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HIGH RESOLUTION TWO-DIMENSIONAL GEL ELECTROPHORESIS OF THE PROTEINS AND GLYCOPROTEINS OF HUMAN BLOOD PLATELETS AND PLATELET MEMBRANES

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Summary

The proteins and glycoproteins of human blood platelets and platelet membranes in both the reduced and the unreduced states have been analysed by isoelectric focusing and sodium dodecyl sulphate-discontinuous polyacrylamide gel electrophoresis in a two-dimensional technique. Gels which had been stained with periodic acid-Schiff's reagent could be counter-stained with Coomassie Brilliant Blue, simplifying the recognition of components which stain with both reagents. The major glycoproteins and some of the proteins have been identified and the characteristics of the membrane and of the whole platelet components established in this system.

Introduction

Platelets play a major role in haemostasis and may well have other important physiological functions [1]. Thus a knowledge of their constituents and simple, efficient methods of analysis of these constituents which can be applied to small samples of platelets with the minimum of preparation would be very useful in studying the relationship between differences in these components and normality and abnormality of function.

It is now well established that most of the surface exposed platelet proteins are glycosylated [2—4]. Investigation of the expression and exposure of glycoproteins on the platelet membrane surface has been carried out using a variety

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of surface-labeling techniques in conjunction with either sodium dodecyl sulphate-phosphate or Laemmli-polyacrylamide gel electrophoresis [2—4].

Recently it has been shown [5] that the platelet membrane glycoproteins are more numerous and complex than the three previously described. This complexity has been confirmed using a variety of techniques and up to 10 glycoproteins have been described [2,4,6,7].

The resolution of proteins and glycoproteins in the one-dimensional gel electrophoresis systems previously used is fairly limited. Two-dimensional unreduced-reduced sodium dodecyl sulphate-polyacrylamide gel electrophoresis gave an improved resolution of the glycoproteins and the existence of peptide chains linked by intermolecular disulphide bonds could be shown [2]. However, this method only resolves those components containing disulphide bonds (predominantly membrane glycoproteins) and by its nature cannot analyse disulphide bond containing proteins in their natural unreduced state, which would be desirable for further investigation of these components.

A method which would allow the clear separation of both the glycoproteins and the proteins in both the reduced and unreduced states would have immediate applications to the study of platelets and their membranes. It would help the study of glycoprotein complexity and purification and also greatly facilitate the study of the non-glycosylated proteins, of which relatively little is known. In particular it should be able to define the characteristics of normal platelets and their membranes and to show if non-glycosylated and/or non-surface exposed proteins are affected in functionally defective platelets.

O'Farrell [8] has described a two-dimensional method for the analysis of proteins which is capable of a remarkable degree of resolution and Ames and Nikaido [9] have adapted this technique for application to membrane proteins. This paper deals with the application of this high resolution, two-dimensional technique, with minor modifications, to the analysis of human platelets and platelet membranes in both the reduced and unreduced states.

Materials and Methods

Human blood platelets. These were isolated within 20 h after collection from citrated blood collected for the Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross in Berne [10]. The buffy coats were syphoned into a buffered glucose solution to give platelet-rich plasma containing about 20 mM glucose, 12 mM phosphate buffer, pH 6.8, and about $4 \cdot 10^9$ platelets per ml [11].

Platelet membranes. These were prepared in batches from the platelet concentrates from 60—100 units of blood. The platelet-rich plasma was made 5 mM in ethylenediamine tetraacetic acid disodium salt (EDTA) and then centrifuged for 20 min at $3000 \times g$. The platelet pellet was washed twice with 129 mM sodium citrate, 30 mM glucose, 120 mM NaCl, 5 mM EDTA buffer, and once with 10 mM Tris, 30 mM glucose, 154 mM NaCl, 5 mM EDTA buffer, pH 7.4, and was finally resuspended in three times the pellet volume of this last buffer. The platelet suspension was cooled in ice to 4° C and sonicated with a B-30 Sonifier (Branson Sonic Power Co., CT, U.S.A.) for 2 min at setting 7, 50% duty cycle, pulsed mode, 1/2 inch horn tip. The membranes were isolated

by centrifugation onto a 30% sucrose cushion or by differential centrifugation.

Washed whole platelets. These were removed from the pool used for the preparation of membranes before sonication. In the case of platelets from individuals, 70—80 ml of blood was drawn and mixed with one part 3.8% sodium citrate solution per nine parts blood. The citrated blood was centrifuged at 180 × *g* for 10 min to give platelet-rich plasma (20—30 ml). The platelet-rich plasma was removed, made 5 mM in EDTA and centrifuged at 3000 × *g* for 20 min to give a platelet pellet which was washed as for the preparation of membranes. After the final wash the platelets were resuspended at about 10^9/ml in the final wash buffer and made 40 mM final concentration in phenylmethylsulphonyl fluoride. Platelets from both sources were stored frozen at −70° C in aliquots of 300 µl.

Isoelectric focusing. Preparation of samples: Unreduced samples: 600 µg of protein, 10 µl 10% (w/v) sodium dodecyl sulphate (SDS), 25 µl 40 mM *N*-ethylmaleimide, water to 100 µl. Reduced samples: 600 µg of protein, 10 µl 10% (w/v) sodium dodecyl sulphate, 25 µl 8% (w/v) dithiothreitol, water to 100 µl. The sample tubes were capped with glass spheres, heated to 100° C for 2 min, cooled and to each was added 100 µl of a solution containing 9.5 M urea, 2% (v/v) ampholytes and 8% (w/v) Nonidet P-40 detergent. The samples were mixed by vortexing and loaded on the gels.

Gels were prepared in 130 × 2.8 mm tubes according to O'Farrell [8]. The quantity given (10 ml) was enough to make 12 gels, 11.5 cm long, leaving an adequate space for the sample. Samples were overlayered with 20 µl of a solution containing 4% (w/v) urea, 2% (v/v) ampholytes, 4% (w/v) Nonidet P-40 and then with the cathode electrolyte. Focusing was carried out at 260 V for 18 h with 20 mM NaOH as cathode electrolyte and 10 mM H_3PO_4 as anode electrolyte. The ampholytes used were Servalyt AG 2-11 (Serva, Heidelberg). The pH gradient obtained was measured by cutting the isoelectric focusing gel into 5-mm sections which were placed in tubes containing 1 ml of degassed, distilled water. The tubes were stoppered and left for 30 min with occasional shaking and the pH was then measured with a pH meter. Under the conditions described a reproducible, linear gradient was obtained from pH 3.5 to 8.0.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The first dimension gels were stored frozen at −70° C in equilibration buffer containing 2.5 mM Tris-HCl, pH 6.8, 10% (w/v) glycerol, and 1% (w/v) sodium dodecyl sulphate. The unreduced gels were equilibrated for 0.5—1 h in this buffer with one change. The reduced gels were equilibrated in this buffer made 2% (w/v) in dithiothreitol for 0.5 h and then without dithiothreitol for a further 0.5 h. The discontinuous sodium dodecyl sulphate-Tris-glycine system of Laemmli [12] was used for the second dimension in the apparatus according to Studier [13]. A 2 cm space was left at the top of the separating gel. After a 1 cm stacking gel (generally 4%) was cast, excess buffer was removed from the isoelectric focusing gel, which was then laid on top of the stacking gel and a further stacking gel cast on top of it. Care was taken to remove trapped bubbles. With reduced gels it was necessary to double the $(NH_4)_2S_2O_8$ content in this second gel because of inhibition of polymerization by traces of dithiothreitol remaining in the gel. Gels were run at 80 mA constant current until the front

(visible as a refractive index difference) reached the bottom of the gel. Running time was about 2.5 h.

Staining. Gels were fixed overnight in 25% isopropanol, 10% acetic acid and 65% water, which also served to remove the bulk of the ampholytes. Gels were washed for 1 h in running water, soaked in 1% $NaIO_4$, 3% acetic acid for 1 h and then washed for 2 h in running water and twice for 0.5 h with degassed, distilled water. The gels were then stained for 2 h with Schiff's reagent prepared from Basic Fuchsin (Merck, Darmstadt) by the method of Segrest and Jackson [14]. Excess Schiff's reagent was removed by soaking in 0.5% sodium metabisulphite (200 ml) with 4—5 changes over 2—3 days. The gels were then soaked for two times 3 h in 7% acetic acid and could then either be counter-stained with Coomassie Blue or dried under vacuum onto filter paper for storage.

Gels stained with Coomassie Blue were soaked in 0.025% Coomassie Brilliant Blue R250 (Merck, Darmstadt) in 25% isopropanol, 10% acetic acid and 65% water for 3 h and were then destained with 25% isopropanol, 10% acetic acid and 65% water for 1 h, 10% isopropanol, 10% acetic acid and 80% water for 2 h and then 10% acetic acid with changes until the gel background was clear.

Purified platelet and plasma proteins. Platelet actin and tubulin and rabbit muscle myosin were the gift of Dr. R. Käser-Glanzmann, Theodor Kocher Institute. Human albumin and fibrinogen were from the Swiss Red Cross Central Laboratory, Berne.

Results

Proteins and glycoproteins from platelet membranes and from whole platelets were analysed by two-dimensional gel electrophoresis, in both the unreduced and the reduced state, with isoelectric focusing as first dimension and discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis as the second dimension. Optimal resolution of the major glycoproteins and proteins was obtained using 7% second dimension Laemmli gels.

Fig. 1a shows a two-dimensional gel run on unreduced platelet membranes and stained with Coomassie Blue. The first dimension is isoelectric focusing between pH 3.5 and 8 and the second is a 7% Laemmli gel. Fig. 1b shows a drawing of the same gel with the components which stain for carbohydrate indicated and the identity of known components shown. Figs. 2a and 2b show the equivalent gel run on reduced platelet membranes and stained with Coomassie Blue.

The major glycoproteins were identified by their properties as defined previously in one-dimensional systems or by comparison with samples purified or partially purified by lectin affinity chromatography [5]. Other proteins were identified by comparison with purified platelet or plasma proteins. In both gels the major spots staining for protein are glycoproteins IIb and IIIa and actin. The major spots staining for carbohydrate can be identified as glycoproteins Ia, IIb, IIIa and IIIb, several other minor glycoproteins are present but a positive identification with the nomenclature established using one-dimensional SDS-phosphate gels has not yet been possible. With regard to their behaviour in the isoelectric focusing dimension the glycoproteins appear to fall into two groups.

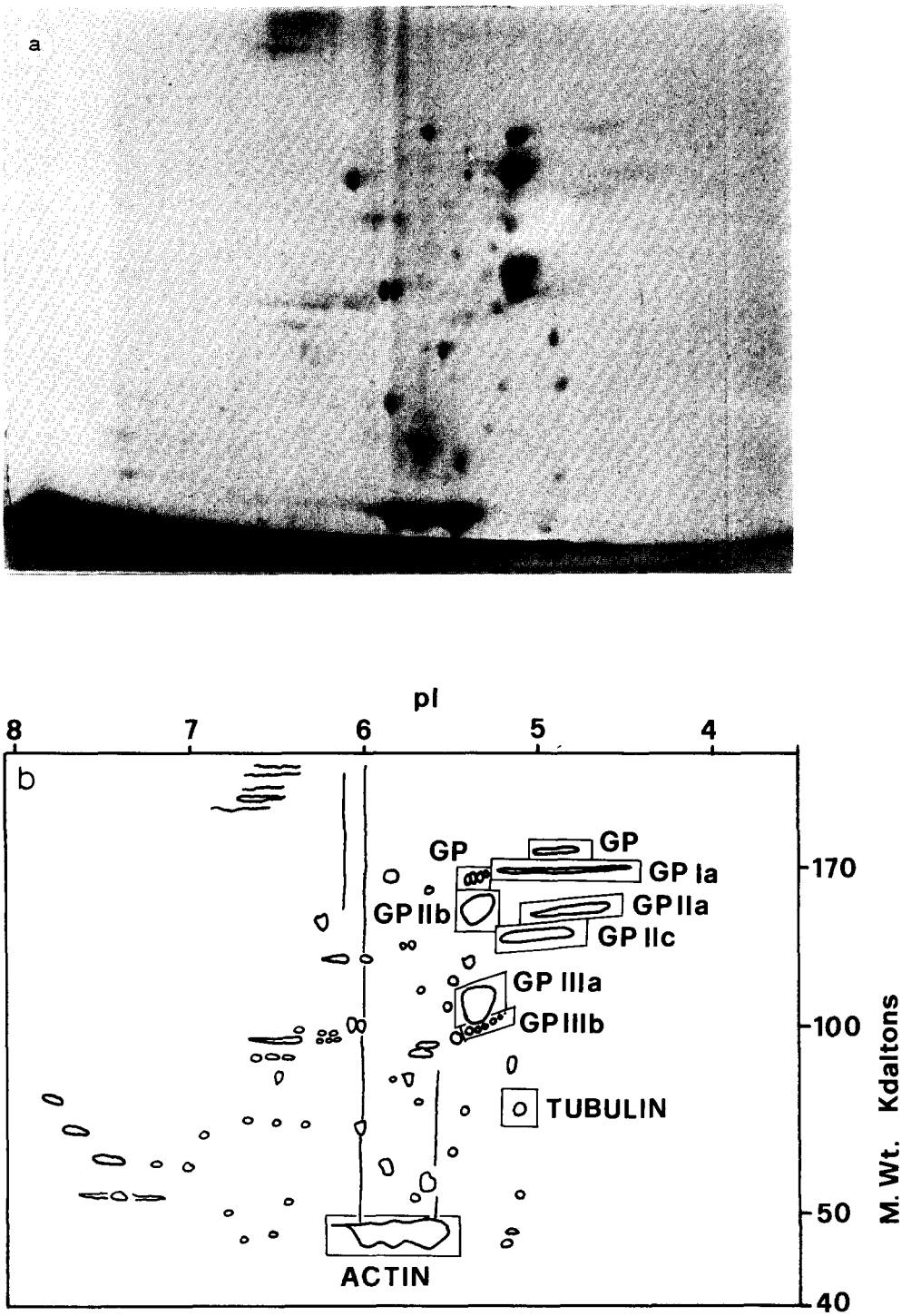
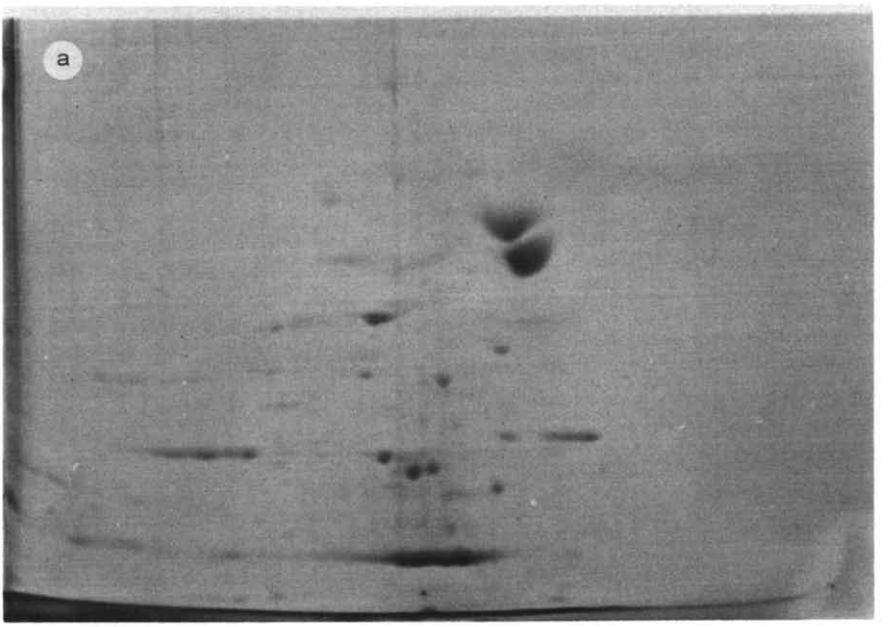


Fig. 1. (a) Two-dimensional gel of human platelet membranes, unreduced. (b) Diagram drawn from gel shown in a, labelled to show the position of identified proteins and glycoproteins (enclosed in frames).



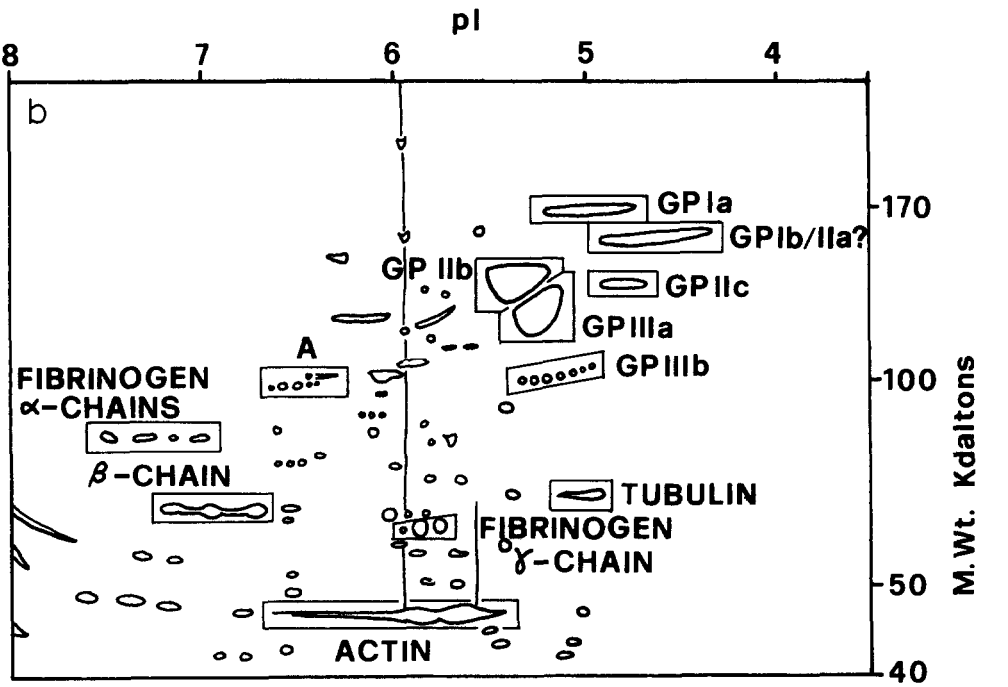


Fig. 2. (a) Two-dimensional gel of human platelet membranes, reduced. (b) Diagram drawn from gel shown in a, labelled to show the position of identified proteins and glycoproteins and other components mentioned in the text (enclosed in frames).

The first group, consisting of glycoproteins IIb, IIIa plus a glycoprotein of higher molecular weight than glycoprotein IIb unreduced, focus as fairly compact spots at a pH of 5.4—5.6. The second group, consisting of glycoproteins Ia, IIIb and the tentatively identified glycoprotein Ib and minor components of molecular weights between IIb and IIIa unreduced, focus over a wider and more acid pH range and appear on the gel as horizontal streaks resolvable sometimes into a series of spots. With glycoprotein IIIb these spots move to a higher molecular weight towards the more acid pH. In the unreduced preparation a spot was seen at the same molecular weight as glycoprotein Ia but at the same pH as IIb and IIIa. This spot stained less strongly than Ia for carbohydrate but more strongly for protein. On reduction it was no longer visible and presumably moved in the same place as IIb on reduction. Two-dimensional electrophoresis of lectin binding and flow-through fractions [5] under the same conditions revealed a glycoprotein spot in the same position as this in the flow-through from wheat germ agglutinin and *Lens culinaris* lectin columns but not present in the lectin-bound fractions. This implies that this glycoprotein is therefore different from Ia, Ib and IIb. There is a minor spot on unreduced gels in the same place as IIb on reduced gels. As its concentration is variable it may well be that it represents a fraction of the IIb molecules which are reduced or where the disulphide bonds have been cleaved in some other way.

When the counter-staining technique was used, staining first with the periodic acid Schiff's reagent method and then with Coomassie Blue, the spots of the first group of glycoproteins, mentioned above, stained predominantly blue, whereas those of the second group remained an overall reddish colour implying that little protein stain had been absorbed.

The proteins which do not stain with periodic acid-Schiff's reagent are much more numerous. The reduced pattern contains more than the unreduced but this may be due to better solubilization under reducing conditions. Actin and tubulin can readily be identified by comparison with pure samples. Problems were encountered with precipitation and perhaps polymerization of the actin in the isoelectric focusing dimension. This caused streaking in the second dimension gel. Platelet actin has been reported to consist of only the β and γ forms in the ratio 5 : 1 [15]. The quantities of membrane protein used here on gels to show minor components and weakly staining glycoproteins was such that some degree of overloading of actin occurred. Heterogeneity in the isoelectric point of the actin induced by heating the samples to 100°C [16] or simply by storage of samples [17] is exaggerated by this overloading and could be greatly reduced by using platelet samples at levels where overloading of actin did not occur (less than 100 μg of protein per gel).

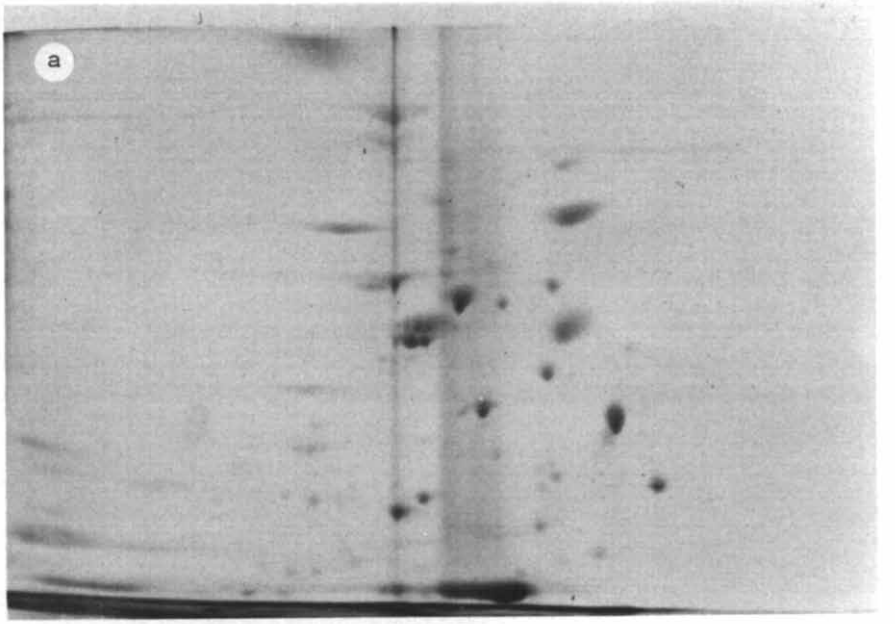
Many of the proteins do not appear to change on reduction and can easily be seen in both gels. There are, however, some exceptions such as a spot staining for protein at a higher molecular weight and slightly more basic pI than tubulin which disappears from this position on reduction. In the more basic region there is a peculiar group of spots (marked A) which appears in the reduced preparation and does not appear in this typical form in the unreduced. They consist of a fan shaped group headed by a single spot on the alkaline side and becoming smaller and spreading over a wider molecular weight range towards the acid side. With Coomassie Brilliant Blue this group stains a

predominantly reddish colour. It is known that Coomassie Brilliant Blue may contain minor red components and that certain proteins stain a definite red colour in this dye. A typical example of this phenomenon is seen with the wheat protein, gliadin, which stains a definite red colour with Coomassie Brilliant Blue (Windemann, H., personal communication). It is known that gliadin has unusual peptide sequences containing large amounts of glutamic acid [18]. It may therefore be the binding of Coomassie Brilliant Blue to peptides rich in acidic amino acids which produces the colour change. Recently, this phenomenon, termed metachromasy, has been reported to occur with collagen polypeptides from peripheral nerves [19].

Whole platelets were also examined by this technique using identical quantities and methods to those used for membranes. Fig. 3a shows a two-dimensional gel of unreduced whole platelets and stained with Coomassie Brilliant Blue. Fig. 3b shows a drawing of the same gel with the components which stain for carbohydrate indicated and the identity of known components shown. Figs. 4a and 4b show the equivalent gel run on reduced whole platelets.

As expected the whole platelets contain many more components than the isolated membranes alone and the spots due to the membrane components are mostly relatively fainter. Nevertheless it is possible to recognise most of the spots seen in the gels run on membranes as present in the gels run on whole platelets. In particular the major glycoproteins are easily distinguished as are also actin and tubulin. The latter is present in higher concentration than in membranes alone. Although it is thought that the internal membranes such as those of the granules also contain glycoproteins [20], these are either identical to those of the plasma membrane or only present in small quantities as only slight differences were observed in the gels stained with periodic-acid-Schiff's reagent of whole platelets compared with membranes. This is in accord with the results of George [6] who found similar glycoprotein patterns with isolated membranes and whole platelets and showed, using a surface-label, that the glycoproteins in the isolated membranes contained 2—3 times more label than those in whole platelets, indicating that the majority of platelet glycoproteins are derived from internal structures. The most noticeable difference was the presence, in the gels run on whole platelets, of a glycoprotein which appears as a long streak between pH 6 and 7 and has a molecular weight of about 180 000 unreduced and 150 000 reduced and which stained very weakly with Coomassie Blue. The so-called thrombin-sensitive protein, which is a granule glycoprotein liberated during the platelet release reaction [21,22] was not observed, either because it did not enter the gel or because it focused outside the pH range used although it could be shown to be present in the samples when SDS gel electrophoresis alone was used. It is possible that the glycoprotein seen only in whole platelets is a protease-modified fragment of thrombin-sensitive protein. However, it was not seen when supernatants from thrombin-treated platelets which contained thrombin-sensitive protein were examined by two-dimensional gel electrophoresis.

Albumin and fibrinogen could also be identified in gels, stained with Coomassie Blue, of reduced samples of both whole platelets and membranes by comparison with purified plasma components. They therefore probably represent, at least partly, plasma proteins bound to the membrane. Platelet fibrinogen



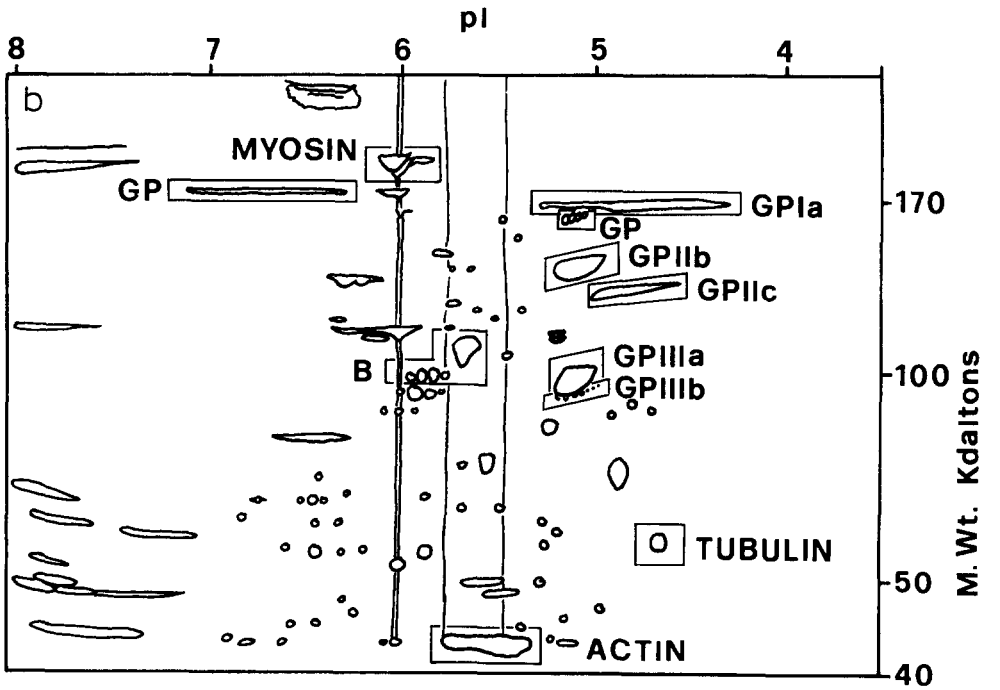


Fig. 3. (a) Two-dimensional gel of whole human platelets, unredured. (b) Diagram drawn from gel shown in a, labelled to show the position of identified proteins and glycoproteins and other components mentioned in the text (enclosed in frames)

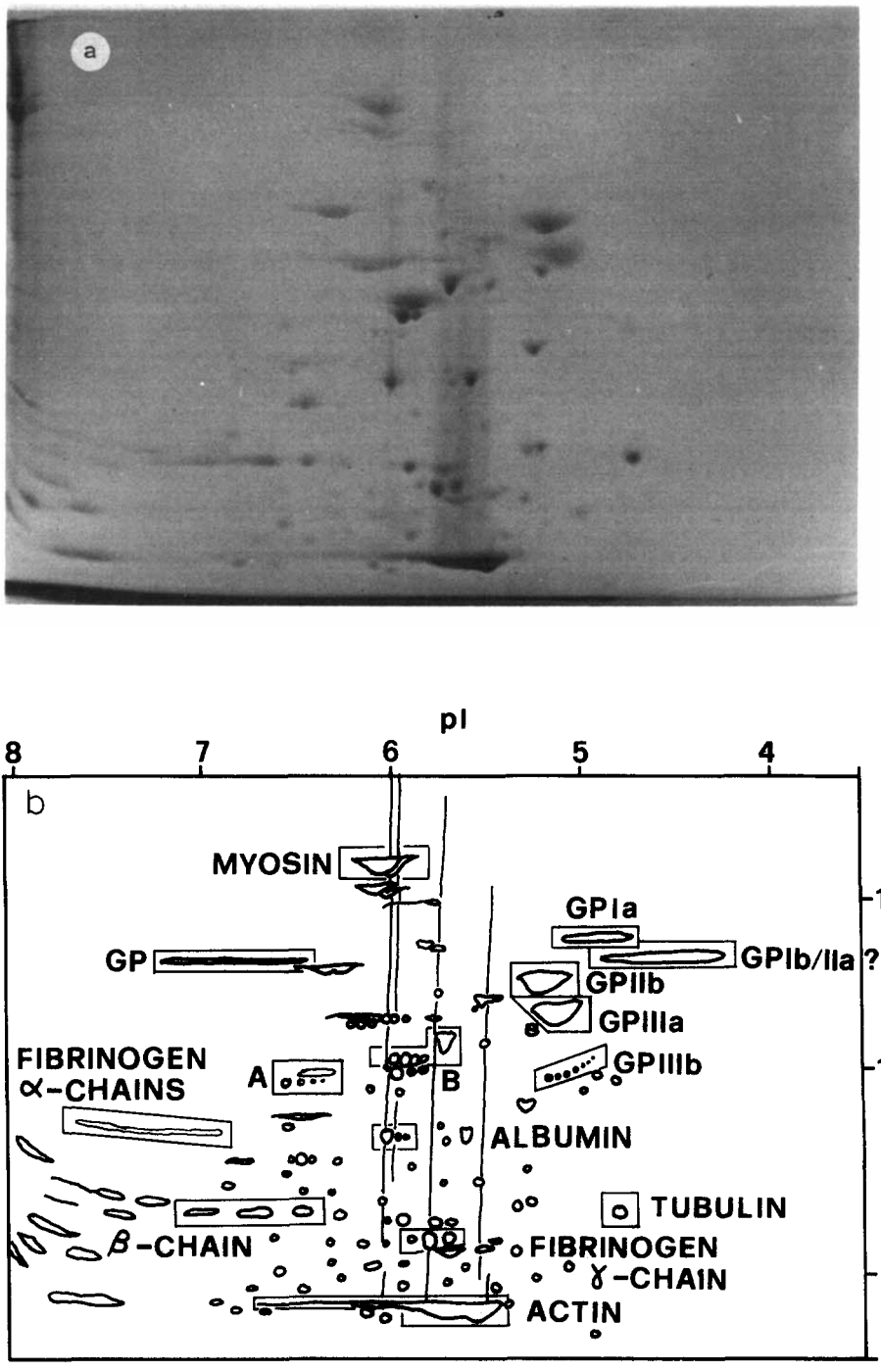


Fig. 4. (a) Two-dimensional gel of whole human platelets, reduced. (b) Diagram drawn from gel shown in a, labelled to show the position of identified proteins and glycoproteins and other components mentioned in the text (enclosed in frames).

has been reported to differ from plasma fibrinogen [23], however no differences were observed in this study. The relatively large amount of fibrinogen found with whole platelets compared to membranes is in accordance with the finding that α-granules contain stored fibrinogen [24,25]. The β- and γ-chains of fibrinogen can be clearly seen in Figs. 2 and 4, the α-chains are somewhat fainter.

Whole platelets isolated from blood drawn from individual normal donors were also examined and compared with the pool platelets. Although the overall pattern of glycoproteins and proteins was extremely reproducible there were quantitative differences found between some individuals. The whole platelet patterns shown in Figs. 3 and 4 are from an individual donor and contain the group of spots marked B which is similar in both reduced and unreduced preparations. This group was either absent or present at very low levels in the preparations from platelets from other donors but was present in a reduced concentration in the preparations made from the pool platelets. This difference is reproducible and not dependent on the method of preparation of the platelets. Additional examinations of fresh preparations from individual donors showed the same difference. The proteins giving rise to these spots appear to be inside the platelet and not membrane components or membrane associated, since they are not present in the membranes prepared from pool platelets, although present when whole pool platelets are studied. The possibility exists that this heterogeneity is a reflection of differences in proteolytic activity during platelet storage. However, no differences were seen for individual donors when freshly prepared or frozen platelets were examined. When platelets from positive and negative donors were mixed 1 : 1 and then examined, the group of spots B were found in half the amount present in the positive donor. This tends to exclude heterogeneous proteolytic activity as the reason for the heterogeneity found. Further work is in progress on the variability in these proteins between individuals to see if any clinical differences exist.

Discussion

The development of highly sensitive gel electrophoretic methods for the analysis of proteins and glycoproteins has opened up new possibilities for the definition of genetic variation and the identification of the molecular changes responsible for defective function. In this paper these high resolution two-dimensional gel electrophoresis methods have been applied to the analysis of the human blood platelet and in particular the platelet membrane, in order to define the distribution of the proteins and glycoproteins. Detection was carried out by staining with the periodic acid-Schiff's reagent method followed by Coomassie Brilliant Blue. The periodic acid-Schiff's reagent method is not particularly sensitive and detects predominantly sialic acid-containing components. Despite this disadvantage it is possible to recognise most of the known glycoproteins in both membranes and intact platelets. The periodic acid-Schiff's reagent method has the advantage that it can be used after separation of components on gels has occurred. The alternative, more sensitive techniques of radiolabelling metabolically followed by autoradiography cannot be applied to human platelets and radiolabelling of isolated platelets or membranes using

chemical methods may affect the pI of the proteins. This is particularly critical in the case of the glycoproteins where selective marking involves either oxidation with periodate followed by reduction with labelled borohydride or treatment with neuraminidase, followed by oxidation of exposed galactose residues with galactose oxidase, which are then reduced with labelled borohydride. These procedures either remove sialic acid residues or change the charge on these residues, in both cases having an effect on the overall charge of the glycoprotein, which is dependent on its sialic acid content.

The separation achieved for the proteins of the blood platelet is much superior to that previously obtained with other methods. However, streaking caused by relative overloading with actin is a problem. This overloading is necessary in order that the less abundant proteins and glycoproteins can be detected.

Recently Anderson and Anderson [26] have applied this technique to the analysis of human plasma proteins, with the intention of developing a screening method for mutagenesis in humans. They found considerable heterogeneity in many components in the isoelectric focusing dimension and put this down to variation in the carbohydrate part of these molecules. Although the same phenomenon is observed with several of the major platelet glycoproteins, it seems less common than with the plasma proteins. This is probably due to the fact that fewer platelet components are glycosylated since solubility in an aqueous medium is only required for the external membrane components or for the granule components which are released on platelet activation. Identification of individual components is considerably easier in the case of plasma proteins since most are well known. In the case of platelets, relatively few components are defined as yet. Gels with an acrylamide concentration of 7% were chosen for the second dimension rather than wide range gradient gels in order to maximise the resolution in the region 40 000—200 000 daltons where the bulk of the major components occurs. The gels are simpler to prepare and in general, more reproducible between batches of gels. There are however, important platelet components which lie outside this region, for example β-thromboglobulin, and the use of high concentration gradient gels to investigate components between 40 000 and 10 000 daltons is now being examined.

The examination of solubilized, whole platelets offers advantages. For large-scale screening purposes, the reduced state would probably be adequate since reproducibility and artifacts are not problems. Despite several precautions, such as the use of *N*-ethylmaleimide to block free thiol groups and prevent cross-linking of proteins by disulphide bridge formation, solubilization was in general less efficient with unreduced samples. Typically, spots such as actin, present in gels of both unreduced and reduced preparations were smaller in gels run on unreduced samples and overall fewer spots were present. Nevertheless, considerable information can still be obtained, in particular where special cases are being examined or the disulphide linkages of individual components investigated. Then it is useful to run gels also on unreduced samples. This method also opens up the possibility of separating components in the unreduced state for further examination, since overlapping of individual components does not seem to be a serious problem.

The excellent resolution of the whole platelet proteins obtained here shows

that for many purposes it is no longer necessary to isolate membranes for analysis. Although minor glycoproteins are less distinct, the other components are easily visible. Thus much smaller samples of blood are required, which is an important consideration when patients, often children, are involved. Also the use of whole platelets simplifies comparisons between laboratories, as the washing required is easier to standardize than the preparation of membranes. Where membranes are used, this method of analysis allows some measure of comparison between preparations and gives some idea of the degree of contamination of membrane preparations with cytosol and organelle components.

The observation of some differences between the patterns obtained with preparations from normal, individual donors is being investigated with larger numbers of donors. Whether or not this proves to be a general phenomenon, it is certainly the case with several normal individuals examined. It is possible that there is a very wide quantitative range in these components without any apparent effect on platelet function. Anderson and Anderson [26] have shown that individuals may exhibit variants of common plasma proteins and a similar phenomenon could account for at least part of the differences.

The possibility that high resolution two-dimensional gel electrophoresis of human platelets may be applicable to screening for patients with a high risk of thrombosis or other platelet-related diseases awaits the acquisition of sufficient data on variations in platelet components observable by this technique and their association with clinical symptoms. Gel electrophoresis has proved an excellent diagnostic tool in some platelet disorders such as Bernard-Soulier syndrome and Glanzmann's thrombasthenia where there are gross changes in the glycoprotein patterns. Previously these methods were not applicable to examination of variation in non-glycosylated proteins because the degree of overlapping of bands was too great. With the development of high resolution techniques it should be possible to establish if more subtle changes in platelet components are also responsible for defects in platelet function.

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