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THE IMMUNOLOGICAL HETEROGENEITY OF THE PROTEINS EXTRACTED FROM *BOS TAURUS* ERYTHROCYTE MEMBRANES BY EDTA

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SUMMARY

The heterogeneity of the EDTA extract of erythrocyte membranes and the protein fractions prepared from this extract by polyacrylamide-gel electrophoresis has been demonstrated by immunological analysis. Antisera against the protein fractions and the whole extract have been raised in rabbits and several common determinants detected in all fractions.

INTRODUCTION

About 40 % of the protein of the mammalian erythrocyte membrane is extracted by dilute solutions of EDTA. Earlier work [1] concluded that the extract contained one protein, "spectrin", but there is now considerable evidence for there being several protein species in the extract. In terms of sodium dodecylsulphate–polyacrylamide-gel electrophoresis it contains major "polypeptides" with molecular weights in the region of 200 000: Peptides 1, 250 000, 2, 200 000 and 2.1, 190 000 in the terminology of Fairbanks et al. [2], and a component around 40 000 daltons (Peptide 5, 45 000). In the 1973 "Protides of the Biological Fluids" conference we reported that N-terminal analysis showed further heterogeneity in both the large and small peptide fractions of the extract from ox (*Bos taurus*) cells and also presented evidence showing that the large polypeptides were aggregates of the smaller ones [3, 4]. At the same meeting Bøg-Hansen and Bjerrum [5] reported that "spectrin" was antigenically heterogeneous with up to 9 different determinants. The N-terminal heterogeneity has subsequently been confirmed by others [6, 7] and ourselves [8] on Peptides 1, 2 and 2.1 as prepared by electrophoresis with dodecylsulphate.

The work of Bøg-Hansen and Bjerrum encouraged us to raise rabbit antisera against the fractions of the ox EDTA extract we prepared by gel electrophoresis in a Tris buffer, i.e. in the absence of any detergent [4, 8]. The mixture is subdivided in this buffer into Fractions α, β, γ, ε and ω (Fig. 1); when these are monitored by electrophoresis in the presence of dodecylsulphate, α and β both contain Peptides 1 and 2, γ consists of 2 or 2.1 (the very small mobility difference between these two bands is insufficient to permit unequivocal identification) and ω contains the 40 000 peptides

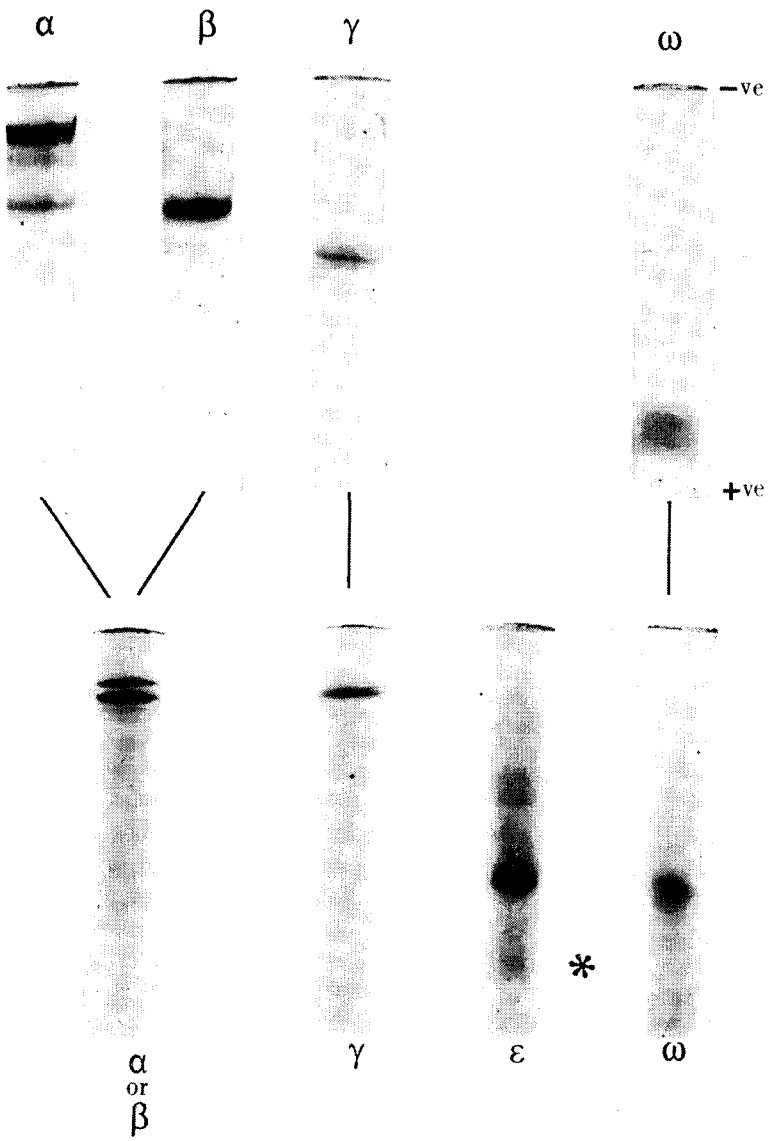

Fig. 1. The electrophoresis of Fractions α, β, γ, ε and ω. α is in fact an aggregate of β and some dissociation into β is apparent in the Tris-polyacrylamide-gel electrophoresis gel of α. Top series, Tris–polyacrylamide-gel electrophoresis; bottom series, dodecylsulphate–polyacrylamide-gel electrophoresis *indicates the trace of haemoglobin present in the extract.

of the dodecylsulphate gels. In the Tris gel there is some protein (Fraction ε) distributed between Fractions γ and ω which consists predominantly of Peptide 5 but is contaminated with some proteins of higher molecular weight (circa 100 000). We here report that each of these fractions is antigenically complex and even more significantly the same antigens are found in all of them.

METHODS

Preparation of extract

Ox erythrocyte ghosts were prepared as previously described [9] and extracted at room temperature for 2 h with 4 vol. of 0.5 mM EDTA adjusted to pH 7.5 with NaOH [8]. The insoluble material was removed by centrifugation for 1 h at 65000 × *g* [4].

Electrophoretic methods

Analytical electrophoresis was carried out using either a Tris–glycine–EDTA buffer system [10] or phosphate buffer system containing dodecylsulphate [11].

Preparative electrophoresis was performed by a vertical slab method [4, 8] using the Tris–glycine–EDTA buffer system or the dodecylsulphate system. The slab was prerun for 6 h at 7 V · cm^{-1} in the case of Tris buffer or for 18 h at 4 V · cm^{-1} in the detergent system, and then 5 ml protein solution (5 mg/ml) were applied and electrophoresed for either 18 h at 6 V · cm^{-1} (Tris) or 72 h at 4 V · cm^{-1} (dodecylsulphate). After electrophoresis the slab was cut up into 0.5 cm slices, the slices homogenised, and the protein extracted as previously described [4, 8].

Samples were prepared for electrophoresis in the dodecylsulphate system by addition of dithioerythreitol (final concn 20 mM) and sodium dodecylsulphate (to 5 %) heated at 100 °C for 3 min and finally made 8 M with respect to urea.

Preparation of antisera

Antisera against the whole EDTA extract and protein fractions α, β, γ, ε and ω were prepared by injecting rabbits intramuscularly with the protein containing acrylamide homogenates emulsified in Freund's complete adjuvant followed by a subcutaneous injection of Pertussis vaccine (Burroughs Wellcome). This procedure was repeated 5 times at three weekly intervals and a large volume of serum was collected 4 weeks after the last immunisation. Two rabbits were injected with each preparation and none failed to elicit a response.

Immunological methods

Two gel precipitation techniques were used to detect the presence of antibodies in each rabbit serum. (a) Immunoprecipitation by double diffusion in one dimension was performed by the modification of Preer [12] of the Oakley–Fulthorpe technique [13] using a 6 mm × 2.7 mm gel of 1 % agar in isotonic phosphate buffered saline, (pH 7.4). (b) Antigenic identity between the precipitation lines produced by one antiserum reacting with all five protein fractions was established using the Crowle refinement [14] of the two-dimensional double diffusion technique of Ouchterlony.

Each antiserum was tested against the whole EDTA extract and all 5 protein fractions derived from it. Several dilutions of the antigen solutions had to be used to obtain a balanced system in which all the precipitin lines were stationary and remained on the gel. Precipitin lines on both types of gel were drawn and photographed with dark ground illumination.

RESULTS

The antigenic heterogeneity and cross-reactivity of all the protein fractions prepared from the EDTA extract is summarized in Table I. Apart from one exception (antiserum against Fraction γ), each antiserum gave a maximum of five groups of precipitin lines when tested against all the protein fractions by the modified Oakley–Fulthorpe test. Some of the groups contained several bands (Fig. 2). Similarly all the protein fractions except γ, gave a maximum of 5 groups of precipitin lines when tested against all the antisera. When protein Fraction γ and its antiserum were tested against the other antisera and protein fractions, respectively, a maximum of 4 groups of precipitin lines was observed. It is interesting to note that the maximum number of lines was not necessarily observed when an antiserum was tested against the immuniz-

TABLE I

SUMMARY OF THE ANTIGEN–ANTIBODY REACTIONS AS OBSERVED USING THE MODIFIED OAKLEY–FULTHORPE TECHNIQUE ON THE PROTEIN FRACTIONS OF THE EDTA EXTRACT AND THEIR ANTISERA

The figures indicate the number of precipitin-line groups observed with each antibody–antigen combination. SDS, sodium dodecylsulfate.

Rabbit antiserum No.	Immunizing protein fraction	Test protein fraction								
		Tris fractions					Whole extract	SDS fractions		
		α	β	γ'	ε	ω		1	2	2.1
243	α	5	5	4	3	5	5	5	5	5
244	β	5	5	3	5	2	4	4	1	4
245	γ'	4	4	3	4	4	4	4	1	3
246	ε	4	5	3	4	2	2	4	3	2
247	ω	5	3	2	3	5	2	2	3	3
AA4	Whole extract	5	3	2	4	2	5	4	3	4

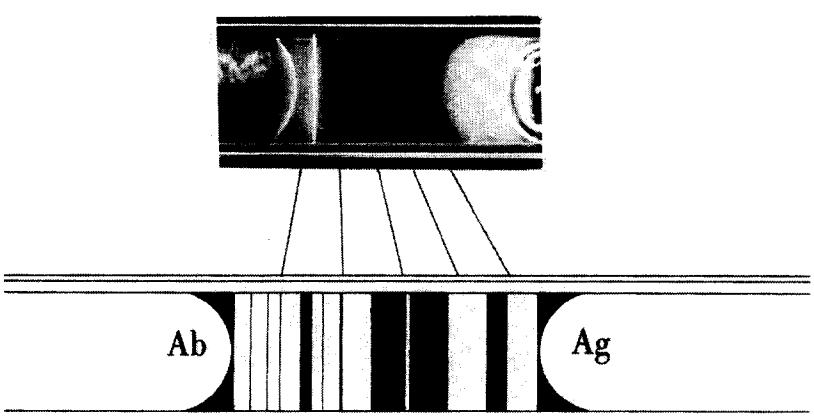

Fig. 2. Oakley–Fulthorpe gel of the interaction between Fraction α and antiserum to Fraction β. 1 % agar in isotonic phosphate buffered saline (pH 7.4).

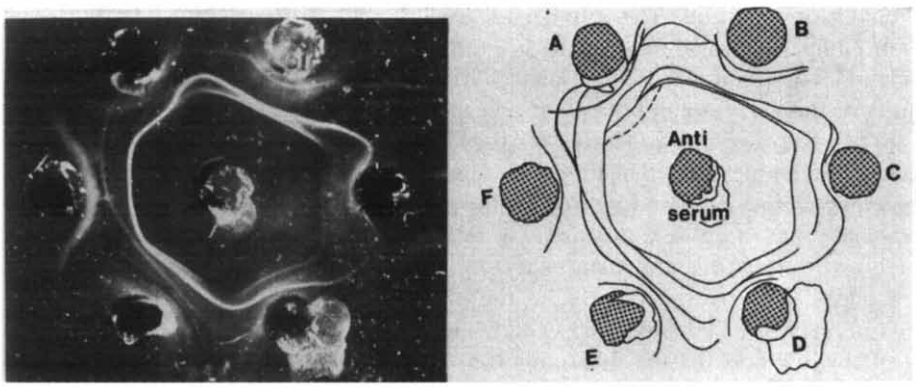

Fig. 3. Comparison by Ouchterlony (Crowle) immunodiffusion of the whole EDTA extract with its Tris-derived fractions. A = whole extract; B = α; C = β; D = γ; E = ε and F = ω. 1 % agar in isotonic phosphate buffered saline (pH 7.4). Well separation = 5 mm.

ing protein fraction or whole EDTA extract. The observed variation when a single antiserum is tested against all the protein fractions, or the converse, most probably arises from a variation in the relative concentration of the antigenic components within each fraction and from a variation in an individual rabbit's ability to respond to all the antigenic species present in any fraction.

Analysis of "spectrin" and its antiserum by the original Ouchterlony method detected only a single precipitin line in our laboratory, thus confirming previous reports [5, 15], and for this reason it is essential to use the more sensitive Crowle modification to demonstrate the antigenic identity of the multiple precipitin lines produced in the Oakley–Fulthorpe test. The identity of several of the antigens in the different protein fractions was confirmed in this way. Several complete rings of precipitate were observed with each antiserum tested against the protein fractions. Furthermore, as the rings were not concentric, they crossed-and re-crossed at several points, the plates also demonstrated the variation in the relative amounts of the antigenic components between each protein fraction (see Fig. 3). The Ouchterlony analysis of the dodecylsulphate derived Fractions 1, 2 and 2.1, was less satisfactory than that of the Tris-derived samples. The precipitin lines from the detergent fractions were diffuse, possibly as a consequence of the presence of residual detergent even though the samples had been exhaustively dialysed before assay. The dodecylsulphate fractions performed adequately in the Oakley–Fulthorpe method. Control experiments using sera of unimmunised rabbits produced no precipitin lines in either technique.

DISCUSSION

These results, in the first place, confirm the antigenic heterogeneity of the proteins extracted from erythrocyte ghosts by a dilute solution of EDTA [5]. Bøg-Hansen and Bjerrum [5] found up to nine components in the extract of human cells by their fused rocket and line immunoelectrophoretic methods. Twelve lines, in five groups, can frequently be discerned by the Oakley–Fulthorpe method in the extract of ox cells and a similar order of complexity is shown by the Crowle modification of

the Ouchterlony method. The number of precipitin lines quoted must be regarded as a minimum which will increase with the application of more sensitive techniques and clearly the immunological approach gives little information of the absolute amounts of the various components it reveals in a mixture.

The present results go further for they show that not only is the EDTA extract antigenically complex, but that an equal degree of complexity exists in the fractions of the whole extract prepared by electrophoresis in either of two buffer systems. This heterogeneity has been demonstrated by raising antisera against the whole extract and against its fractions and then analysing the interaction of each antiserum with each protein. These fractions have, in the presence of dodecylsulphate, molecular weights between 250 000 and 45 000 and while there is some doubt on the total reliability of this method for the determination of the molecular weights of membrane proteins [8, 16], in this particular instance the size distribution of α, β, and γ is confirmed by their analysis in the Tris buffer, i.e. in the absence of detergent, using the Ferguson procedure [17]. Although we do not have an independent molecular weight estimation of the protein(s) of high mobility in the Tris buffer, i.e. independent of the 45 000 obtained by the dodecylsulphate method, their high mobility strongly suggests that in the absence of detergent they remain small relative to α, β and γ. This molecular weight distribution presumably persists during diffusion in the agar media used for the immunological tests.

From these considerations it therefore follows that (a) a protein solution containing only one or two components by electrophoresis in the presence of dodecylsulphate can be antigenically considerably more heterogeneous, and (b), the same antigens can exist in several different molecular weight forms.

The immunological similarities between the various fractions suggests that they are composed of the same polypeptide chains in different states of aggregation. This would be consistent with our previous report that the 45 000 dalton protein can form 200 000 to 250 000 aggregates that are not dissociated by dodecylsulphate [3, 4] and also with the results obtained by N-terminal analysis of the α, β, γ and ω [8]. It was necessary to postulate one type of aggregate of each molecular weight to explain the N-terminal results but this immunological data points to there being several aggregates of each size. A possible, but not necessary, explanation of the immunological similarity and heterogeneity of the fractions of different sizes is that each fraction consists of homopolymers of each member of a basic set of haptens.

The nature of the forces holding the aggregates together is unknown. In our earlier paper [8] we argued that resistance to dodecyl sulphate does not necessarily demonstrate there being covalent links as several non-covalent associations which can resist the detergent are known [2, 18–21], but the possibility of the aggregation arising from covalent cross links between relatively small polypeptides is supported by the recent reports of the existence of such bonds between membrane polypeptides [7, 22, 23]. Whatever the mechanism, it is possible that these fractions represent different stages in the assembly of the proteins into the membrane matrix.

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