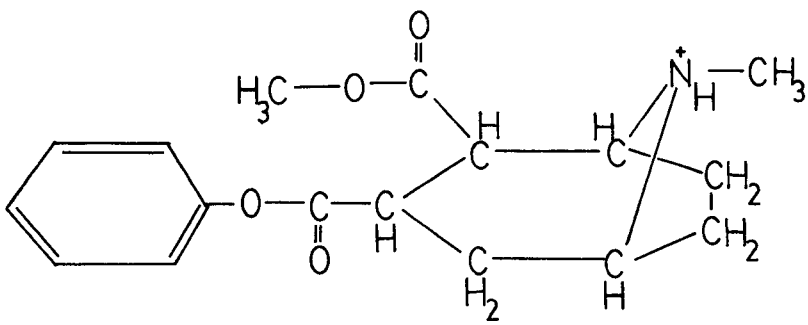
3.4.4 Cocaine Spheres

Incubating the platelet discocyte with 10 mM of cocaine for half an hour produces a "cocaine sphere" with a surface area of $26.4 \times 10^{-8} cm^2$. The electrophoretic mobility increases dramatically from $-1.08 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ for the discocyte to $-1.40 \times 10^{-4} cm^2.sec^{-1}.V^{-1}$ for the cocaine sphere. This corresponds to an apparent 30% increase in charge density (charge per cm^2) and a 108% increase in the number of charges per platelet (Table 3) at the plane of shear. Having a surface area slightly larger than that of the echinocyte, it has 68% more charge than the echinocyte. This change cannot be attributed to increase in surface area alone (see below).

Treatment of the cocaine spheres (unfixed) with neuraminidase resulted in a 52.6% reduction in mobility representing 1.39×10^6 sialic acid groups per platelet removed from the plane of shear. This is 66.4% more than in the fixed echinocytes. The chemistry data, on the other hand, shows that the total amount of sialic acid removed from the platelet surface is about the same for the fixed echinocyte and unfixed cocaine sphere. In other words, the

percentage of sialic acid groups on the platelet surfce situated at the plane of shear whose loss is seen electrophoretically, has increased from 2.9% to 5.1%. Similarly the percentage for the phosphate groups went up from 3.9% to 6.0% (Table 11). Since the total enzyme susceptible sialic acid and phosphate groups on the surfaces of the cocaine sphere and the echinocyte are similar it is reasonable to believe the total number of surface molecules bearing these groups is similar in the two forms. The change in surface architecture associated with cocaine binding apparently allows a larger fraction of the sialic acid and phosphate groups to contribute to the mobility, hence the increase in surface charge.

Cocaine has the molecular structure:



Its mechanism as a local anesthetic is still unclear, however it is commonly believed to act on the membrane of nerve cells interfering with their function (Papahadjopoulos, 1972; Mather and Cousins, 1979). The lipophilic benzene ring buries itself into the membrane lipid layer and the hydrophilic end remains in solution. It causes expansion of the membrane and conformation changes (Mather and Cousins, 1979). Although cocaine has a positively charged tertiary amine group, it is doubtful that this will contribute to the electrokinetic properties of the platelet at the plane of shear. It is more

likely to interact with the negative charges of the phospholipid head groups (Papahadjopoulos, 1972). It is thought that this molecule's unique position in the cell membrane may somehow interfere with Na^+/K^+ fluxes and the transmission of nerve signals. Cocaine and related local anesthetics also transform biconcave erythrocytes into cup-shaped forms by disrupting the inside/outside ionic environment of the erythrocytes (Deuticke, 1968). By displacing Ca^{++} from anionic sites on the membrane and in the cytoplasm, cocaine and related anesthetics also cause the breakdown of microfilaments, microtubules and cytoskeleton in cells (Poste et al., 1975; Nicolson et al., 1976) and platelets (Nachmias et al., 1977; Nachmias et al., 1979; Davies and Palek, 1982). No matter which is the mechanism for the sphering of the platelet, the change in surface electrokinetic properties is overwhelming.

3.4.5 Calcium and Magnesium Ion Binding

It was estimated that 2.10×10^5 Ca^{++} binding sites are present on the elctrokinetic surface of each discocyte, assuming that equation [3] and [5] model the situation adequately. ADP stimulation of the discocyte increases this figure twelve-fold to 2.58×10^6 sites. The density of binding sites increases eight-fold from 1.28×10^{12} per cm^2 on the discocyte to 1.09×10^{13} per cm^2 on the echinocyte. The average binding free energy (G) at the same time drops from -4.09 to -2.89 Kcal/mole (Table 12). Using similar procedures Seaman et al. (1969) found the Ca^{++} binding site density to be 4.97×10^{12} per cm^2 for the human erythrocyte and 3.84×10^{12} per cm^2 for the polymorphonuclear leukocyte (Table 15). Their binding free energies were -3.8 and -4.0 Kcal/mole respectively. The density of Ca^{++} binding sites on the discocyte is therefore comparatively low, although the average binding free

energy is similar to those of the erythrocyte and leukocyte. After transformation into the echinocyte the density becomes much higher than on the RBC and leukocyte but the binding is less strong. The estimated distance between adjacent sites decreases from about 9nm to 3nm after echinocyte transformation. Data obtained for Mg^{++} binding were not significantly different from those of Ca^{++} although the number of sites is somewhat lower (Table 13 and 14). After transformation of discocytes into spherocytes by hypotonic shock the density of the Ca^{++} binding sites decreased by over 80% representing apparently a drastic change in platelet membrane properties.

Using the $^{45}Ca^{++}$ binding techniques, Taylor and Heptinstall (1980) found 2.20×10^5, Brass and Shattil (1982) found 4.85×10^5, and Peerschke et al. (1980) found 1.66×10^6 sites per discocyte. Peerschke et al. (1980) found no increase in $^{45}Ca^{++}$ binding after ADP stimulation while Brass and Shattil (1982) found a 28% increase. The range of values shows the susceptibility of the $^{45}Ca^{++}$ experiments to calcium internalization. However the microelectrophoresis method used here also has some drawbacks. It only estimates Ca^{++} and Mg^{++} binding to the electrokinetic surface of the platelets. It is therefore likely to underestimate the total Ca^{++} and Mg^{++} binding to the whole plasma membrane surface. The estimates obtained from microelectrophoresis depend on an idealized model. They ignore, by depending on equation [3], the distributed nature of the surface charge throughout the glycocalyx. Despite these uncertainties, differences in the binding free energy and the number of binding sites on various platelet forms are much less likely to be in error than the individual absolute values. Given their limitations, comparisons of the $^{45}Ca^{++}$ and the electrophoresis results are difficult to interpret.

Table 15. Comparison of Calcium Ion Binding on Platelets, Erythrocytes and Polymorphonuclear Leukocyte

	Density of Binding Sites (cm^{-2})	- G (Kcal/mole)
Discocyte	1.28×10^{12}	4.09
Echinocyte	1.09×10^{13}	2.89
Spherocyte	2.22×10^{11}	4.13
RBC*	4.97×10^{12}	3.82
PMN*	3.84×10^{12}	4.01

*From Seaman et al. (1969)

The necessity for fibrinogen binding to precede platelet aggregation and the requirement of Ca^{++} and Mg^{++} for fibrinogen binding to the surface of the activated platelet has already been discussed in previous chapters and will be discussed further in the next chapter. It has been estimated that between 40,000 (Bennett and Valarie, 1979; Plow and Marguerie, 1980) and 50,000 (Hawiger et al., 1980) fibrinogen molecules bind specifically to surface of each echinocyte. This in effect gives a binding ratio of about 50 to 60 Ca^{++} per fibrinogen molecule. The fibrinogen molecule has been estimated to be about 45nm long and 6.5nm in diameter (Weisel et al., 1981). The maximum area per Ca^{++} binding site on the echinocyte surface is about $9nm^2$ (i.e. the reciprocal of the sites density of $1.09x10^{13}/cm^2$). Therefore on the average only a maximum of 30 Ca^{++} could be associated with each fibrinogen molecule. From this estimatin about half of the Ca^{++} on the echinocyte could be associated with fibrinogen providing fibrinogen molecules maximize thier area of contact with the surface and the Ca^{++} binding sites are uniformly distributed.

The above discussion is based on the assumption that all the Ca^{++} binding sites are saturated. Obviously, under physiological conditions this is not the case. The optimum Ca^{++} concentration for platelet aggregation is 4mM (Chapter 2). One can back calculate using Fig. 23 and equation [5] that only $5.7x10^4$ Ca^{++} ions bind to each echinocyte at this concentration. Only about 2% of the binding sites are therefore occupied. This also gives a relationship of 1 to 1.4 Ca^{++} ion per fibrinogen molecule.

3.4.6 A Hypothesis Regarding Membrane Exposed by ADP Stimulation or Hypotonic Swelling

Using the above data, a table outlining the surface properties of the platelet discocyte, echinocyte and spherocyte as well as their "new membranes" can be constructed (Table 16). "New membrane" is defined as the increased or evaginated surface areas of the echinocyte and spherocyte. Total negative charge is the apparent net negative charge (Table 3) corrected by adding the number of positively charged amino groups (Table 4). The specific properties of the "new membrane" are calculated by assuming that the surface properties of the original "old membrane" of the platelet remain unchanged during shape transformation and that the additional charged groups which appear are distributed uniformly over the exposed area added during formation of the echinocyte or spherocyte.

There seem to be only small differences between the discocyte and echinocyte membrane considering their respective sialic acid/total charge, phosphate/total charge and sialic acid/ phosphate ratios, although absolute quantitative differences are obvious. The spherocyte, and especially the spherocytic "new membrane," on the other hand, are quite different from the others both quantitatively and qualitatively.

It was proposed at the beginning of this work that the newly exposed, evaginated membrane of the echinocyte might be responsible for the sticky nature of the platelet surface. In other words, the non-sticky platelet discocyte has a cloistered sticky surface that can be unfolded to produce a sticky platelet. The data presented in this chapter argue against this interpretation. Firstly, the unfolding of the evaginated canalicular membrane system using hypotonic shock produces a spherocyte with completely

Table 16a. Platelet Surface Properties by Microelectrophoresis

	Discocyte	Spherocyte	Spherocyte New Membrane	Echinocyte	Echinocyte New Membrane
Area (cm^2)	16.4×10^{-8}	36.7×10^{-8}	20.3×10^{-8}	23.7×10^{-8}	7.3×10^{-8}
Total negative charge -					
per platelet	1.50×10^{6}	2.39×10^{6}	0.89×10^{6}	1.88×10^{6}	0.38×10^{6}
per cm^2	9.13×10^{12}	6.50×10^{12}	4.38×10^{12}	7.92×10^{12}	5.21×10^{12}
Amino groups -					
per platelet	2.18×10^{5}	3.97×10^{5}	1.79×10^{5}	2.82×10^{5}	0.64×10^{5}
per cm^2	1.33×10^{12}	1.08×10^{12}	0.88×10^{12}	1.19×10^{12}	0.88×10^{12}
Sialic acid groups -					
per platelet	6.53×10^{5}	7.80×10^{5}	1.27×10^{5}	8.34×10^{5}	1.18×10^{5}
per cm^2	3.98×10^{12}	2.12×10^{12}	0.62×10^{12}	3.52×10^{12}	2.48×10^{12}
Phosphate groups -					
per platelet	3.74×10^{5}	7.19×10^{5}	3.45×10^{5}	4.62×10^{5}	0.88×10^{5}
per cm^2	2.28×10^{12}	1.96×10^{12}	1.70×10^{12}	1.95×10^{12}	1.20×10^{12}
Sialic acid/Total charge	43.5%	32.6%	14.3%	44.4%	47.6%
Phosphate/Total charge	25.0%	30.1%	38.8%	24.6%	23.1%
Sialic acid/Phosphate	1.75	1.08	0.37	1.80	2.07

Table 16b. Platelet Surface Enzyme Released Sialic Acid and Phosphate Group (Chemistry Data)

	Discocyte	Spherocyte	Spherocyte New Membrane	Echinocyte	Echinocyte New Membrane
Sialic acid groups -					
per platelet	2.26×10^{7}	2.65×10^{7}	0.39×10^{7}	2.67×10^{7}	0.41×10^{7}
per cm^2	1.38×10^{14}	0.72×10^{14}	0.19×10^{14}	1.13×10^{14}	0.56×10^{14}
Phosphate groups -					
per platelet	9.44×10^{6}	18.52×10^{6}	9.08×10^{6}	12.48×10^{6}	3.04×10^{6}
per cm^2	5.75×10^{13}	5.04×10^{13}	4.47×10^{13}	5.28×10^{13}	4.17×10^{13}
Sialic acid/Phosphate	2.39	1.43	0.43	2.14	1.34

Table 16c. Platelet Surface Ca^{++} and Mg^{++} Binding Sites

	Discocyte	Spherocyte	Echinocyte	Echinocyte New Membrane
Ca^{++}				
per platelet	2.10×10^{5}	8.16×10^{4}	2.58×10^{6}	2.37×10^{6}
per cm^2	1.28×10^{12}	2.22×10^{11}	1.09×10^{13}	3.25×10^{13}
Mg^{++}				
per platelet	1.77×10^{5}	-	1.56×10^{6}	1.38×10^{6}
per cm^2	1.08×10^{12}	-	6.60×10^{12}	1.89×10^{13}

different surface properties. Secondly, it does not seem possible that the original discocyte surface ("old membrane") remains unchanged during echinocyte transformation. This becomes most evident if the platelet surface Ca^{++} binding data is considered (Table 16c). Under the sticky invaginated membrane model almost all of the Ca^{++} binding sites on the echinocyte surface must originate from the "new membrane." Yet during spherocyte transformation the total number of sites actually decreases. Not only do no contributions appear from the "new membrane", but in this case Ca^{++} binding sites are disappearing.

This contradiction implies that the spherocyte membrane cannot consist of unchanged echinocyte membrane plus "new membrane." Rather, the average properties of the whole surface are likely to have changed during sphering.

Finally, it was mentioned earlier that about 40,000 fibrinogen molecules bind specifically onto the surface of an activated platelet. Considering the fibrinogen molecule as a cylindrical rod of 45nm long and 6.5nm in diameter (Weisel et al., 1981), 40,000 molecules will cover about $11.7 \times 10^{-8} cm^2$ if the molecules lie flat, about half of the total echinocyte surface area. If they are standing up on ends, the 40,000 fibrinogen molecules could occupy an area as small as $1.3 \times 10^{-8} cm^2$. Since the surface area of the echinocyte "new membrane" is about $7.3 \times 10^{-8} cm^2$, it is possible, given a favourable orientation for the fibrinogen molecules, that only "new" membrane could be involved in the binding. However, the Ca^{++} binding site density in this region would be extremely high. It remains to be seen whether independent evidence, perhaps ultrastructural, in favour of this model will appear.

In conclusion, although it appears that there are significant differences between the discocyte membrane and the "new membrane" of the echinocytes and spherocytes, it is not clear that the "new membrane" is responsible for the stickiness of the echinocyte surface. The changes in Ca^{++}, Mg^{++} and

fibrinogen binding sites could just as well involve the whole platelet and not just the "new membrane."

CHAPTER 4

RADIOCHEMICAL LABELLING OF PLATELET SURFACE STRUCTURES

4.1 INTRODUCTION

Polyacrylamide gel electrophoresis (PAGE) of radiolabelled platelet membranes dissolved in sodium dodecyl sulphate (SDS) has been an important tool in the understanding of the surface chemistry of platelets. The gel electrophoresis patterns and their interpretations have been discussed and reviewed extensively elsewhere (Jenkins et al., 1979; Phillips, 1979; McGregor et al., 1979; Nurden et al., 1981; Connellan et al., 1982; Bowles and Brunton, 1982; Toor et al., 1982). Three major platelet membrane glycoproteins were originally found by radiolabelling techniques and SDS-PAGE (Phillips, 1979; Phillips, 1980; Okumura and Jamieson, 1976; George et al., 1980). They are glycoprotein I (GP-I) with an apparent molecular weight of between 130,000 and 160,000; glycoprotein II (GP-II) with a molecular weight of between 110,000 and 130,000 and glycoprotein III (GP-III) with a molecular weight of between 90,000 and 110,000. Subsequent work revealed another glycoprotein with a molecular weight of between 150,000 and 170,000. This glycoprotein ran very near the GP-I peak and was resolved only through improved SDS-PAGE techniques. This last glycoprotein was named GP-Ia and the original GP-I was then called GP-Ib. Careful SDS-PAGE experiments demonstrated more minor glycoprotein peaks below the GP-III peak: GP-IV (sometimes referred to as GP-IIIb) was found with a molecular weight of between 85,000 and 100,000; GP-V has a molecular weight of between 68,000 and 89,000. If one uses the nomenclature of GP-IIIb for GP-IV, then GP-V becomes GP-IV. A variety of nomenclature systems have been developed in different laboratories. Wide ranges of molecular weight have also been reported in the literatures for the glycoproteins species, resulting in some difficulty and confusion in the interpretation of data. The nomenclature system using GP-Ia, GP-Ib, GP-II, GP-III,

GP-IV, GP-V and GP-VI generally referred to as the Phillips system (Phillips, 1979) will be used throughout this work.

SDS-PAGE has proved useful for the separation of proteins according to their molecular weights (Reynold and Tanford, 1970; Lasky, 1978). The usefulness of SDS-PAGE for molecular weight determination depends on the ability of the anionic detergent SDS to interact with and denature a wide variety of proteins in a similar manner. Native proteins having a wide difference in charge, size and shape characteristics are converted, upon disulfide bond reduction and SDS-binding, to SDS-protein complexes of their constituent polypeptide chains. The complex is a rod-like structure, the length of which varies with the molecular weight of the protein. One interesting aspect of this complex is that different proteins bind identical amounts of SDS on a gram per gram basis. The charge per unit mass of protein is therefore approximately constant. In other words, the charge densities of different SDS-protein complexes are about equal and the electrokinetic properties of the complex will be mainly a function of the protein's molecular weight. The PAGE gel is formed by the cross-linking of acrylamide and bis-acrylamide to give a three-dimensional polymer meshwork. The degree of cross-linking depends on the gel (acrylamide) concentration. This three-dimensional meshwork forms a molecular sieve. During electrophoresis, the smaller proteins will encounter less resistance from the gel matrix than the larger ones and hence migrate farther along the gel. When the distances migrated by different proteins are plotted vs. the logarithm of their molecular weights, it is generally found that the proteins fall on a straight line. Ideally, the molecular weight of an unknown protein can be read off from such a calibration plot, providing identical experimental conditions are used. However, errors in determining

molecular weights can occur (Lasky, 1978). First, the semi-logarithmic calibration plot may not be linear, especially towards the higher molecular weight region of the plot (top of the gel). Second, the action of SDS may not be perfect (uniform) towards all proteins and errors of as much as 15% in the determination of molecular weight can occur (Lasky, 1978). Finally, little is known about how the carbohydrate moieties of glycoproteins may affect the molecular weight determination.

Three methods are commonly used for the radiolabelling of membrane glycoproteins (Phillips, 1979 and McGregor et al., 1979). The first method is lactoperoxidase-catalyzed iodination which covalently attaches the radioactive iodine to tyrosine residues of the glycoproteins. This method gives a strong GP-III and a moderate GP-II peak on SDS-PAGE. The second method requires the removal of terminal sialic acid residues from the surface glycoproteins with neuraminidase. Galactose oxidase is then used to oxidize the hydroxyl groups at the C-6 position of the exposed galactose residues into aldehyde groups. Next, ^{3}H-borohydride is used to reduce the aldehyde groups, resulting in the incorporation of ^{3}H into the galactose molecules. The third method uses periodate to cleave oxidatively the carbon-carbon bonds between C7-C8 and C8-C9 positions of the terminal sialic acid residues. The aldehyde groups so formed are then reduced with ^{3}H-borohydride resulting in ^{3}H being incorporated into the sialic acid molecules. The second and the third labelling methods give similar patterns on SDS-PAGE. GP-Ib has the strongest peak while peaks of GP-Ia, GP-II and GP-III are moderately strong. All three methods will be used here to detect possible changes in platelet surface glycoproteins after transformation to echinocytes and spherocytes.

Bunting et al. (1978) using the periodate-tritiation method found no differences between the gel patterns of normal and ADP activated platelets. George et al. (1980) using the diazotized ^{125}I-diiodosulfanilic acid labelling technique found no difference. This last compound reacts with and labels amino groups on the platelet surface. Preliminary experiments by us using ^{125}I-lactoperoxidase iodination also failed to demonstrate any difference between discocyte and echinocyte SDS-PAGE patterns. Therefore double label experiments were undertaken to try to improve the resolution. Sorg and Greczy (1976) have used double radiolabelling in conjunction with SDS-PAGE in lymphocyte studies. They have shown that this can be a valuable technique in detecting small differences between test and control experiments.

Double labelling with ^{125}I and ^{131}I was used with lactoperoxidase-catalyzed iodination. This was done in two ways. Firstly, discocytes were labelled with ^{125}I and echinocytes or spherocytes were labelled with ^{131}I. They were then mixed, dissolved in SDS and run on the same gel. This method helps to eliminate gel to gel differences that may have masked small differences in the patterns. Secondly, ^{125}I-labelled discocytes were transformed into echinocytes or spherocytes. Then these ^{125}I-labelled echinocytes and spherocytes were relabelled with ^{131}I. Any ^{131}I peak in the gel represents new glycoprotein species exposed after ^{125}I labelling. Controls were done by double labelling the discocytes without the transformation step. A similar double radio-iodination technique was used by Phillips and Agin (1974) to find the platelet surface thrombin proteolytic site.

For the tritiation experiments, double labelling is achieved by first "labelling" the discocytes with non-radioactive borohydride using the periodate or neuraminidase-galactose oxidase method. Then after transformation into

echinocytes or spherocytes, the platelets are relabelled with ^{3}H-borohydride using similar methods. Any ^{3}H peak in the gel greater than that in the control represents glycoproteins exposed during the transformations. These are termed pseudo-double label experiments.

Lactoperoxidase iodination was also used to study the surface component involved in the binding of fibrinogen onto the echinocyte surface. The requirement for fibrinogen in ADP induced aggregation has been discussed earlier. Activation of platelets results in the exposure or appearance of receptors for fibrinogen. Fibrinogen molecules then bind onto the surface of the activated platelets. Calcium or magnesium ions are required for the binding (Chapter 2). The number of specific receptor sites per activated platelet ranges from about 40,000 (Bennett and Vilaire, 1979; Plow and Marguerie, 1980) to 50,000 (Hawiger et al., 1980). Peerschke et al. (1980) found 4000 high affinity and 9000 low affinity sites.

Recent studies suggest that the fibrinogen receptor is associated with the GPII-GPIII complex. Four lines of evidence point to this fact. Firstly, patients with Glanzmann's thrombasthenia have platelets that fail to bind fibrinogen when activated. Glanzmann's thrombasthenia platelets lack the GPII-GPIII complex. Platelets from patients with Bernard-Soulier syndrome bind fibrinogen normally when activated. They have the GPII-GPIII complex but lack GPIb (Mustard et al., 1979; Bennett and Vilaire, 1979; Peerschke et al., 1980; Lee et al., 1981). Secondly, antibodies to the GPII-GPIII complex block fibrinogen binding (Lee et al., 1981; Coller, 1981; DiMinno et al., 1981). Thirdly, isolated GPII, GPIII and GPII-GPIII complex when adsorbed to plastic microtitre plates bind to fibrinogen (Nachman and Leung, 1982). The glycoproteins were isolated using lentil lectin affinity chromatography and were

found to react with mono-specific anti-GPIIa and IIIb antibodies. Fourthly, by using fibrinogen coupled to a photoreactive agent Peerschke et al., (1981) and Bennett et al. (1981) were able to demonstrate the association of fibrinogen with GPII and GPIII respectively. This agent (methyl-4-azidobenzoimidate) reacts with organic molecules covalently on exposure to light. It can therefore link fibrinogen with its receptor, or to structures near the receptor, when the experiment is done in darkness and then exposed to light. When fibrinogen is bound, a fibrinogen-protein supercomplex is formed and identified as a high molecular weight band on SDS-PAGE. The molecular weight of the putative receptor is the difference between the molecular weight of the supercomplex and that of fibrinogen. This method does not demonstrate a ligand-receptor relationship but only a close proximity between the fibrinogen molecule and GPII/III molecule.

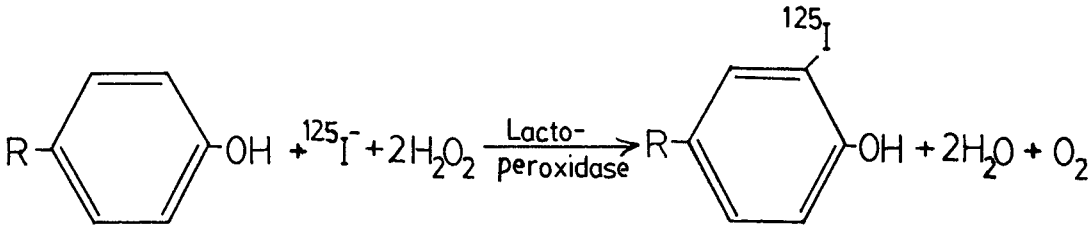
Morrison and Bayse (1970) have shown that the lactoperoxidase enzyme molecule must form a complex with tyrosine in a stereospecific manner before iodination can occur. It will be shown here that the presence of fibrinogen interferes with the iodination of GPIII in the activated platelet, demonstrating a close relationship between the adsorbed fibrinogen and GPIII.

4.2 MATERIALS AND METHODS

4.2.1 Surface Labelling of Platelets

4.2.1.1 Lactoperoxidase Iodination

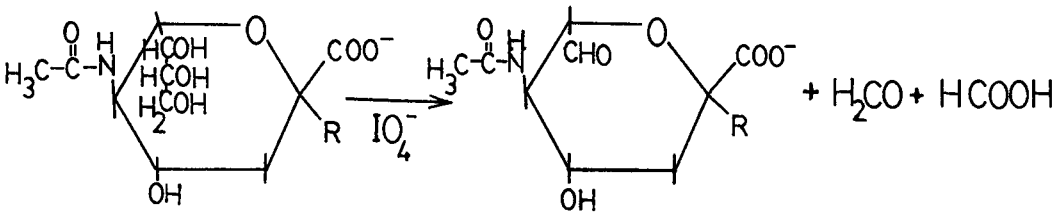
Lactoperoxidase iodination of platelets was carried out according to the methods of Phillips (1972).

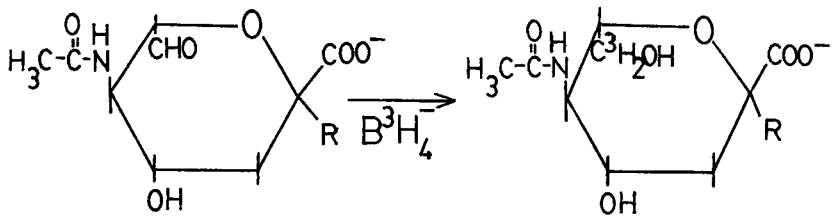


Platelets were isolated and adjusted to 2.5×10^8 per ml with Tyrode's solution as in Chapter 2. They were mixed with 0.1 mCi/ml of carrier-free $Na^{125}I$ or $Na^{131}I$ and 0.1 mg/ml of lactoperoxidase. Hydrogen peroxide was then added at 0.5 mM final concentration. Reaction was almost instantaneous at room temperature and after 2 min the platelets were washed free of excess radiolabel. Spherocytes were labelled in hypotonic Tyrode's solution under the same conditions and reagent concentrations. Whole platelets were dissolved in 1% SDS and reduced with 40 mM dithiothreitol (DTT) according to Fairbanks (1971). Double labelling was as described above in the Introduction. In situations where ^{125}I labelled discocytes had to be transformed and relabelled with ^{131}I, the platelets were first washed free of excess ^{125}I, transformed and then reincubated with fresh lactoperoxidase, H_2O_2 and $Na^{131}I$. After labelling, platelets were counted in a LKB 1282 Dual-channel Compugamma counter before membrane isolation.

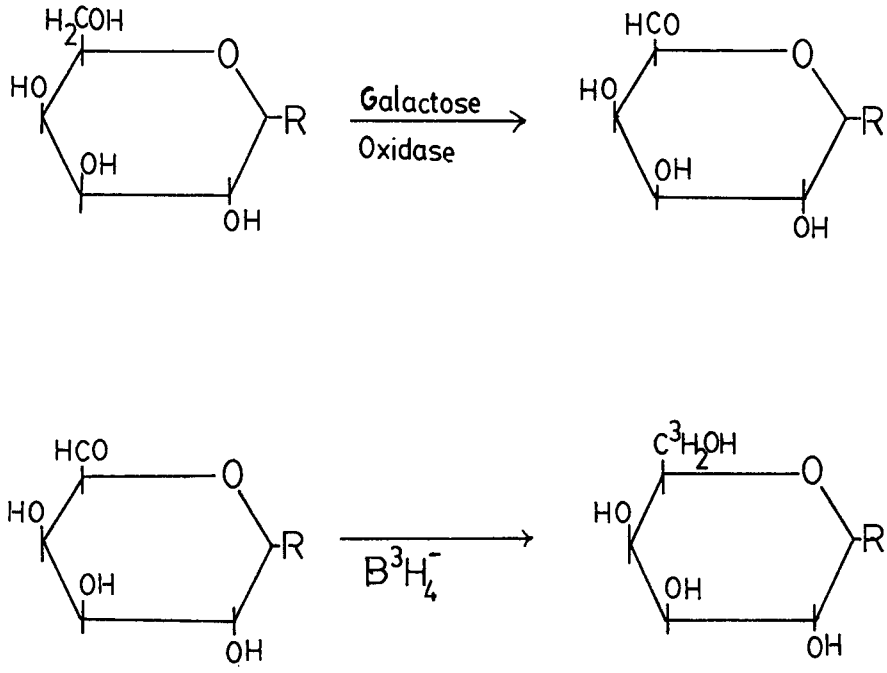
4.2.1.2 Borohydride Tritiation

Platelets ($2.5x10^8$/ml) in Tyrode's solution were first incubated with 1 mM sodium metaperiodate for 10 min at room temperature to selectively oxidize sialic acid residues. Then, 1 mCi/ml (4.4 mM) sodium ^{3}H-borohydride was used to reduce and label the sialic acid at room temperature for 30 min (McGregor et al., 1979).





Other platelets ($2.5x10^8$/ml) were treated with neuraminidase as outlined in Chapter 3 to release sialic acid and then incubated with galactose oxidase (20 U/ml) at 37°C for 30 min at pH 7.5 to oxidize the exposed galactose residues. Reduction using ^{3}H-borohydride was as above (McGregor et al., 1979).



The pseudo-double label experiments were done as outlined in the Introduction using cold (5 mM) and then hot borohydride. In these cases "cold labelled" platelets were washed and then transformed with ADP or hypotonic shock. They were treated with metaperiodate or the enzymes under the same conditions as used for the discocyte before relabelling with ^{3}H-borohydride. Spherocytes were handled and labelled in hypotonic Tyrode's solution but otherwise under the same conditions and reagent concentrations as above. After labelling, a portion of each of the tritiated platelet samples was dissolved in Amersham NCS-Tissue Solubilizer and diluted in an OSC-Organic Counting Scintillant (Amersham) fluid. The samples were then counted with a Beckman LS-233 scintillation counter. Changes in counting efficiency were monitored using the

external standard ratio output of the counter. Other portions of the platelet samples were used for membrane isolation.

4.2.2 Isolation of Membrane

Isolation of membranes from labelled platelets was according to the method of Jamieson et al. (1979). Platelets ($2.5x10^8$/ml) in Tyrode's solution were disrupted using a Braunsonic 1510 sonicator (A. Braun, South San Francisco, California) at 100W for about 30 seconds. Membranes were then isolated using differential centrifugation. The homogenates were initially centrifuged at 20,000xg for 20 min at 4^oC. The pellets containing platelet debris and undisrupted organelles were discarded. The remaining supernatant was again centrifuged at 150,000xg for 1 hr at 4^oC. The pellets containing membranes were washed in Tyrode's solution and dissolved in 20 mM Tris-HCl pH 8 buffer containing 2% SDS and reduced with 40 mM dithiothreitol (DTT) according to Fairbanks (1971). Both centrifugation steps were done with a Beckman L5-65 Ultracentrifuge and a SW41 Swinging Bucket Rotor.

4.2.3 Gel Electrophoresis

SDS-PAGE was run on 5% cylindrical gels cast according to the method of Fairbanks (1971). The gel solution consists of 5% acrylamide, 0.18% bis-acrylamide, 0.2% SDS, 0.025% tetramethylethylenediamine (TEMED) and 0.15% ammonium persulfate in a buffer system containing 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA at pH 7.4. The gel solution was cast into 7x12.5 mm glass tubes and allowed to harden at room temperature for an hour. About 20 μg of dissolved protein material together with a small amount of bromophenol blue as tracking dye were loaded into each tube. The electrophoresis buffer (pH 7.4) consists of 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA and 2 gm/L of

SDS. A Hoefer Scientific Instrument (San Francisco, California) gel electrophoresis chamber model DE102 was used. Electrophoresis was run at 5 mA/tube in a 4°C refrigerator until the tracking dye reached bottom. The gels were removed from the tubes and fixed with 3.5% perchloric acid.

BDH protein molecular weight standards were run alongside the membrane samples. They have molecular weights of 53,000; 106,000; 159,000; 212,000 and 265,000. The molecular weight standard gels were stained with coomassie blue according to Fairbanks (1971).

Radioactive gels were cut into 1 mm slices using a BioRad gel slicer model 195 (BioRad Laboratories, Richmond, California). Iodinated gel slices were counted with the LKB Dual-channel Compugamma counter. Tritiated gel slices were incubated with 0.6 ml NCS-Tissue Solubilizer at 50°C for 2 hr. Radioactivity was leached out of the gel into solution which was then diluted with 6 ml of OSC-Organic Counting Scintillant fluid for counting in a Beckman LS-233 scintillation counter. These procedures were recommended by the manufacturer Amersham Corp. (Arlington Heights, Illinois). Leaching was complete after 2 hrs, further incubation of the gel with fresh NCS overnight failed to remove any more radioactivity from the gel.

4.2.4 Surface Labelling in the Presence of Fibrinogen

In this series of experiments fibrinogen at 0.5 mg/ml was included during iodination of the platelets. Fibronectin and albumin at the same concentration were added to other samples as controls. Calcium ions at 1 mM were added together with the proteins. Labelling with ^{125}I-lactoperoxidase followed. Membrane isolation and SDS-PAGE were then carried out in the same way as before.

4.2.5 Materials

Fibrinogen used was the same as mentioned in Chapter 2. Fibronectin was prepared and kindly supplied by Mr. Johan Janzen using the method of Vuento and Vaheri (1978). Fibronectin from fresh human citrated plasma was first bound to a gelatin-Sepharose 4B affinity column and then eluted with 1.0M L-arginine. Bovine serum albumin was from Miles Laboratories (Elkhart, Indiana).

Enzymes: neuraminidase (Vibrio cholerae) was from Calbiochem (La Jolla, California), lactoperoxidase from Sigma (St. Louis, Missouri) and galactose oxidase (Dactylium dendroides) was from Worthington (Freehold, N.J.).

Carrier-free sodium iodide-^{125}I and ^{131}I as well as tritiated sodium borohydride were obtained from Amersham (Arlington Heights, Illinois). NCS-Tissue Solubilizer and OCS-Organic Counting Scintillant were also from Amersham.

SDS-PAGE reagents including SDS, acrylamide, bis-acrylamide, TEMED and DTT were all from BioRad (Richmond, California). The molecular weight standard was from BDH (Poole, England).

4.3 Results

4.3.1 Iodination Experiments

Gel electrophoresis of lactoperoxidase iodinated whole platelet discocytes and echinocytes revealed one major glycoprotein peak (GP-III) with an apparent molecular weight of 100,000 (Fig. 24). A smaller peak with molecular weight of 120,000 corresponding to GP-II was also present. The gel pattern of the spherocyte showed two major peaks, the GP-III peak and an additional peak with a molecular weight similar to that of GP-II (Fig. 24). When the plasma membrane from the iodinated platelets was isolated and electrophoresed, the patterns from the discocyte, echinocyte and spherocyte were all similar (Fig. 25). The large 120,000 molecular weight peak in the spherocyte pattern had apparently disappeared although the smaller underlying peak similar to that in the discocyte and echinocyte patterns remained. Gel patterns in Fig. 24 and 25 were typical of seven experiments. Washing of the spherocytes after iodination had no effect on the 120,000 molecular weight protein which was apparently removed during plasma membrane isolation. It appears that two proteins with similar molecular weights are involved: GP-II with a smaller peak size, and a second protein with a larger peak size appearing only on the intact spherocyte. The most likely explanation for the observation is that this second protein is not native to the platelet plasma membrane but an artifact originating from inside the spherocyte. It apparently leaks out and attaches itself to the surface of the spherocyte during hypotonic shock. The attachment is strong enough to withstand the washing procedure but the rigors of membrane isolation can dislodge this protein from the spherocyte membrane. This observation will be discussed further in section 4.4.1. In light of this phenomenon, all SDS-PAGE experiments throughout the rest of this work were done using plasma membranes isolated from radiolabelled platelets.

Fig. 24. SDS-PAGE of ^{125}I-labelled platelets. Whole platelet (a) discocytes; (b) echinocytes and (c) spherocytes were dissolved in 1% SDS and reduced in 40 mM DTT. They were then run on 5% gels.

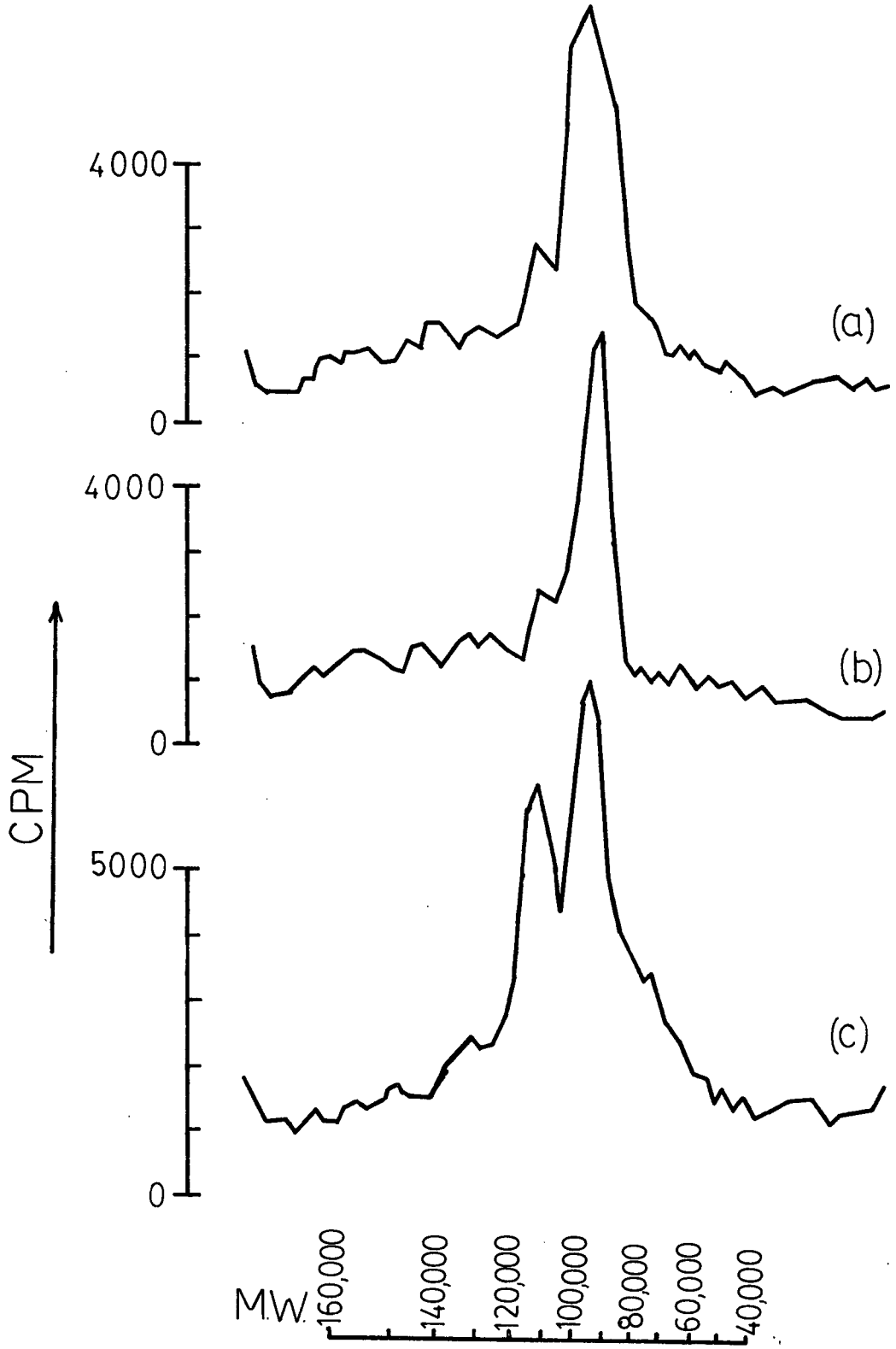
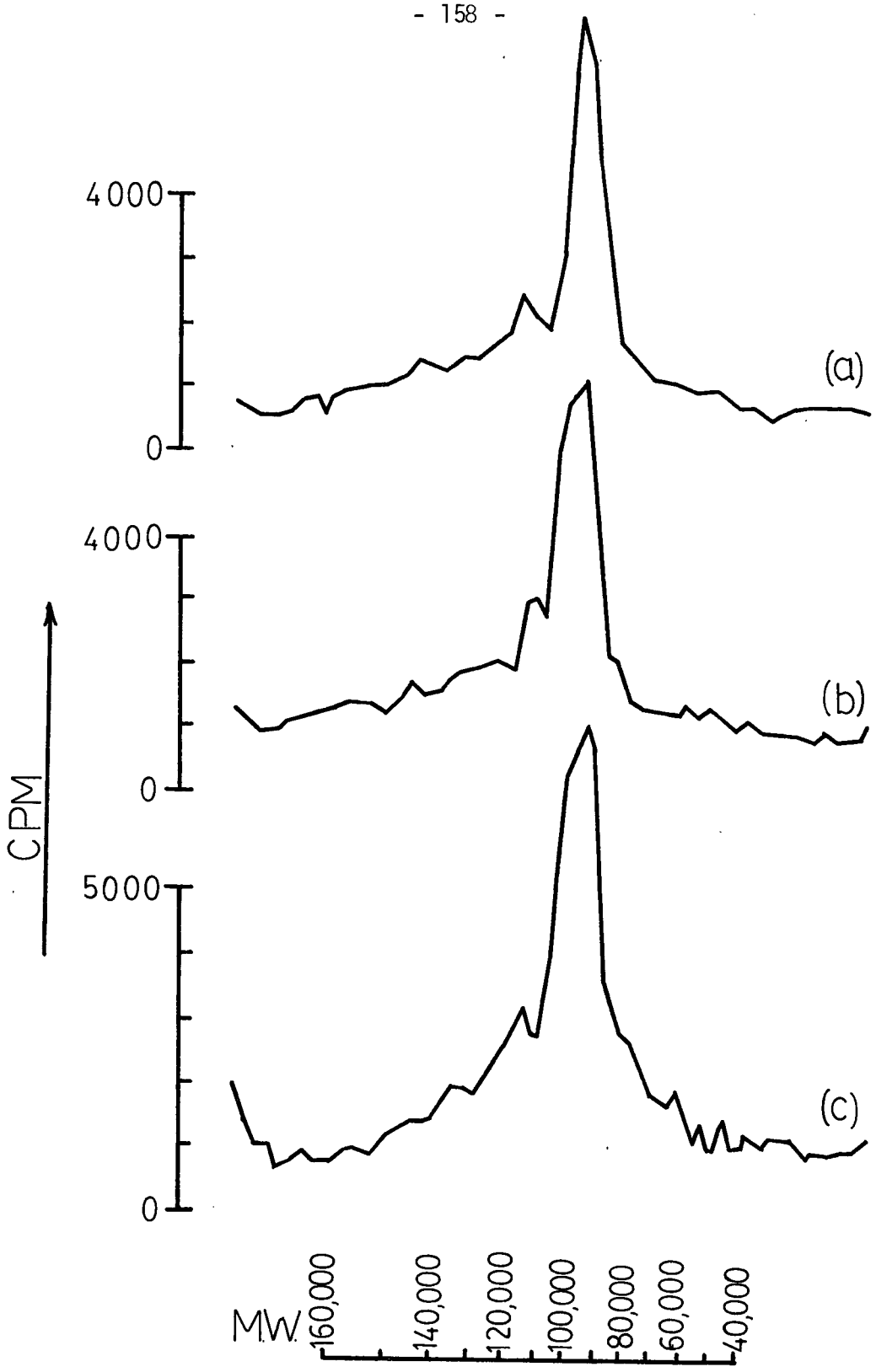

4000
0
(a)
4000
0
(b)
CPM
5000
0
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 25. SDS-PAGE of plasma membrane isolated from ^{125}I-labelled platelet (a) discocyte; (b) echinocyte and (c) spherocyte. Isolated membranes were dissolved and reduced as in Fig. 24. They were then run on 5% gels.


4000
0
(a)
4000
0
(b)
CPM
5000
0
(c)
MW
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Gel patterns of isolated platelet membranes from the double iodination experiments are shown in Figs. 26-31. Fig. 27 and 28 demonstrate the first kind of double labelling experiment in which discocytes were labelled with ^{125}I. Echinocytes or spherocytes derived from unlabelled discocytes were separately labelled with ^{131}I and then run on the same gels as the ^{125}I-discocytes. Fig. 26 is a control in which ^{125}I labelled discocytes were mixed with ^{131}I labelled discocytes and run on the same gel. Figs. 29-31 show the second kind of double labelling experiment in which ^{125}I labelled discocytes were transformed into echinocytes or spherocytes and then relabelled with ^{131}I. Fig. 29 is the control in which ^{125}I labelled discocytes were relabelled with ^{131}I without being transformed. The ratio, R, of the ^{125}I to ^{131}I counts is shown under each pair of the gel patterns in Fig. 26-31. They will be referred to as R-plots.

No major differences can be observed between the patterns in any of these figures. They all show one major (GP-III) peak at molecular weight of about 100,000 and a smaller peak (GP-II) with molecular weight of about 120,000. Although some small variations are shown in the R-plots, they are not significant enough to demonstrate unequivocal differences between the ^{125}I-^{131}I patterns. The brackets (S-S) in all the R-plots in Figs. 26-31 represent ± 2 standard deviations of their respective means. In theory, if the ^{125}I and ^{131}I patterns are identical the R-plot will be a straight line. If there is a significant difference between the patterns it will show up as a large peak or valley in the R-plot (see under the fibrinogen section below). The above patterns are typical of three series of similar experiments.

Quantitation of the counts revealed that during the activation of platelets from discocyte to echinocyte there was a 21.7% ($\pm 1.5\%$) increase in the iodine

Fig. 26. SDS-PAGE of membranes (reduced) from a mixture of ^{125}I-labelled discocytes and ^{131}I-labelled discocytes run on the same 5% gel. (a) ^{125}I-pattern; (b) ^{131}I-pattern and (c) ratio, R, of ^{125}I to ^{131}I activity in each slice (R-plot). Dotted line marks the mean of the R values and the bracket (s-s) represents ± 2 standard deviation.

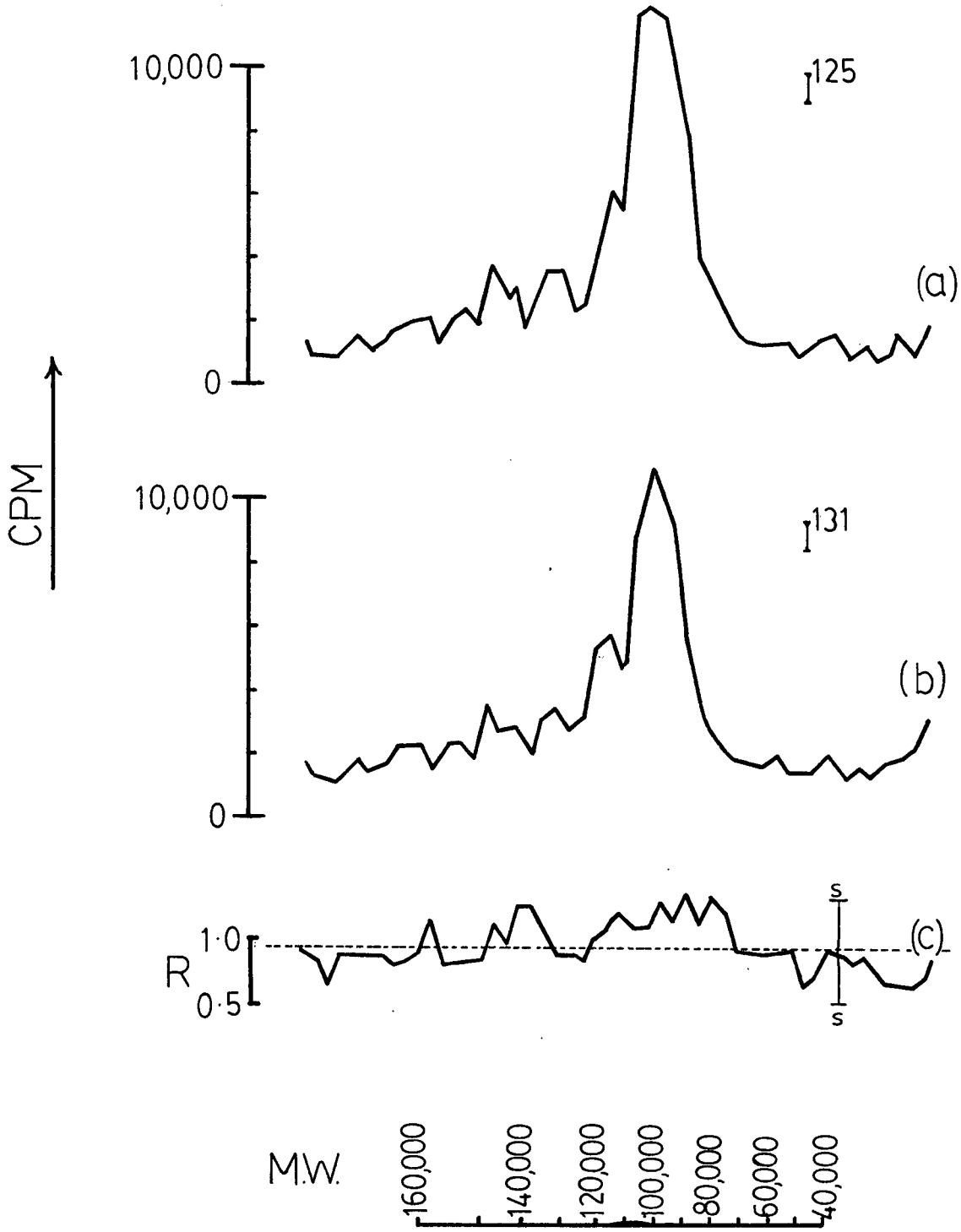

10,000
0
I^{125}
(a)
CPM
10,000
0
I^{131}
(b)
R
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 27. SDS-PAGE of membranes (reduced) from a mixture of ^{125}I-labelled discocytes and ^{131}I-labelled echinocytes run on the same 5% gel. (2) ^{125}I-pattern; (b) ^{131}I-pattern and (c) R-plot, as defined for Fig. 26.

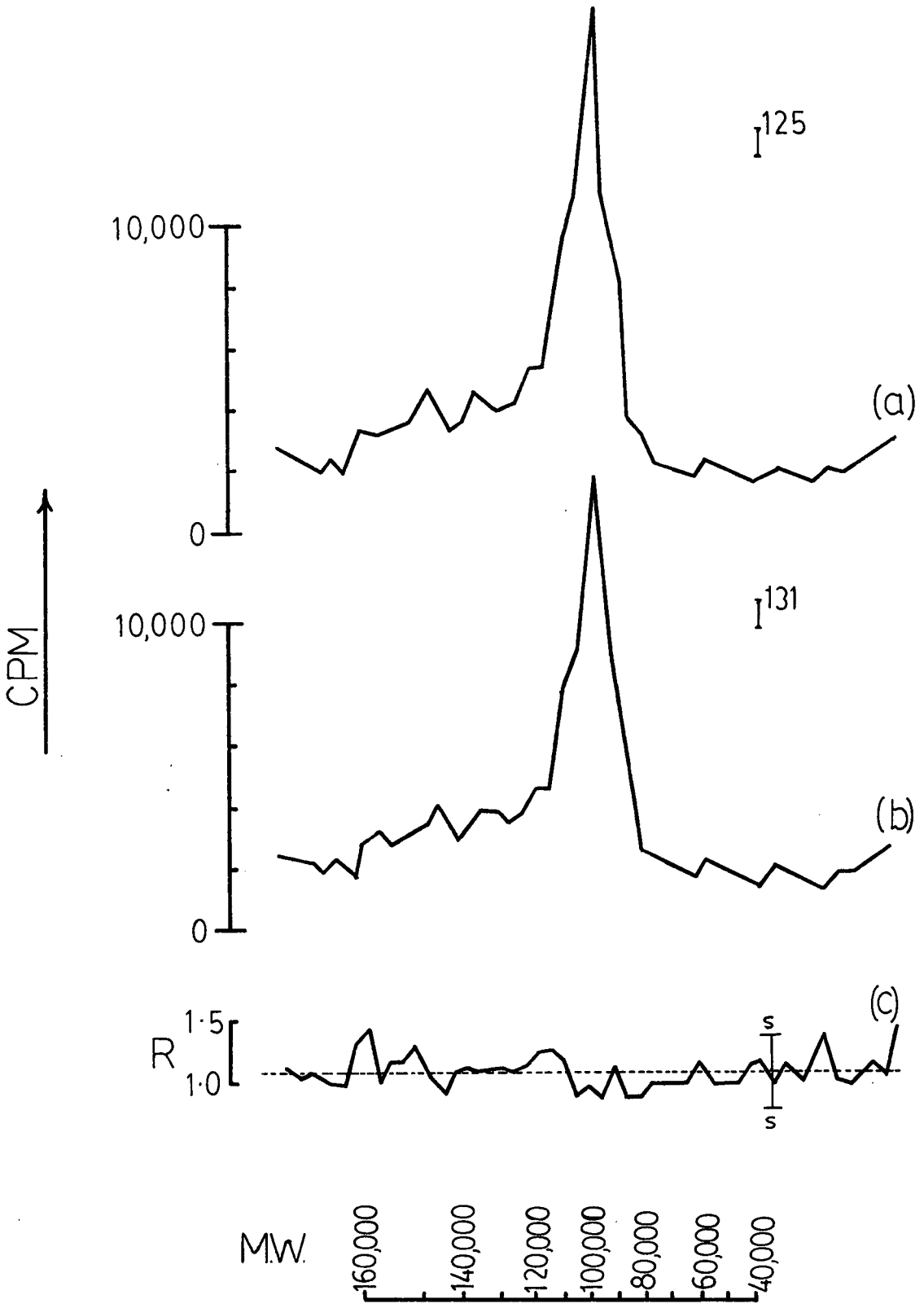

I125
10,000
0
(a)
CPM
I131
10,000
0
(b)
(c)
R
1·5
1·0
s
s
MW.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 28. SDS-PAGE of membranes (reduced) from a mixture of ^{125}I-labelled discocytes and ^{131}I-labelled spherocytes run on the same 5% gel. (a) ^{125}I-pattern; (b) ^{131}I-pattern and (c) R-plot, as defined for Fig. 26.

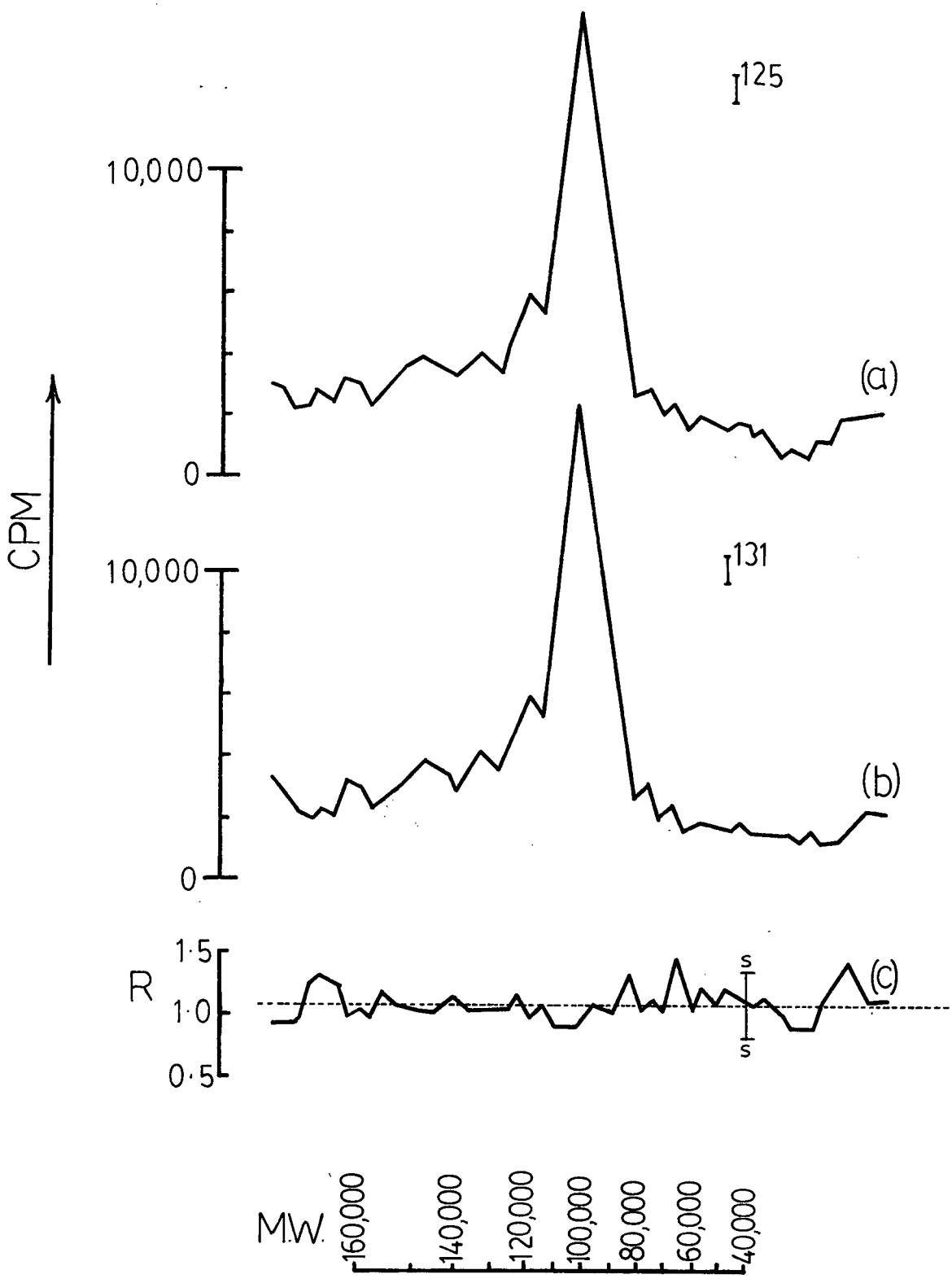

I125
10,000
0
(a)
CPM
I131
10,000
0
(b)
R
1·5
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 29. SDS-PAGE of membranes (reduced) from ^{125}I-labelled discocytes which were washed and then relabelled with ^{131}I. The membranes were run on 5% gel. (a) ^{125}I-pattern; (b) ^{131}I-pattern and (c) R-plot, as defined for Fig. 26.

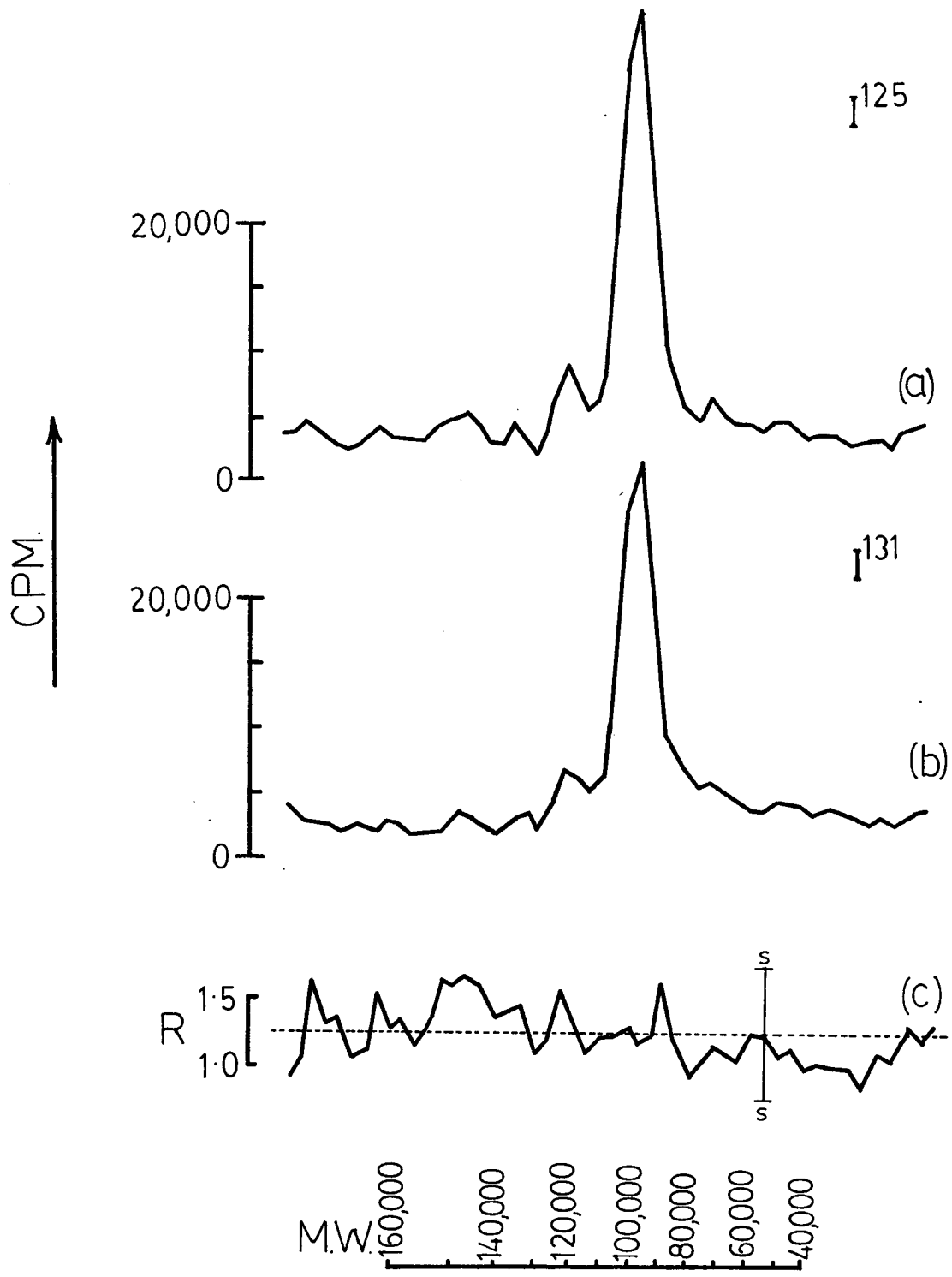

I125
20,000
0
(a)
CPM.
I131
20,000
0
(b)
R
1·5
1·0
S
S
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 30. SDS-PAGE of membranes (reduced) from ^{125}I-labelled discocytes which then transformed into echinocytes and re-labelled with ^{131}I. The membranes were run on 5% gel. (a) ^{125}I-pattern; (b) ^{131}I-pattern and (c) R-plot, as defined for Fig. 26.

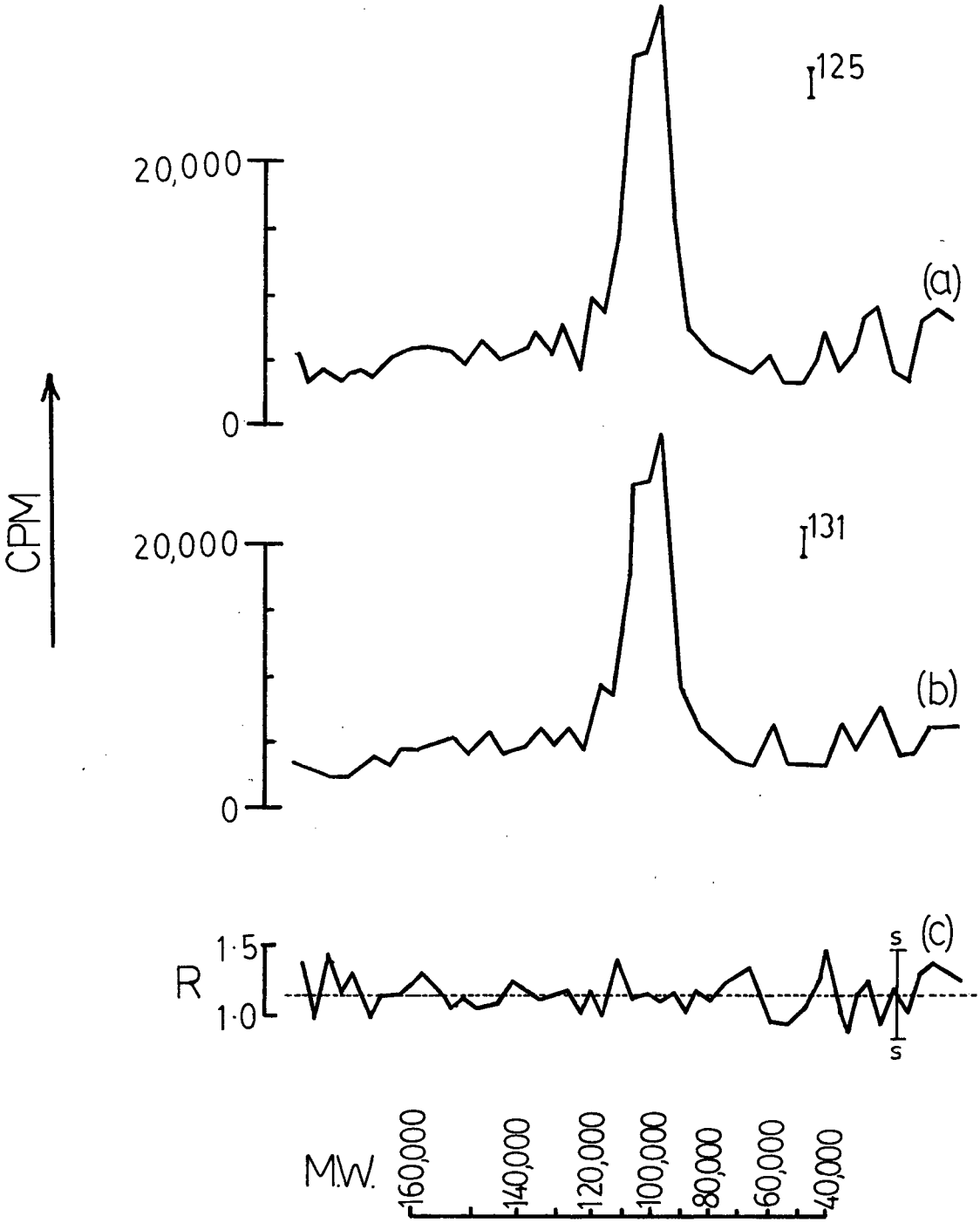
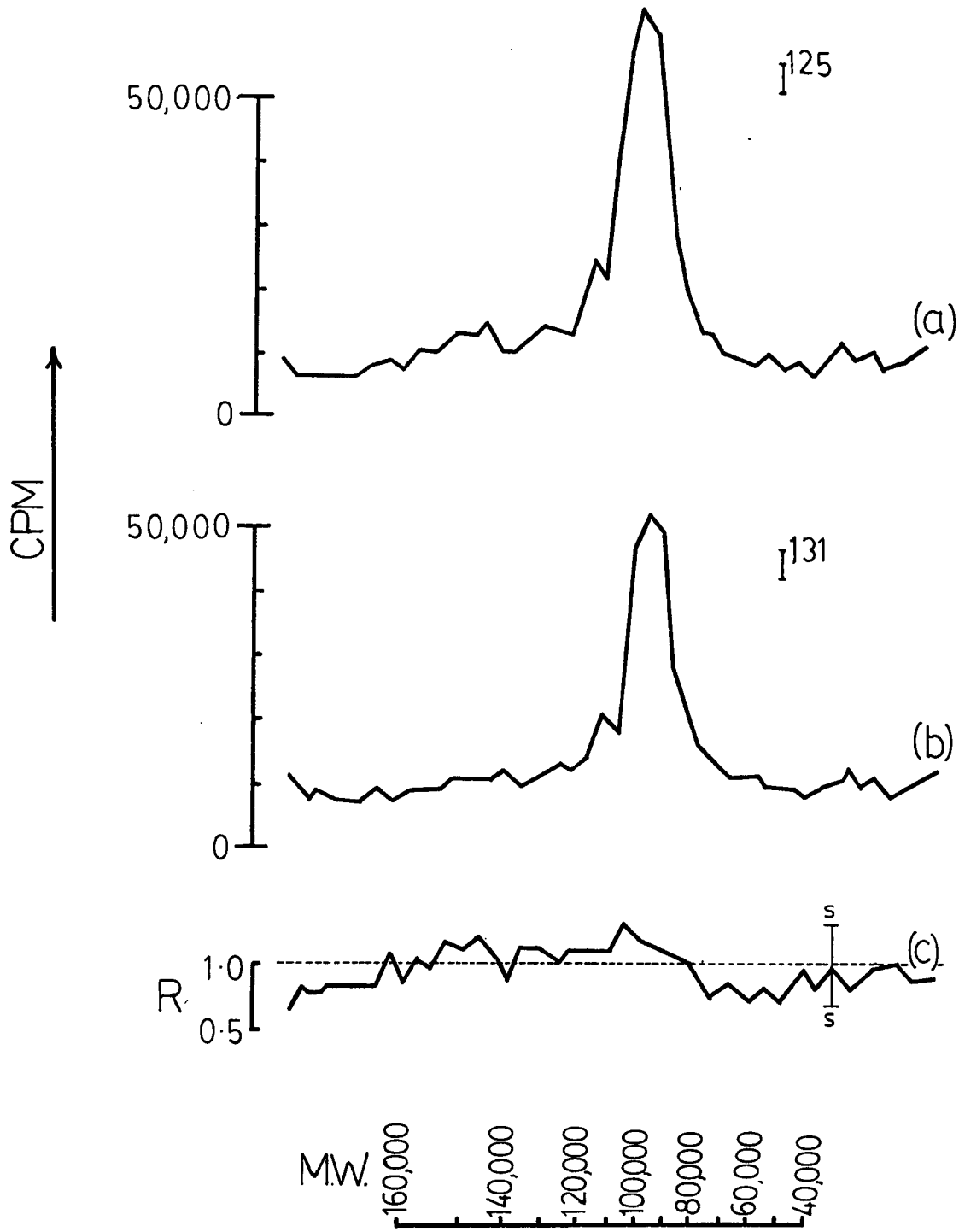

I125
20,000
0
(a)
CPM
I131
20,000
0
(b)
R
1·5
1·0
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 31. SDS-PAGE of membranes (reduced) from ^{125}I-labelled discocytes which then transformed into spherocytes and re-labelled with ^{131}I. The membranes were run on 5% gel. (a) ^{125}I-pattern; (b) ^{131}I-pattern and (c) R-plot, as defined for Fig. 26.


50,000
0
I125
(a)
CPM
50,000
0
I131
(b)
R
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

incorporation. Transformation from discocytes to spherocytes resulted in a 39.8% (±1.3%) increase. These were whole platelet ^{125}I counts from parallel single label experiments. The percentages were means of four series of such experiments.

Morphological examinations of the platelets under phase microscopy were done before and after labelling to ensure they were in their proper discoid, echinoid or spheroid shapes as desired. There were no problems in this respect. No aggregation was encountered during or after labelling.

4.3.2 Tritiation Experiments

Fig. 32 shows the gel patterns of platelet discocytes, echinocytes and spherocytes labelled with the periodate method. Fig. 33 shows them labelled using the neuraminidase-galactose oxidase method. More peaks are revealed in these patterns. The four major ones are GP-Ia; GP-Ib; GP-II and GP-III with apparent molecular weights 150,000, 140,000, 120,000 and 100,000 respectively. A few minor peaks can also be seen. They are GP-IV (which appears as a shoulder on the right side of GP-III); GP-V and GP-VI with molecular weights of 90,000, 80,000 and 65,000 respectively. However the overall patterns for the discocytes, echinocytes and spherocytes do not differ in either tritiation labelling procedure. These patterns are typical of two series of experiments.

Quantitation of whole platelet counts showed that the echinocyte and the spherocyte had 0.4% and 0.9% more counts respectively than the discocyte using the periodate tritiation method. While using the neuraminidase-galactose oxidase labelling method the echinocyte had 16.2% and the spherocyte had 21.1% more counts than the discocyte. These percentages were the means of two series of experiments.

Fig. 32. SDS-PAGE gel patterns of membranes (reduced) from tritiated (a) discocyte; (b) echinocyte and (c) spherocyte. Tritiation was by the periodate method.

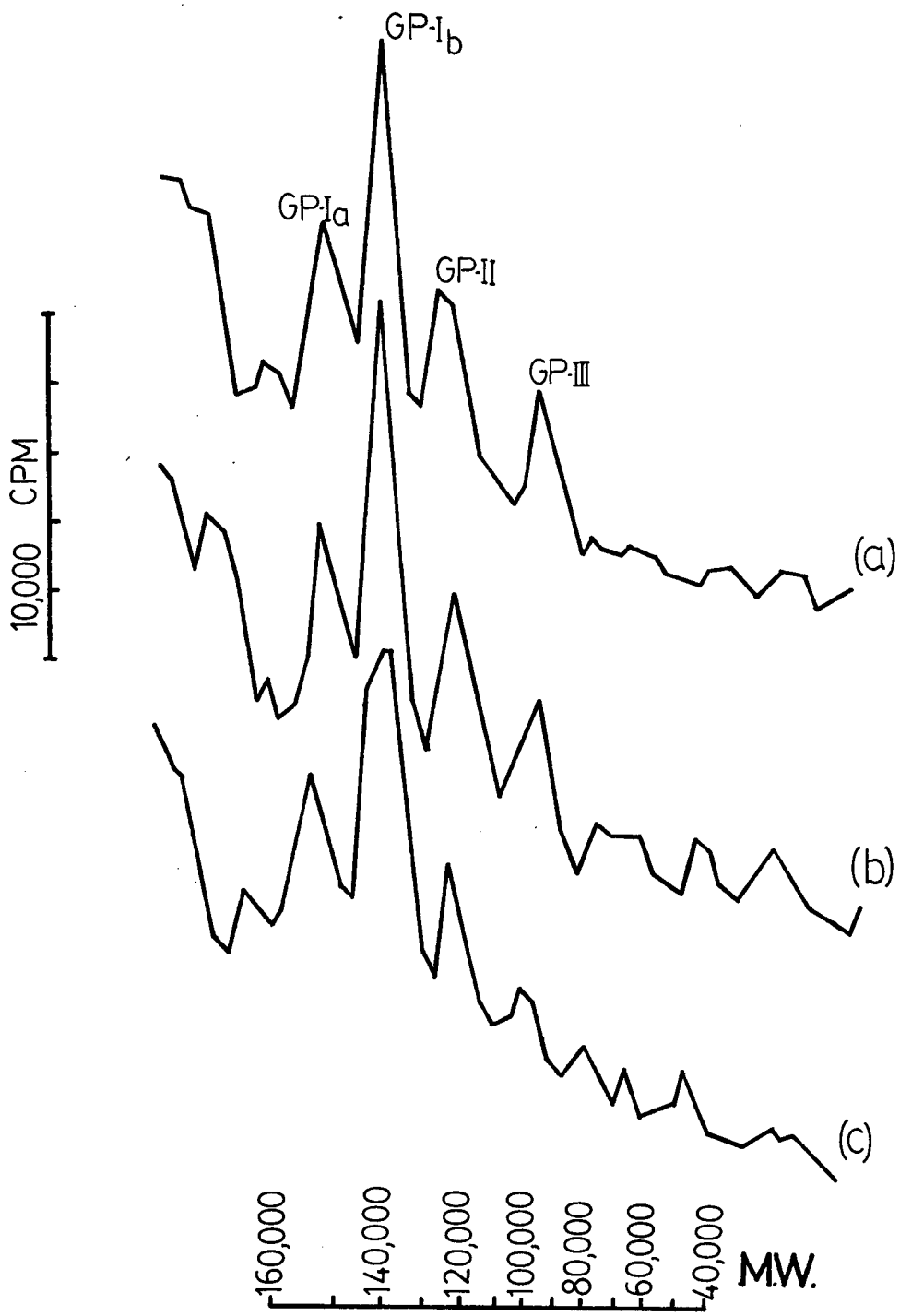
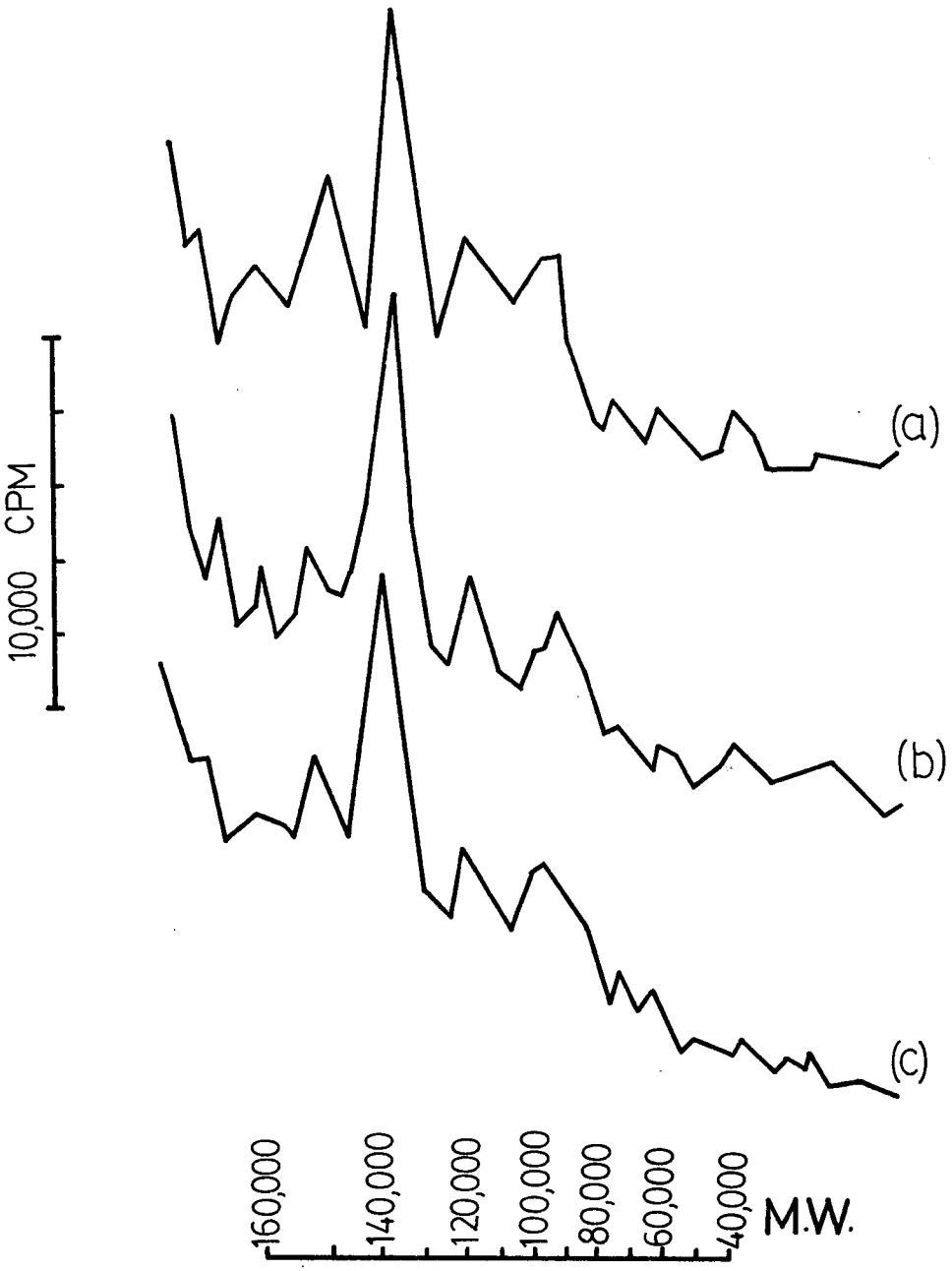

GP-Ib
GP-Ia
GP-II
GP-III
10,000 CPM
(a)
(b)
(c)
160,000
140,000
120,000
100,000
80,000
60,000
40,000
M.W.

Fig. 33. SDS-PAGE gel patterns of membranes (reduced) from tritiated (a) discocyte; (b) echinocyte and (c) spherocyte. Tritiaton was by the neuraminidase/galactose oxidase method.


10,000 CPM
(a)
(b)
(c)
160,000
140,000
120,000
100,000
80,000
60,000
40,000
M.W.

The gel patterns of the pseudo-double label experiments are shown in Figs. 34 and 35. Using the periodate method, little or no labelling by ^{3}H (the 2nd label) has taken place (Fig. 34). With the neuraminidase-galactose oxidase method, small peaks are present in the gel patterns of the echinocytes and spherocytes (Fig. 35). They correspond to GP-Ia, GP-Ib, GP-II and GP-III. In both Figs. 34 and 35, the patterns of the discocytes served as controls. Here "cold labelled" discocytes were relabelled with ^{3}H-borohydride without being transformed. Theoretically, if the cold reaction went to completion, no ^{3}H labelling should be observed.

Morphological examinations of the platelets under phase microscopy were done as in the iodination experiments above. Again, no problems were encountered.

4.3.3 Surface Labelling in the Presence of Fibrinogen

The influence of fibrinogen (0.5 mg/ml) on lactoperoxidase catalyzed iodination of the platelet forms is shown in Figs. 36-38. The fibrinogen was added together with the lactoperoxidase and therefore was present during the whole process of iodination. Profiles of the gels of the discocyte (Fig. 36) and spherocyte (Fig. 38) show that fibrinogen did not influence iodination of the discocyte and spherocyte surface. On the other hand, a drop in the peak size of GP-III in the patterns of the echinocytes labelled in the presence of fibrinogen can be seen (Fig. 37). This is also reflected by a peak in the R-plot demonstrating a difference between the control (without fibrinogen) and the test gel at that location. Fibrinogen therefore impaired the labelling of GP-III on the echinocyte but not on the spherocyte or discocyte. Fibronectin (Fig. 40) and albumin (Fig. 39), tested also at 0.5 mg/ml, had no influence on the gel patterns of the echinocytes. This impairment in labelling was also

Fig. 34. SDS-PAGE gel patterns of membranes (reduced) from the pseudo-double labelled (a) discocyte; (b) echinocyte and (c) spherocyte. Platelet discocytes were first labelled with cold borohydride, then transformed and relabelled with ^{3}H-borohydride using the periodate method both times.

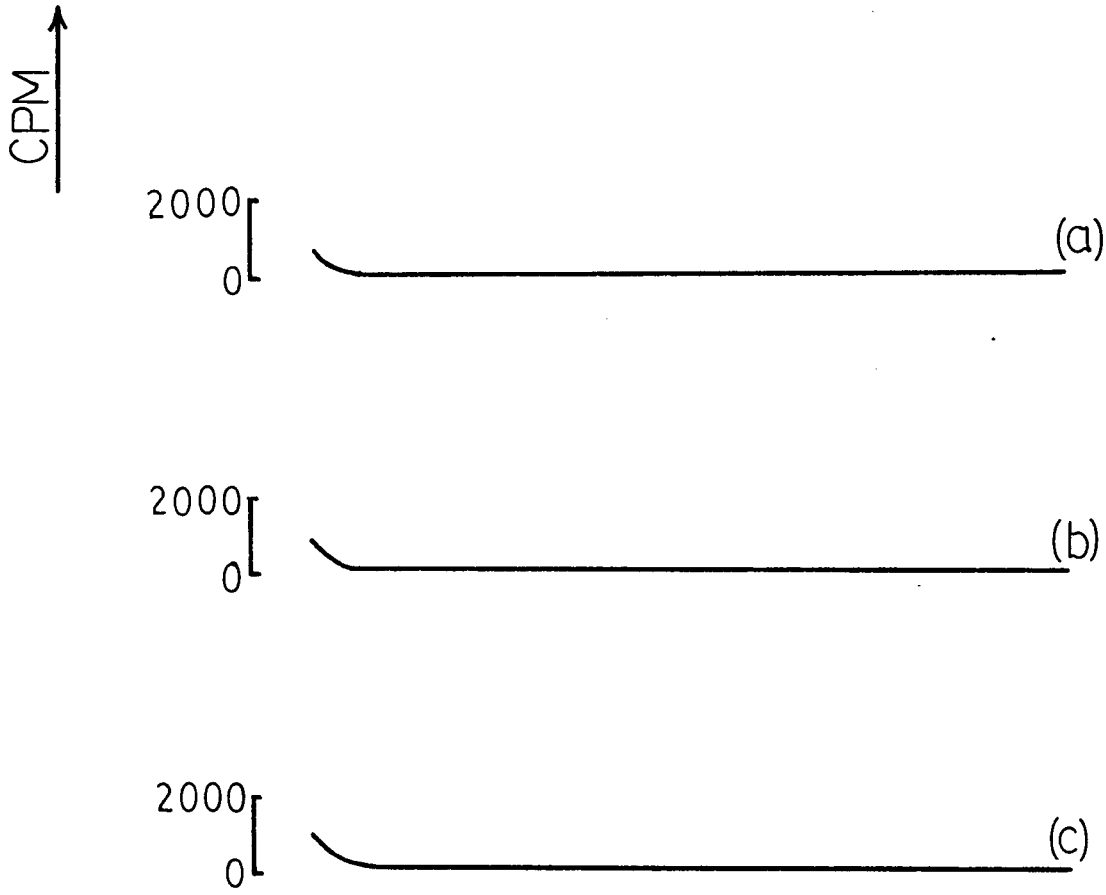

CPM
2000
0
(a)
2000
0
(b)
2000
0
(c)

Fig. 35. SDS-PAGE gel patterns of membranes (reduced) from the pseudo-double labelled (a) discocyte; (b) echinocyte and (c) spherocyte. Platelet discocytes were first labelled with cold borohydride, then transformed and relabelled with ^{3}H-borohydride using the neuraminidase/galactose oxidase method both times.

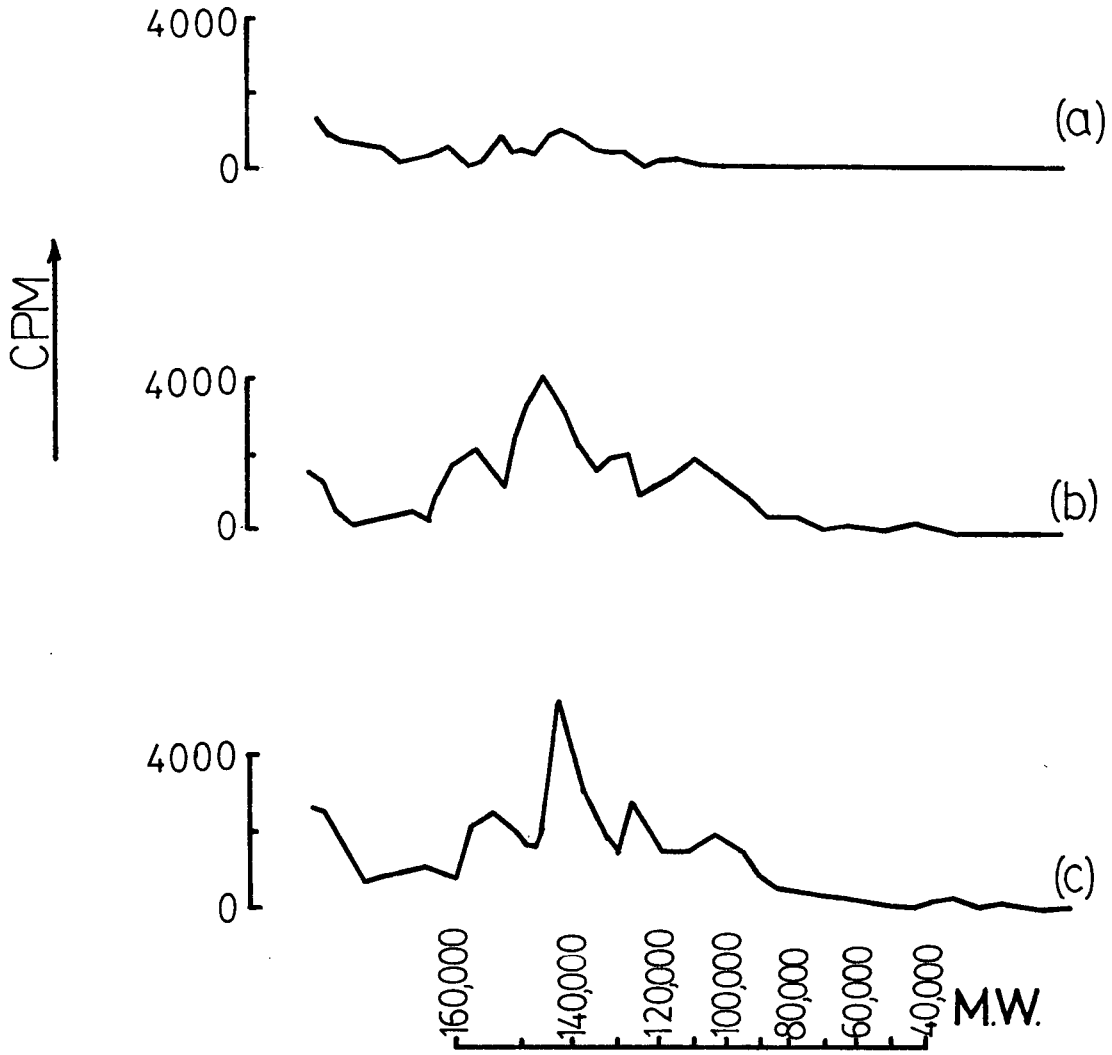

4000
0
(a)
CPM
4000
0
(b)
4000
0
(c)
160,000
140,000
120,000
100,000
80,000
60,000
40,000
M.W.

Fig. 36. SDS-PAGE gel patterns of membranes (reduced) from discocyte ^{125}I-lactoperoxidase labelled (a) without and (b) with fibrinogen present (0.5 mg/ml). (c) Ratio, R, of the radioactivity in (a) to that in (b) for each gel slice (R-plot). Dotted line marks the mean of the R values and the bracket (s-s) represents $\pm$2 standard deviation.

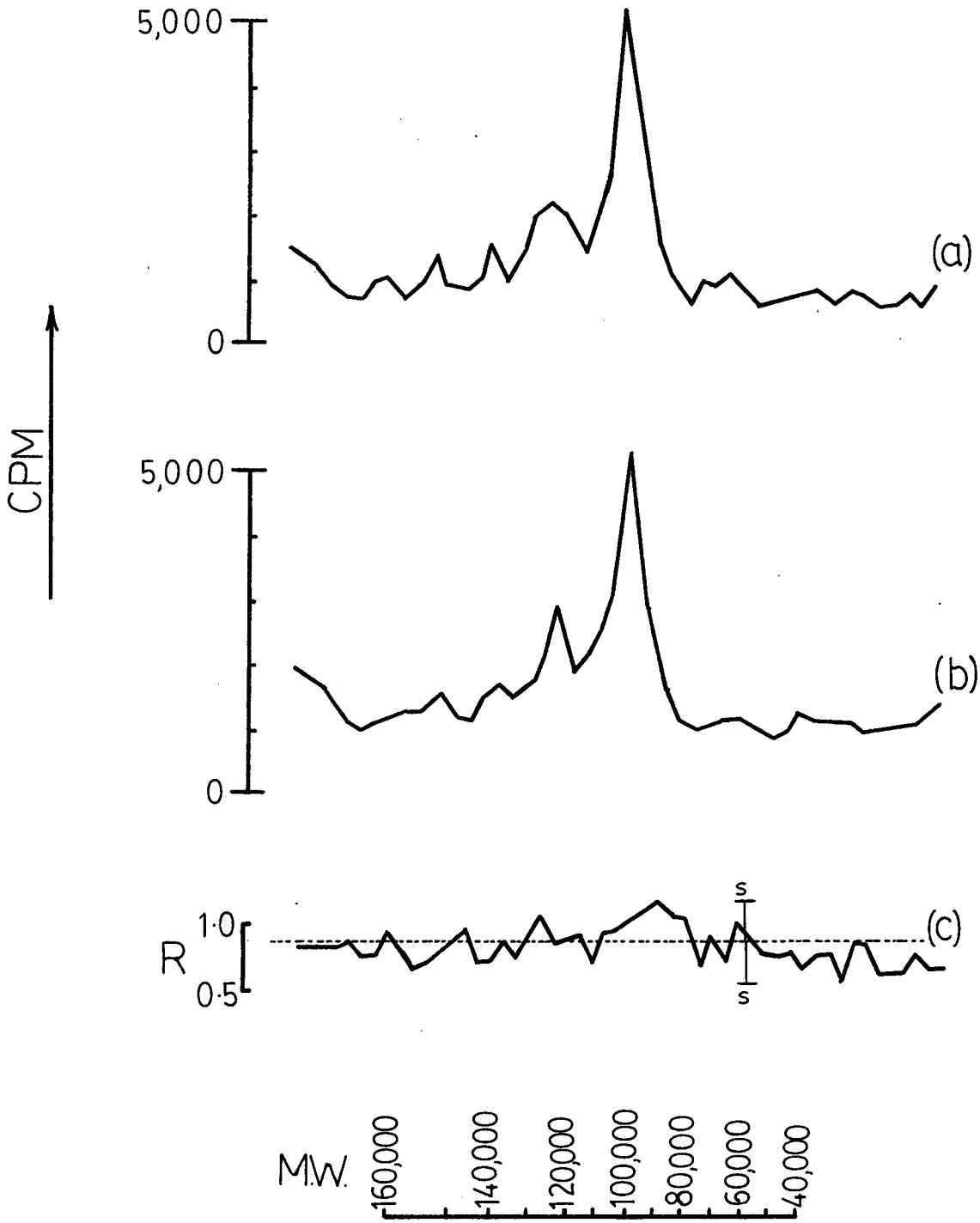

5,000
0
(a)
CPM
5,000
0
(b)
R
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 37. SDS-PAGE gel patterns of membranes (reduced) from echinocytes ^{125}I-lactoperoxidase labelled (a) without and (b) with fibrinogen present (0.5 mg/ml); (c) R-plot, the bracket (S-S) is the same as that of the control in Fig. 36.

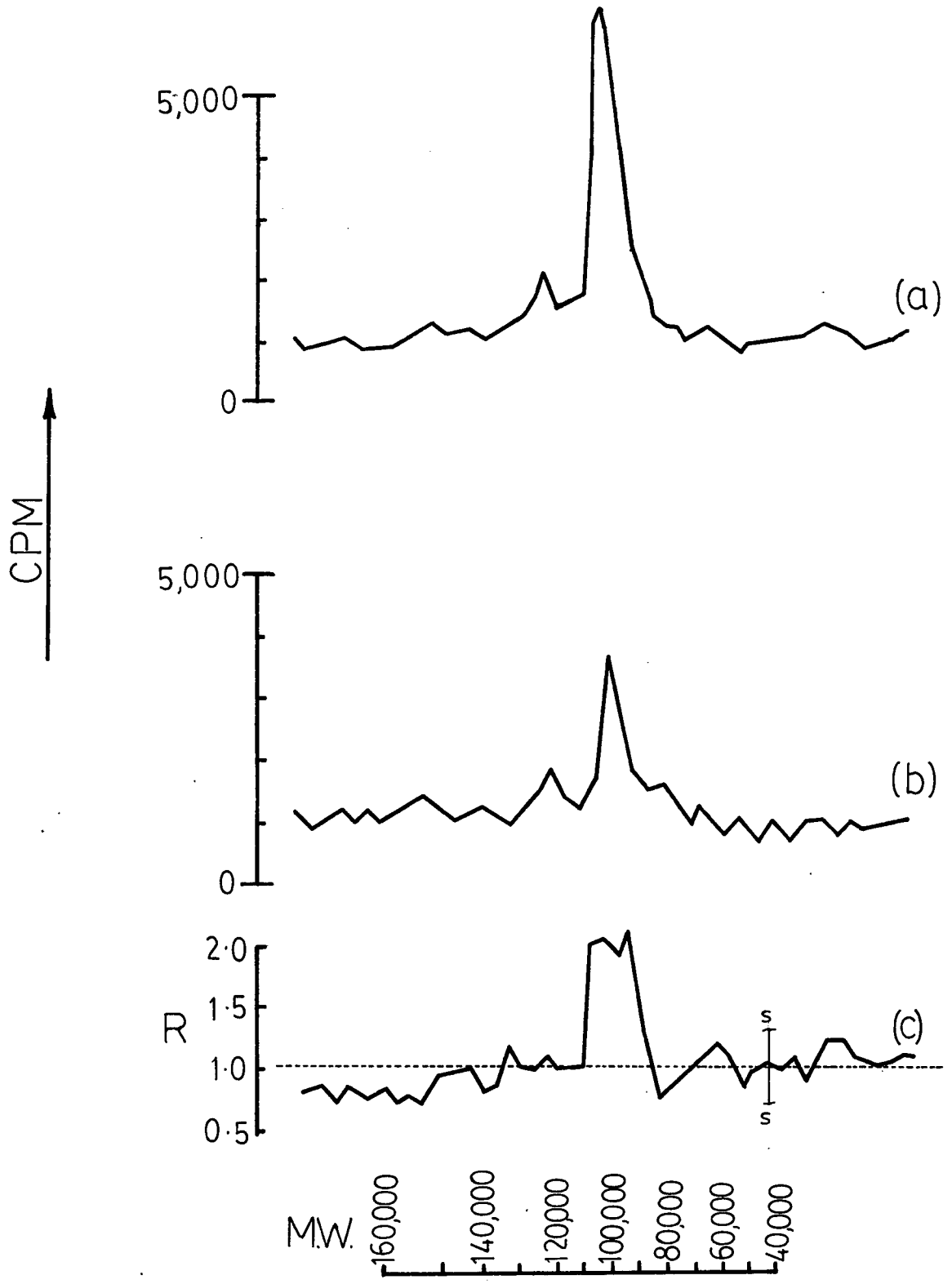

5,000
0
(a)
CPM
5,000
0
(b)
R
2·0
1·5
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 38. SDS-PAGE gel patterns of membranes (reduced) from spherocytes ^{125}I-lactoperoxidase labelled (a) without and (b) with fibrinogen present (0.5 mg/ml); (c) R-plot, as defined for Fig. 36.

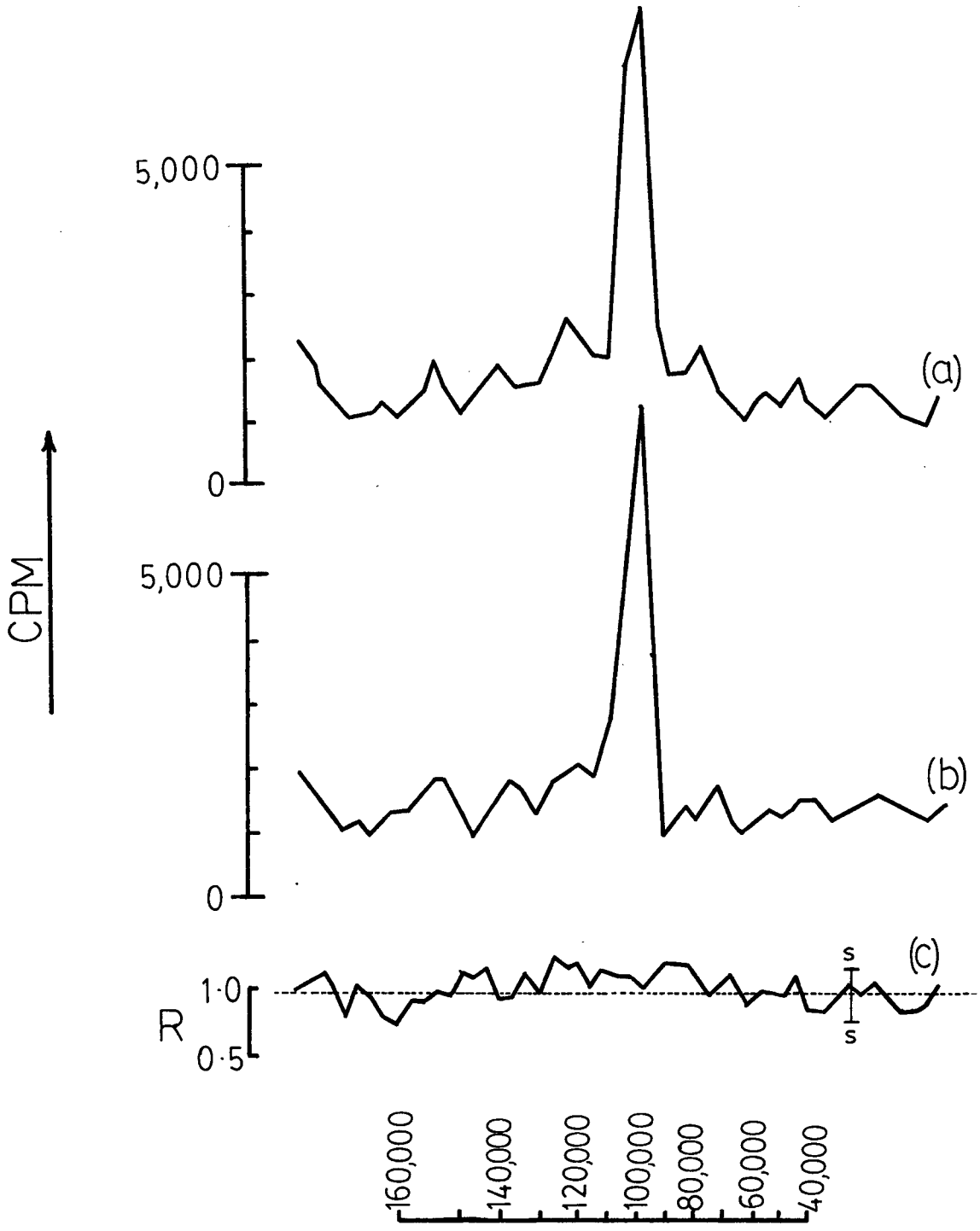

5,000
0
(a)
CPM
5,000
0
(b)
(c)
s
s
R
1·0
0·5
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 39. SDS-PAGE gel patterns of membranes (reduced) from echinocytes ^{125}I-lactoperoxidase labelled (a) without and (b) with albumin present (0.5 mg/ml); (c) R-plot, as defined for Fig. 36.

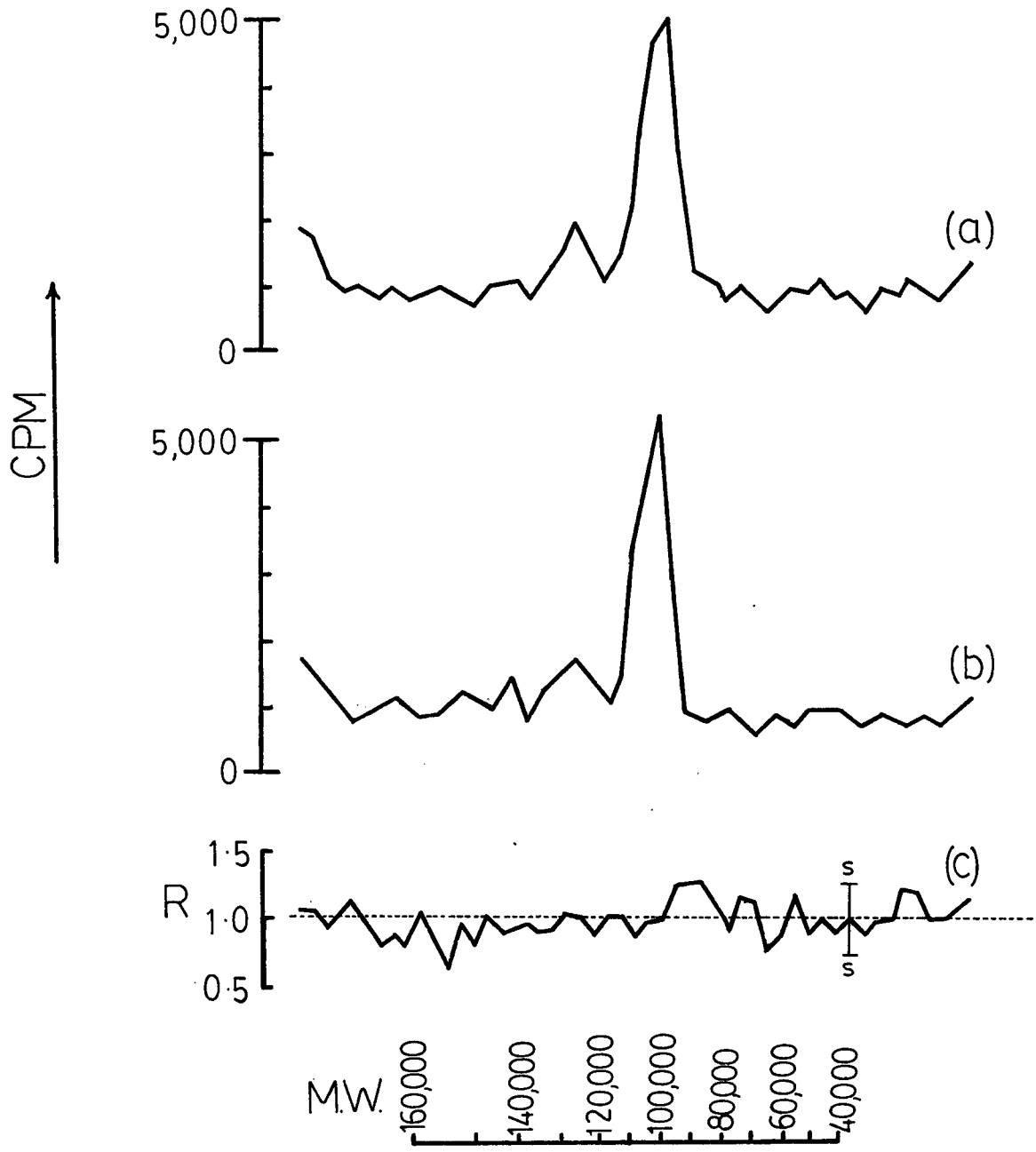
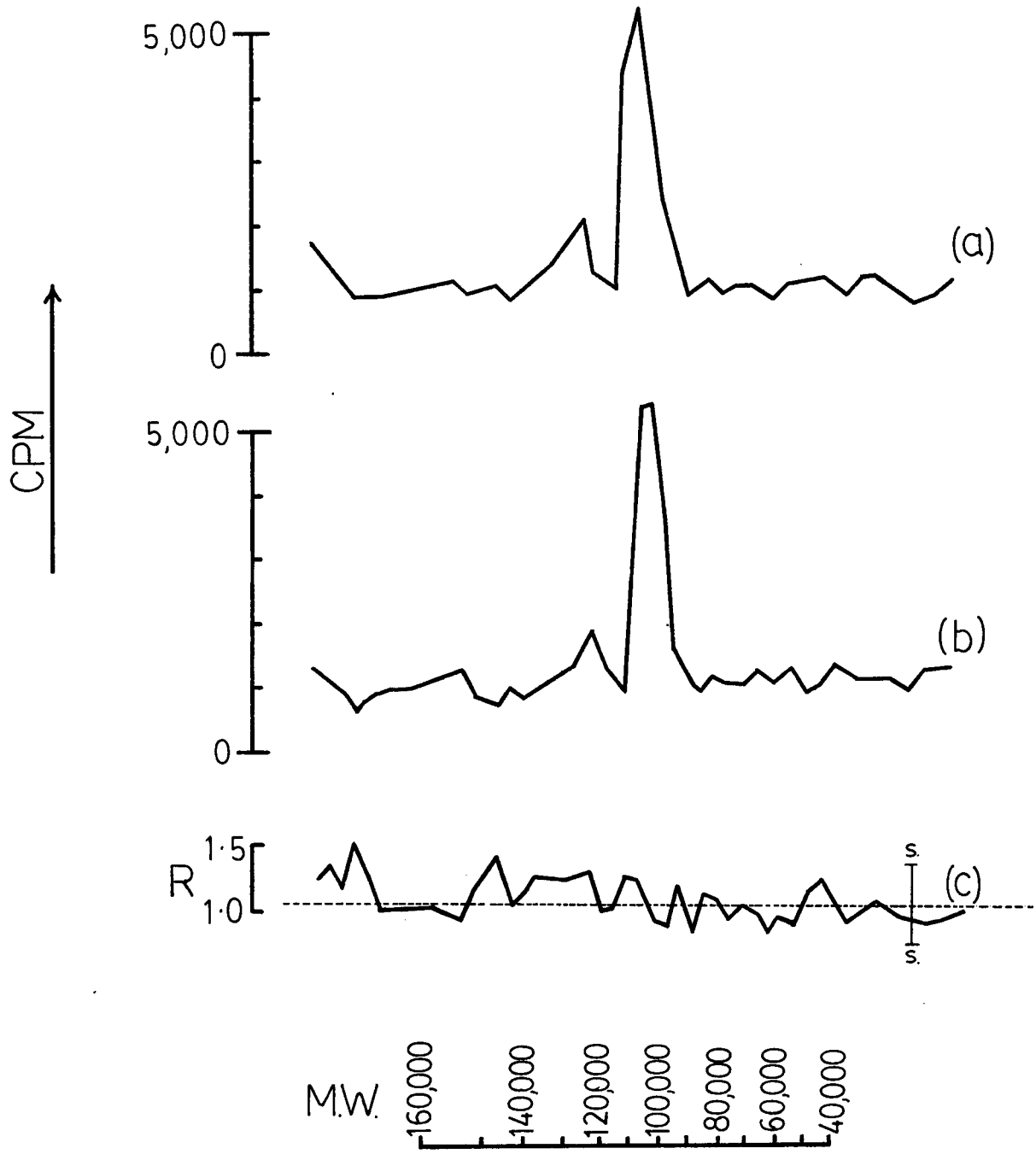
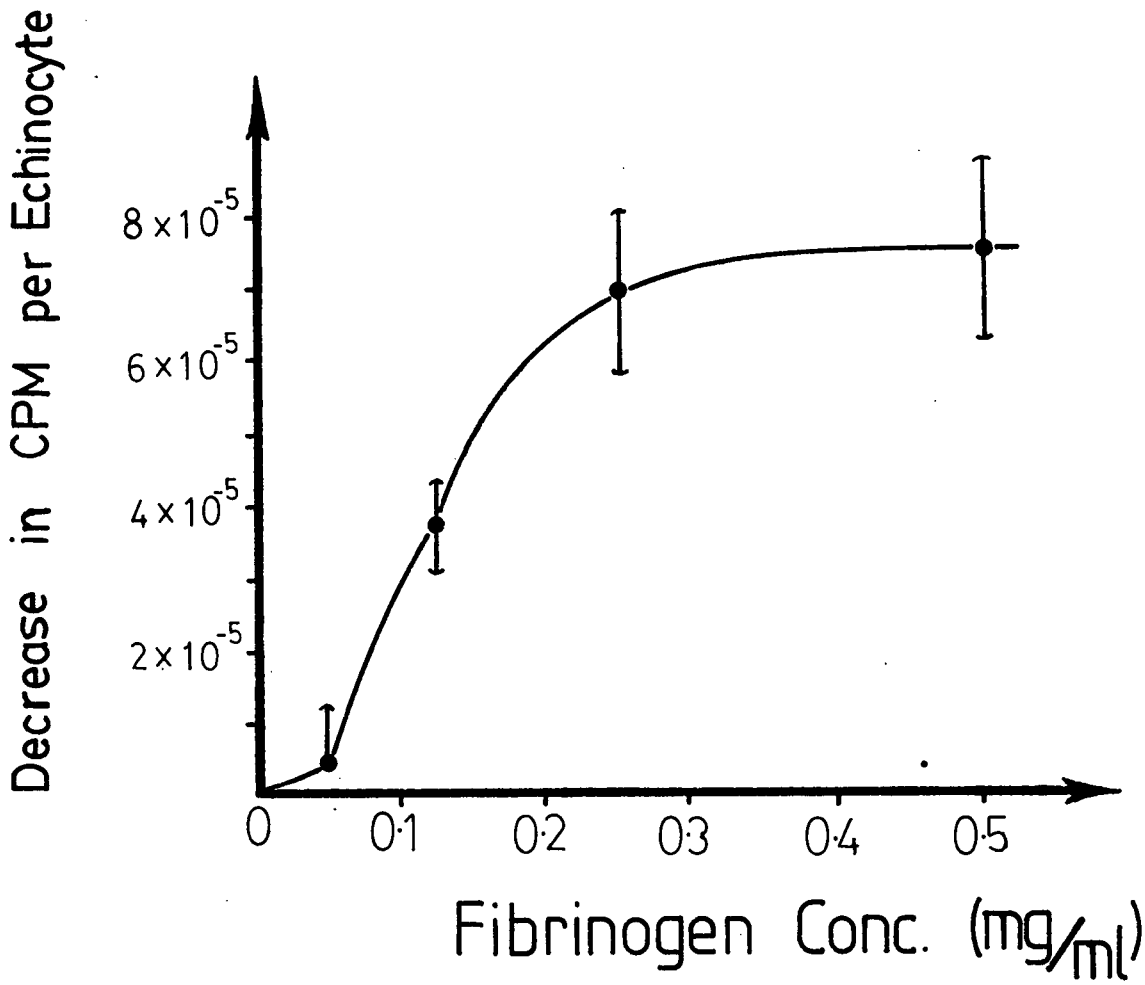

5,000
0
(a)
CPM
5,000
0
(b)
1·5
R
1·0
0·5
s
s
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

Fig. 40. SDS-PAGE gel patterns of membranes (reduced) from echinocytes ^{125}I-lactoperoxidase labelled (a) without and (b) with fibronectin present (0.5 mg/ml); (c) R-plot as defined for Fig. 36.


5,000
0
(a)
CPM
5,000
0
(b)
R
1·5
1·0
s.
s.
(c)
M.W.
160,000
140,000
120,000
100,000
80,000
60,000
40,000

dependent on the concentration of fibrinogen. The decrease in the number of counts (cpm) from the control (echinocyte labelled with no fibrinogen present) increases in magnitude with fibrinogen concentration (Fig. 41). A plateau was reached at about 0.5 mg/ml. Gel patterns are typical of three series of similar experiments. Values in Fig. 41 are also from three sets of experiments.

Fig. 41. The effect of fibrinogen concentraion on the ^{125}I-labelling of echinocytes. The decrease in the number of counts (cpm) per echinocyte from the control (echinocyte labelled with no fibrinogen present) is plotted against fibrinogen concentration.


Decrease in CPM per Echinocyte
8 x 10-5
6 x 10-5
4 x 10-5
2 x 10-5
0
0·1
0·2
0·3
0·4
0·5
Fibrinogen Conc. (mg/ml)

4.4 DISCUSSION

4.4.1 Iodination Experiments

The gel patterns in Figs. 24-31 are not unlike those found by others for lactoperoxidase iodination (Phillips, 1972; Okumura and Jamieson, 1976; Connellan et al., 1982). GP-III is most dominant while the GP-II peak is usually small. Those of the GP-Ia and GP-Ib are less obvious and better demonstrated in the tritiation gel patterns.

Little difference can be seen between the gel patterns of single iodinated discocytes and echinocytes (Figs. 24 and 25). However the difference between the gel patterns of the iodinated intact spherocytes (Fig. 24) and that of plasma membrane isolated from iodinated spherocytes (Fig. 25) is obvious. The large peak with an apparent molecular weight of 120,000 had disappeared during the membrane isolation process leaving behind only a small peak similar to that of GP-II observed in the discocyte and echinocyte patterns. Apparently two proteins with similar molecular weights are involved here. The smaller peak belongs to GP-II. The identity of the other remains uncertain although it must be a product of hypotonic shock. The fact that it was removed during membrane isolation makes it less likely to be a native component of the membrane. It it believed to be a protein originating from the inside of the platelet and released to the outside during hypotonic treatment. Presumably it was then adsorbed onto the surface of the spherocyte and iodinated. Removal from the platelet surface occurred during membrane isolation. The adsorption would be facilitated by low ionic strength condition of the hypotonic Tyrode's solution (Heard and Seaman, 1960). After membrane isolation they were washed in isotonic Tyrode's solution and desorption might have occurred assisted by the higher salt concentration.

In 1974, Phillips and Agin reported the presence of a thrombin sensitive protein with a molecular weight similar to that of GP-II in SDS-PAGE. They were not able to confirm the observation in a latter study (Phillips and Agin, 1977) and attribute it to an artifact. In their initial 1974 observation, it was found that only 30% of the 120,000 molecular weight peak was susceptible to thrombin indicating the presence of two overlapping components with similar molecular weights in the gel. Several ideas were entertained at that point concerning the nature of the thrombin sensitive component. One of the suggestions was platelet factor XIII.

Platelet factor XIII accounts for approximately 50% of total factor XIII activity in human whole blood and almost all of it is carried in the platelet cytoplasm (Lopaciuk et al., 1976; Walsh, 1981). Leakage of platelet contents such as beta-thromboglobulin and LDH to the outside during hypotonic shock has already been discussed in Chapter 2. Leakage of some factor XIII out of the spherocyte is therefore not totally unexpected. Factor XIII was known to adsorb strongly onto platelet surfaces (Born, 1968). Platelet factor XIII is a dimer of two identical chains each having a molecular weight of about 110,000 (Loewy et al., 1961; Ganguly, 1971; Schwartz et al., 1971). In an in vitro environment the dimer spontaneously dissociates into its two monomer subunits (Loewy et al., 1961). Platelet factor XIII monomers appear to be a likely candidate for the protein in question here. Unlike plasma factor XIII, platelet factor XIII contains no carbohydrate (Schwartz et al., 1971) and therefore will not influence sialic acid results in Chapter 3. On the other hand, the presence of this adsorbed protein may physically interfere with the neuraminidase molecule resulting in underestimation of sialic acid density on the spherocyte surface. However, this seems unlikely because the density of

the phosphate groups on the spherocyte and the echinocyte surfaces are similar (Table 9) and the molecular weight of alkaline phosphatase is much higher than neuraminidase.

In light of the artifact involving intact spherocytes, the rest of the SDS-PAGE experiments were done using plasma membranes isolated from radio-labelled platelets. During the preliminary single iodination experiments (Figs. 24 and 25) no significant difference could be observed between the gel patterns of the discocyte and the echinocyte. The decision was therefore made to utilize double label procedures in hope that it might bring out more subtle differences.

The gel patterns of the double iodination experiments are shown in Figs. 26-31. It seems clear that the patterns are again similar to each other, since in no case did the ratio R vary by more than two standard deviations from its mean value. Apparently no new platelet surface species are labelled on the echinocyte or spherocyte. Fig. 29 shows that iodination of the tyrosine residues is less than complete. After the initial ^{125}I-iodination, there was considerable tyrosine left over to be labelled with ^{131}I for the second iodination. Fig. 29 represents the control experiment in which discocytes were first labelled with ^{125}I and then relabelled with ^{131}I without transformation. However, failure of the first iodination reaction to go to completion will not affect the outcome of the echinocyte and spherocyte experiments since the experimental conditions for both ^{125}I and ^{131}I iodinations were kept the same. New species appearing on the echinocyte or spherocyte surface after transformation would be labelled by ^{131}I together with "old" species not yet labelled by ^{125}I.

Quantitative experiments showed that the echinocyte bound 21.7% more ^{125}I label than the discocyte. The estimated increase in surface area, on the other hand, is 44% (Chapter 3). There are a number of possible explanations for this discrepancy. Firstly, the echinocyte area estimated in Chapter 3 may be too high. This seems unlikely because the areas obtained by neuraminidase and alkaline phosphatase experiments mutually agree with each other and with the Frojmovic estimation. Secondly, lactoperoxidase may have entered into part of the invaginated surface-connected canalicular system of the discocyte to catalyze iodination there. In this case the difference betwwen values (counts) obtained for the discocyte and echinocyte will be smaller than expected with respect to the estimated difference in surface areas. Lactoperoxidase has a molecular weight of 93,000, larger than neuraminidase but smaller than alkaline phosphatase. As discussed in Chapter 3 for neuraminidase and alkaline phosphatase, it is unlikely that lactoperoxidase will enter the canalicular system. Thirdly, if the only change in the biochemical nature of the echinocyte surface is the appearance of "new membrane" from the canalicular system, this membrane may be deficient, relative to the discocyte surface, in iodinatable tyrosine residues. It has already been shown in Table 16 that the echinocyte "new membrane" has lower quantities of terminal sialic acid, phosphate and amino groups. Fourthly, it is also possible that the chemical character of the platelet surface may have changed due to ADP-induced transformation such that the overall labelling is reduced. Membrane changes involving whole platelet surfaces such as cation and fibrinogen binding sites have been discussed in Chapter 3. However it is uncertain if the third, the fourth, or both explanations best fits the discrepancy.

The spherocyte showed a 39.8% increase in labelling while the geometric surface area actually increased by 123% (Chapter 2). The increase in labelling due to the appearance of the 120,000 molecular weight protein (factor XIII), on the hypothetical "new membrane" is therefore minimal. Again one can use reasons given above for echinocytes to explain this situation.

4.4.2 Tritiation Experiments

Gel patterns of tritiated platelets show more peaks than the iodinated samples. Again, there is no apparent difference between the gel profiles of the discocyte, echinocyte, or spherocyte. The patterns of platelets labelled using the periodate method and those using the neuraminidase-galactose oxidase method are also similar (Figs. 32 and 33). They are not unlike those shown elsewhere (McGregor et al., 1979; Mosher et al., 1979; Marchesi and Chasis, 1979; Phillips and Agin, 1977)

The pseudo-double label experiments using the periodate tritiation method showed no ^{3}H-labelling at all (Fig. 34). Here the discocytes were first labelled with cold borohydride following periodate oxidation. They were then transformed into echinocytes or spherocytes and relabelled with the periodate method using ^{3}H implies that all the terminal sialic acid residues on their surfaces had already reacted. In other words, the periodate crossed the membrane or entered the canalicular system and oxidized all the sialic acid in the membrane. Both borohydride and metaperiodate are very small molecules and should easily enter the surface-connected canalicular system. They also cross membranes and label the interior materials of intact cells (Gahmberg and Andersson, 1977).

The pseudo-double label experiments using the neuraminidase-galactose oxidase method present another picture (Fig. 35). Small peaks were found corresponding to the surface glycoproteins already demonstrated in the single label gels. Since the molecular weight of neuraminidase is 90,000 and galactose oxidase is 42,000 (Worthington, 1972), they would not be expected to enter the canalicular system significantly. Therefore the glycoproteins on the canalicular surface were probably not cold labelled in the discocyte. Even though the cold borohydride had access to the canalicular system, the enzymes required to catalyze the reaction should not have been able to enter. After transformation from discocyte to echinocyte or spherocyte the glycoproteins from these surfaces became available for enzymatic oxidation and ^{3}H labelling. The fact that the peaks here are relatively small compared to those of the direct labelling experiments (Fig. 33), suggests that the amount of glycoproteins cloistered in the discocyte canalicular system is rather small.

Quantitative single label experiments using the periodate method showed that the echinocyte and the spherocyte had only 0.4% and 0.9% more counts respectively than the discocyte. Using the neuraminidase-galactose oxidase method, the echinocyte exhibited 16.2% and the spherocyte 21.1% more ^{3}H label than the discocyte. The discrepancies can be explained using the same argument as for the pseudo-double labelling experiments discussed above. The periodate had ready access to the invaginated canalicular system. Therefore the amount of membrane surface available for labelling is the same in the discocyte, echinocyte and spherocyte. On the other hand, the enzymes will not be able to enter the canalicular system. More surface will therefore be made

available for labelling after evagination of the canalicular system in the echinocyte and spherocyte.

The quantitative results from neuraminidase-galactose oxidase ^{3}H labelling were quite similar to those of the lactoperoxidase ^{125}I-labelling experiments. The percent increases in the tritiation of the echinocyte and spherocyte surfaces were much lower than the percent increases in their surface areas estimated in Chapter 2 and 3. This again suggests either that the density of the glycoproteins on the invaginated membrane system is much lower than on the external plasma membrane or that the average surface properties of the transformed cells have changed.

It has been reported by Cazenave et al. (1976) that periodate (1-10 mM) treatment of rabbit platelets in Tyrode's solution caused them to aggregate. However, we found no aggregation during the experiments with human platelets. The chief concern here is that the discocytes may turn into echinocytes during the labelling procedures. Microscopic examination demonstrated that this did not happen.

4.4.3 Surface Labeeling in the Presence of Fibrinogen

It was found here that fibrinogen impairs the iodination of echinocyte surface GP-III. It seems that the binding of fibrinogen onto the echinocyte surface brings it into close association with GP-III, close enough for fibrinogen (molecular weight 340,000) to interfere with the enzyme lactoperoxidase which catalyzes the iodination. While this does not mean that the fibrinogen receptor is actually GP-III, it certainly adds further evidence to support the idea that GP-III is the receptor. The phenomenon is specific only to the echinocyte (activated platelet) and not the discocyte or spherocyte. It is

specific to fibrinogen and does not occur with albumin or fibronectin. This last result is consistent with the work of Harfenist (1980) who found that fibronectin is not involved in platelet aggregation and will not bind to activated platelets. It has already been shown in Chapter 2 that ADP induced platelet aggregation requires fibrinogen. Fig. 10 and Fig. 41 are extremely similar to each other. They imply a close relationship between platelet aggregation and fibrinogen binding to or near GP-III on the platelet surface.

This experiment failed to demonstrate any association of fibrinogen with the spherocyte surface. It is possible that there is no fibrinogen receptor on the spherocyte or that the receptors have been destroyed during the hypotonic treatment. Presence of the 120,000 molecular weight protein on the spherocyte might also have physically blocked the fibrinogen receptors and/or the Ca^{++} binding sites. This might have caused the drastic drop in the Ca^{++} binding sites on the spherocyte (section 3.3.5).

4.4.4 Summary of Chapter 4

Different methods have been used to label the platelet discocyte, echinocyte and spherocyte but none showed any qualitative differences between the gel patterns. Quantitative differences were demonstrated, however, but it was found that the labelling density on the evaginated surfaces of the canalicular system was lower than on the discocyte surface, presuming the latter was unchanged during shape transformation. A close association between fibrinogen and GP-III was also demonstrated in the echinocyte, the concentration dependence of which closely parallels the fibrinogen dependence of aggregation.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The purpose of this project was to investigate the surface properties of the platelet discocyte, echinocyte and spherocyte. A model is examined here in which an echinocyte attains its stickiness properties by evagination of the surface-connected canalicular system. Platelets also evaginate their canalicular system upon hypotonic shock treatment to form spherocytes. By comparing the properties of different forms of platelets some insight into the nature of "stickiness" was sought. The following conclusions can be drawn from the results obtained.

5.1 The surface areas of the discocyte and spherocyte were found microscopically to be $16.4 \times 10^{-8} cm^2$, and $36.7 \times 10^{-8} cm^2$ respectively (Chapter 2). The surface area of the echinocyte was estimated to be $23.7 \times 10^{-8} cm^2$ using surface chemical and cell electrophoresis techniques (Chapter 3). The surface area of the echinocyte is therefore much smaller than that of the spherocyte.

5.2 Electron microscopic examination showed that the surface-connected canalicular system may not have totally evaginated in the echinocyte, therefore supporting the surface area calculations.

5.3 Aggregometry studies demonstrated a biphasic requirement for the divalent cations Ca^{++} or Mg^{++} to support ADP induced aggregation. The optimum concentration is 4 mM for both cations but Ca^{++} is more efficient than Mg^{++} in supporting aggregation. Sr^{++} does not support aggregation while Mn^{++} inhibits aggregation.

5.4 Microelectrophoresis studies revealed an eight fold increase in the density of Ca^{++} binding sites on the platelet surface during discocyte to

echinocyte transformation. The increase in the density of Mg^{++} binding sites was over six fold. The spherocyte which has lost its ability to aggregate also lost over 60% of its Ca^{++} binding sites.

5.5 ADP induced aggregation requires the presence of fibrinogen (Chapter 2). The presence of fibrinogen also interferes with the lactoperoxidase labelling of GP-III on the ADP activated platelet surface (Chapter 4). Similar concentration dependences were found between the two phenomena.

5.6 Although spherocytes have lost their ability to aggregate they can still be agglutinated with ristocetin and a plasma component, presumably von Willebrand's factor.

5.7 Neuraminidase treatment has no effect on ADP induced aggregation but the platelet's responses to different lectins are altered. The removal of terminal sialic acid residues and the exposure of galactose by the enzyme decreases the aggregation response to WGA and increase the responses to RCA and JBA.

5.8 Electrokinetic analysis of live, fixed and neuraminidase or alkaline phosphatase treated platelets showed major differences in charge densities as well as amino, sialic acid and phosphate group densities on the discocyte, echinocyte and spherocyte. The evaginated "new" membrane surfaces of the echinocyte and spherocyte seem also considerably different. Data are summarized in Table 16.

5.9 SDS-PAGE of platelets radiolabelled using a variety of methods including lactoperoxidase (single and double) iodination, periodate-borohydride tritiation and neuraminidase/galactose oxidase-borohydride tritiation failed

to show any significant differences in the gel patterns of the discocyte, echinocyte and spherocyte. No new glycoprotein species appeared after the transformations but quantitative differences among the three forms of platelets were found in the radiolabelling experiments.

5.10 Considering the amounts of sialic acid removed from the platelet forms by neuraminidase and the quantitative differences in radiolabelling experiments, it appears that the "new membrane" surfaces of the echinocyte and spherocyte have lower densities of glycoproteins than the discocyte surface.

In the original hypothesis, it was proposed that the discocyte surface-connected canalicular membrane system is inherently "sticky". The apparent increase in surface area associated with the discocyte to echinocyte transformation was thought to occur via the evagination of this membrane system. Since there is no evidence that platelets synthesize membrane, the "pre-formed" membrane of the canalicular system seems to be the most likely, if not the only, choice for the source of the added area. However, whether this cloistered membrane system is also inherently "sticky" is another matter. Evagination of the canalicular membrane system by hypotonic shock failed to create a spherocyte that was capable of fibrinogen-dependent aggregation, even though it was found that the spherocyte had a much larger surface area than the echinocyte, indicating a greater degree of evagination.

It is shown in Table 16 that the echinocyte "new membrane" has lower densities of the various charge groups than the discocyte surface. Its evagination will therefore cause a dilution effect resulting in the lowering of the average surface charge density on the platelet echinocyte.

Lowering of surface charge in principle could facilitate platelet-platelet contact and aggregation. However, the spherocyte has an even lower negative charge density than that of the echinocyte but its surface is non-sticky and the spherocytes do not aggregate. Therefore it seems unlikely that "stickiness" results simply from the lowering of platelet surface charge density. Two other properties that can be considered as a characteristic of "stickiness", i.e. the appearance of Ca^{++}/Mg^{++} binding sites and of fibrinogen binding sites were observed here. They are unique to the echinocyte and were not observed on the discocyte or spherocyte. GP-III was demonstrated in all three forms of platelets but only those on the echinocyte surface associate with fibrinogen molecules. This suggests that a conformational change in GP-III itself, or in molecules associated with it, is a consequence of platelet activation and not necessarily a result only of shape change and area increase.

In conclusion, a number of differences were demonstrated here between the surface properties of the platelet discocyte, echinocyte and spherocyte (Table 17). However, the data fail to support the model that platelet "stickiness" is a result only of canalicular system evagination or that the cloistered membrane of the canalicular system is inherently "sticky". It appears more likely that changes occur over the whole platelet surface during activation and make them capable of participating in aggregation reactions.

Table 17. Highlights of Differences* Between Platelet Discocyte, Echinocyte, and Spherocyte

	Discocyte	Echinocyte	Spherocyte
Area (cm^2)	16.4×10^{-8}	23.7×10^{-8}	36.7×10^{-8}
Negative charge density per cm^2	9.13×10^{12}	7.92×10^{12}	6.50×10^{12}
Amino groups per cm^2	1.33×10^{12}	1.19×10^{12}	1.08×10^{12}
Sialic acid molecules per cm^2	3.98×10^{12}	3.52×10^{12}	2.12×10^{12}
Phosphate groups per cm^2	2.28×10^{12}	1.96×10^{12}	1.96×10^{12}
Sialic acid/Total charge	43.5%	44.4%	32.6%
Phosphate/Total charge	25.0%	24.6%	30.1%
Sialic acid/Phosphate	1.75	1.80	1.08
Ca^{++} binding sites per cm^2	1.28×10^{12}	1.09×10^{13}	2.22×10^{11}
Fibrinogen association with membrane	No	Yes	No
Aggregation	No	Yes	No
Membrane "stickiness"	No	Yes	No

*No differences found in their SDS-PAGE patterns.

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APPENDIX

STATISTICAL CONSIDERATIONS FOR THE Ca^{++} AND Mg^{++} BINDING EXPERIMENTS*

The densities of the divalent cationic (C^{++}) binding sites on platelets were determined according to the following equation presented in Chapter 3+:

$$\frac{1}{\Delta\sigma} = \frac{1}{2en} + \frac{1}{2enK} \cdot \frac{1}{[C^{++}] \cdot \exp(2e\zeta/kT)}$$

$$K = \frac{\exp(\Delta G/kT)}{55.6}$$

$1/\Delta\sigma$ was plotted against $1/[C^{++}] \cdot \exp(2e\zeta/kT)$. Linear regression was used to obtain a line whose slope represents $1/2enK$ and the intercept with the ordinate gives $1/2en$. The coefficient of variation (c.v.) for a particular $1/\Delta\sigma$ was equal to the c.v. of the mean mobility for that point, i.e:

$$\text{c.v.}(1/\Delta\sigma) = \frac{\text{s.d.}(1/\Delta\sigma)}{(1/\Delta\sigma)} = \text{c.v.}(\mu)$$

where s.d. = standard deviation

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* All statistical methods are according to Kalbfleisch (1974).

+ Details of the equation and a full list of the symbols can be found in Chapter 3.

This is derived by calculating the absolute value of the first derivative of $\frac{1}{\Delta\sigma}$:

$$\delta\left(\frac{1}{\Delta\sigma}\right) = \left|\frac{-\delta(\Delta\sigma)}{(\Delta\sigma)^2}\right|$$

Therefore $$\frac{\delta\left(\frac{1}{\Delta\sigma}\right)}{\left(\frac{1}{\Delta\sigma}\right)} = \frac{\delta(\Delta\sigma)}{\Delta\sigma} = \frac{\delta(\mu)}{\mu} = \text{c.v.}(\mu)$$

since

$$\sigma = \text{constant} \times \mu$$

Hence, the uncertainty in $\left(\frac{1}{\Delta\sigma}\right)$ is given by the uncertainty in (μ), provided both uncertainties are expressed as fractions of the appropriate mean.

In situations like the present one, where reciprocal values are plotted against each other, linear regression is valid only if the variances of the data points are not significantly different from each other. To test this, the variance of the first $\frac{1}{\Delta\sigma}$ data point on each regression line was compared to the variances of all the other $\frac{1}{\Delta\sigma}$ data points on the same line point by point. Pairs of variances were tested using the F-distribution and a confidence interval of 95%. It was determined that the variances for the data points along each regression line were not significantly different from each other. The regression lines in Fig. 23 are therefore valid.

Statistical comparisons for the binding site densities (N) and binding free energy (ΔG) between different platelet forms and cations were achieved by comparing the regression lines concerned using analysis of variance tables. They are shown below. The number of degrees of freedom is based on the number of points on each line illustrated in the figures, each point representing the average of at least 100 individual mobility determinations. Significance between the two intercepts reflects the significance between the binding site densities while that between the slopes reflects the significance between the ΔG.

Ca^{++} binding; comparing discocyte vs echinocyte :-

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	19	3.33×10^{-5}	-
Intercept	1	1.64×10^{-6}	6.14
Slope	1	4.77×10^{-6}	17.86
Residual	16	4.27×10^{-6}	-

Intercept $0.025 < p < 0.05$; significant difference.
Slope $0.001 < p < 0.0005$; significant difference.

Mg^{++} binding; comparing discocyte vs echinocyte :-

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	19	1.63×10^{-5}	-
Intercept	1	1.32×10^{-6}	14.23
Slope	1	5.18×10^{-6}	55.69
Residual	16	7.44×10^{-7}	-

Intercept $0.005 < p < 0.01$; significant difference.
Slope $p < 0.0005$; significant difference.

Discocytes; comparing Ca^{++} vs Mg^{++} binding :-

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	15	2.46×10^{-5}	-
Intercept	1	5.33×10^{-8}	0.1525
Slope	1	6.78×10^{-8}	0.1941
Residual	12	4.19×10^{-6}	-

Intercept $0.50 < p < 0.75$; no significant difference.
Slope $0.50 < p < 0.75$; no significant difference.

Echinocyte; comparing Ca^{++} vs Mg^{++} binding:—

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	15	2.20×10^{-5}	-
Intercept	1	2.91×10^{-5}	0.04
Slope	1	7.36×10^{-8}	1.07
Residual	12	6.88×10^{-8}	-

Intercept $0.50 < p < 0.75$; no significant difference
Slope $0.25 < p < 0.50$; no significant difference.

Ca^{++} binding; comparing discocyte vs spherocyte:—

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	15	4.80×10^{-4}	-
Intercept	1	3.71×10^{-4}	345.57
Slope	1	4.48×10^{-5}	41.68
Residual	12	1.29×10^{-5}	-

Intercept $p < 0.005$; significant difference
Slope $0.01 < p < 0.025$; significant difference

Ca^{++} binding; comparing echinocyte vs spherocyte:—

Source of Variance	Degree of Freedom	Sum of Squares	Mean Square Ratio
Total	15	5.21×10^{-4}	-
Intercept	1	4.15×10^{-4}	510.96
Slope	1	4.92×10^{-5}	60.58
Residual	12	9.75×10^{-6}	-

Intercept $p < 0.005$; significant difference
Slope $0.005 < p < 0.01$; significant difference.