Biochimica et Biophysica Acta, 646 (1981) 275–282
Elsevier/North-Holland Biomedical Press

BBA 79356

AN IMMUNOLOGICAL ANALYSIS OF THE EFFECTS OF BROMELAIN ON THE MOUSE ERYTHROCYTE MEMBRANE

DAVID A. CUNLIFFE and K.O. COX

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia, 5042 (Australia)

(Received November 28th, 1980)
(Revised manuscript received April 17th, 1981)

Key words: Erythrocyte membrane; Bromelain modification; Antigenic change; (Mouse)

The technique of crossed immunoelectrophoresis was used to analyse the effects of bromelain on the antigens contained within the membranes of mouse erythrocytes. Immune sera were obtained from goats that had been injected with membranes prepared from either normal erythrocytes or bromelain-modified erythrocytes. Precipitation patterns obtained by crossed immunoelectrophoresis showed that treatment of erythrocytes with bromelain changed the electrophoretic mobility of a major protein antigen and increased the solubility of two membrane glycoproteins. The possibility that enzymic modification of the erythrocytes led to the creation of entirely new antigenic determinants was not supported by the precipitin patterns obtained using sera adsorbed with various membrane preparations.

Introduction

The spleens of untreated mice contain a relatively high number of cells secreting antibodies directed against antigens within the membranes of autologous erythrocytes [1]. These antigens, which cannot be detected on the surface of normal erythrocytes, are exposed by treating the cells with proteolytic enzymes such as bromelain [1,2]. It has been proposed that bromelain-modification leads to the digestion of structures on the erythrocyte surface thereby exposing antigens that are normally hidden [1,3]. Physical analyses of erythrocyte membranes have shown that bromelain-treatment removes both protein and glycoprotein from the surface of normal cells [3]. However, little is known about the associated immunochemical changes within the erythrocyte membranes.

Crossed immunoelectrophoresis has been shown to have great potential for the immunochemical analysis of membrane components [4,5]. In particular, it has been used to characterize individual proteins from human erythrocyte membranes [6,7]. In the investigations reported here, the technique of crossed immunoelectrophoresis has been used to analyse the effects of bromelain-treatment on mouse erythrocyte membranes. The aims were to examine antigenic changes caused by the proteolysis and to determine whether any new antigenic structures were produced.

Materials and Methods

Materials. Inbred C_3H mice aged 6–12 weeks of either sex were from the Institute of Medical and Veterinary Sciences in Adelaide. Bromelain was obtained from Sigma Chemicals, neuraminidase (*Vibriocholerae*) from Calbiochem and Agarose C from Pharmacia Fine Chemicals. Phosphate buffered saline with glucose with a pH of 7.4 and an osmolarity of 325 mosM was used [3].

Preparation of antisera. Goats weighing 25–30 kg, were given 15–20 mg of membrane proteins from normal or bromelain-modified erythrocytes subcutaneously in multiple sites at intervals of 21 days. For the initial inoculum 1 ml of the membrane proteins was mixed with 1 ml of Freund's complete adjuvant.

In three subsequent inocula given at 21-day intervals, 1 ml of the proteins was mixed with 1 ml of Freund's incomplete adjuvant. Blood was collected from the goats one week after the final injection and the immunoglobulin fraction was prepared by precipitation of the serum at a 50% saturation of $(NH_4)_2SO_4$.

Adsorption of goat antibodies. Two adsorptions of the fractionated serum were performed by incubation with an equal volume of a 50% suspension of erythrocytes at 37°C for 30 min followed by centrifugation at 2 000 × *g* for 5 min. Erythrocyte membranes in a volume equivalent to 2.0 ml of packed cells were incubated at 37°C for 30 min with 1.0 ml of goat antiserum. The serum was recovered after centrifugation at 20 000 × *g* for 40 min.

One ml of goat serum was preincubated at 37°C for 30 min with solubilized membrane proteins equivalent to 2 ml packed erythrocytes.

Erythrocytes and bromelain-treatment. One volume of mouse erythrocytes collected into Alsever's solution was washed three times, each time in at least 50 vol. of phosphate buffer. Erythrocytes were treated with bromelain at a final concentration of 10 mg/ml [3]. Phenylmethylsulfonylfluoride [3] was added to erythrocytes from which membranes were prepared.

Neuraminidase treatment. Mouse erythrocytes and solubilized membrane proteins were digested as described [6]. In contrast to human membrane proteins those from mice did not aggregate at pH 5.5 [6].

Membrane preparation and solubilization. Erythrocyte membranes were prepared by the method of Dodge et al. [8] and solubilized by incubation at 20°C for 40 min in the presence of Triton X-100 added to a final concentration of 2% (w/v).

Crossed immunoelectrophoresis. The technique has been described [3]. The buffer used was 24 mM 5,5'-Diethylbarbituric acid, 73 mM Tris, 0.4 mM calcium lactate, 1% (v/v) Triton X-100, pH 8.6.

Estimation of protein. Protein was estimated by the method of Lowry et al. [9] as modified by Markwell et al. [10]. Bovine serum albumin was used as the reference standard.

Results

The crossed immunoelectrophoresis was performed principally using sera from goats that had been injected with membranes of normal mouse erythrocytes. Diagrammatic representations of the precipitation patterns obtained by crossed immunoelectrophoresis of solubilized membrane proteins from either normal erythrocytes or bromelain-modified erythrocytes are shown in Fig. 1. The identity of precipitates from the two patterns and the observed properties are listed in Table I.

Bromelain-modification of the erythrocytes clearly altered the patterns of precipitation (Fig. 1). The changes in part were caused by digestion of membrane components and by increasing the solubility of the membranes in Triton X-100.

Identity of precipitates in the two patterns was established by crossed immunoelectrophoresis of mixtures of solubilized membrane components from both normal erythrocytes and bromelain-modified erythrocytes (Fig. 2). The patterns produced showed that peaks 1 and 6 representing membrane proteins from normal cells joined with peaks 23 and 23b, respectively, formed by a membrane protein from bromelain-modified cells to produce double peaks.

TABLE I

CHARACTERISTICS OF THE MAJOR PRECIPITATES OBSERVED IN CROSSED IMMUNOELECTROPHORESIS OF MEMBRANE PROTEINS FROM NORMAL ERYTHROCYTES AND BROMELAIN-MODIFIED ERYTHROCYTES

A, sialoglycoproteins; B, digested by bromelain; C, surface component.

Precipitates of normal erythrocytes	A	B	C	Corresponding precipitate(s) of bromelain erythrocytes
1	+	+	+	23
2				26
3				25
4				24
5				28
6		+		23b
8			+	
13			+	
Heat soluble antigens	+			21, 22

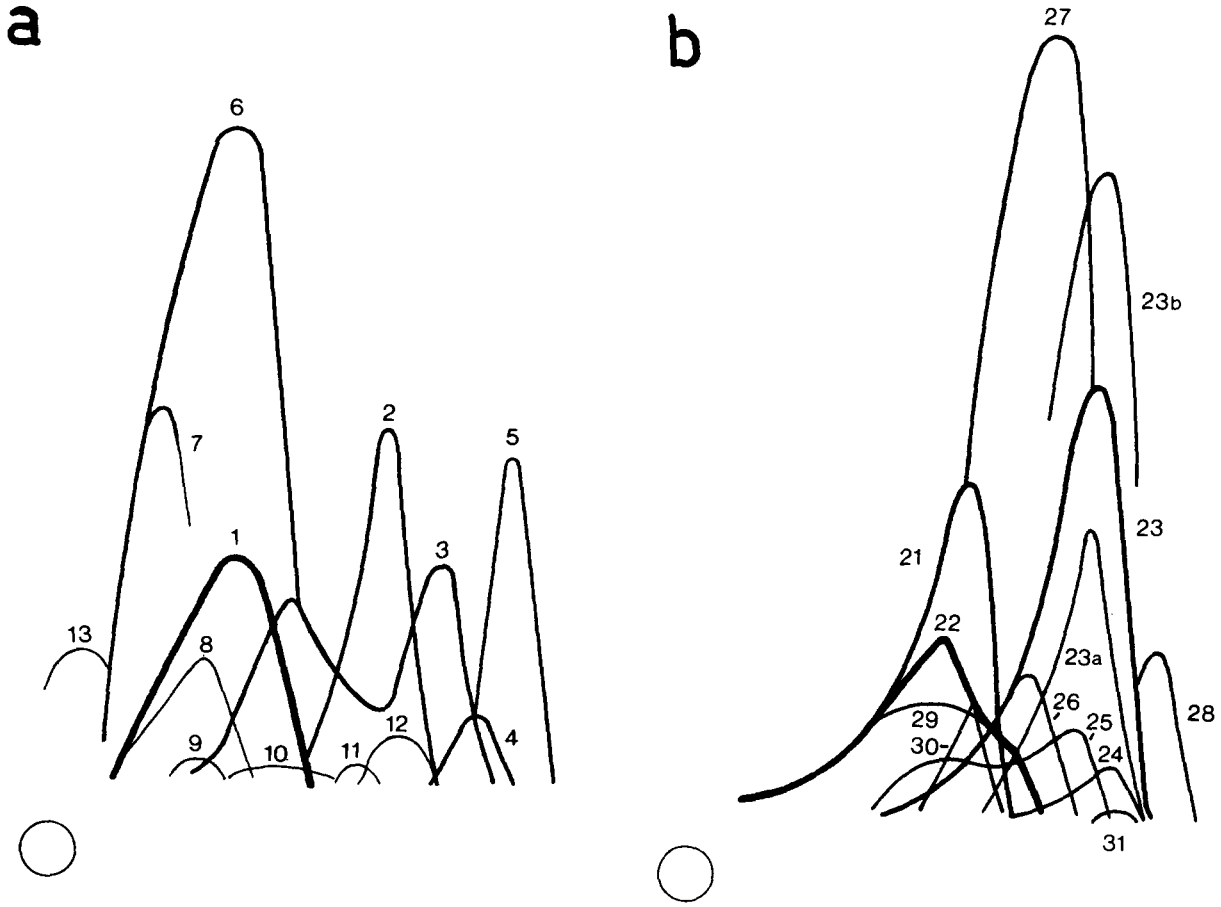


Fig. 1. Diagrammatic representations of crossed immunoelectrophoresis of Triton-solubilized membranes from normal erythrocytes and bromelain-modified erythrocytes using goat serum against normal erythrocyte membranes. In (a) 200 μg of normal membrane proteins and in (b) 100 μg of membrane proteins from bromelain-modified erythrocytes were electrophoresed into serum used at a final dilution of 1/10. First dimension electrophoresis was performed for 1 h at 80 V/cm, anode to the right. Second dimension electrophoresis was performed at 15 V/cm for 12 h, anode at the top. The gels were stained using Coomassie brilliant blue R. The bands 23, 23a and 23b were labelled to signify the possibility of three antigens on the same protein which always comigrated and were often linked.

This indicates that one effect of bromelain-modification was the partial digestion of the protein represented by peaks 1 and 6. The mixed crossed immunoelectrophoresis also demonstrated identity between peaks 2 and 26, 3 and 25, 4 and 24, and 5 and 28 (Fig. 2).

Comparison of the two membrane types revealed that those from normal cells were less susceptible to solubilization in Triton X-100 at 20°C. Incubating the insoluble fraction at 63°C for 10 min in the presence of Triton X-100 (2% w/v) solubilized two antigens as detected by crossed immunoelectrophoresis. Electrophoresing mixtures of the heat soluble proteins and solubilized membrane proteins from bromelain-modified erythrocytes demonstrated identity between precipitates 21 and 22 and the heat soluble proteins (results not shown).

Bromelain-modification did not lead to major changes in the immunogenicity of the erythrocyte membranes in goats. When goat serum raised against membranes prepared from bromelain-modified erytrocytes, rather than from normal erythrocytes, was used in the crossed immunoelectrophoresis there were few changes to the precipitation patterns obtained

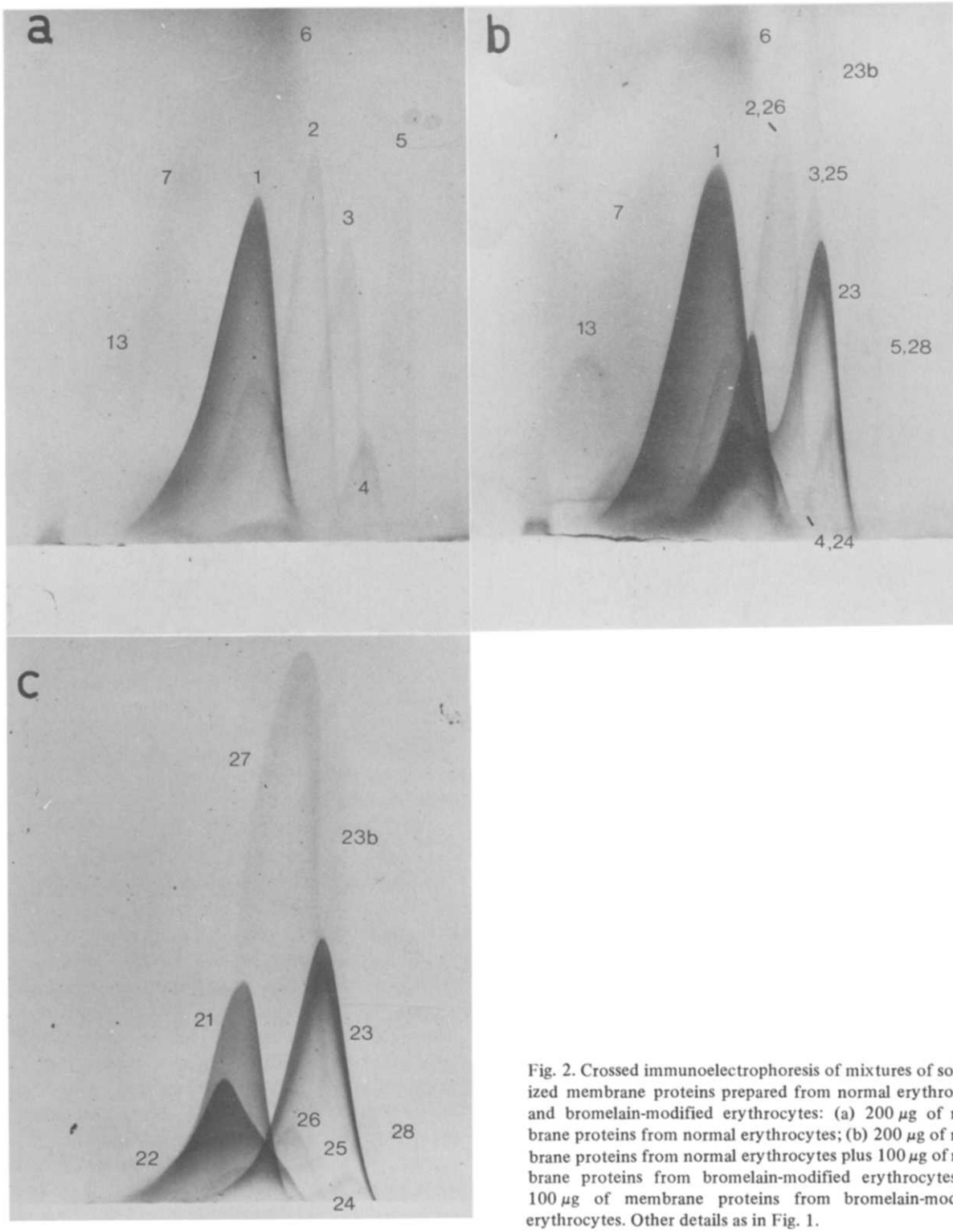


Fig. 2. Crossed immunoelectrophoresis of mixtures of solubilized membrane proteins prepared from normal erythrocytes and bromelain-modified erythrocytes: (a) 200 μg of membrane proteins from normal erythrocytes; (b) 200 μg of membrane proteins from normal erythrocytes plus 100 μg of membrane proteins from bromelain-modified erythrocytes; (c) 100 μg of membrane proteins from bromelain-modified erythrocytes. Other details as in Fig. 1.

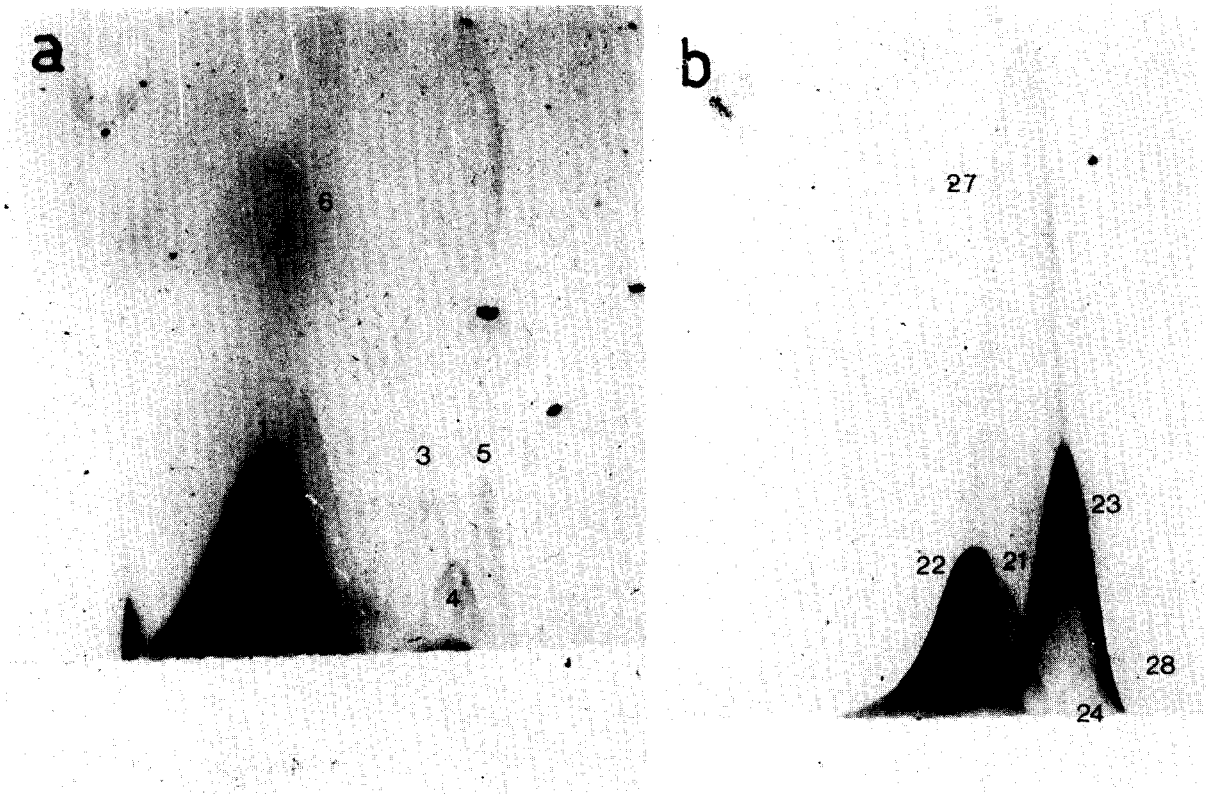
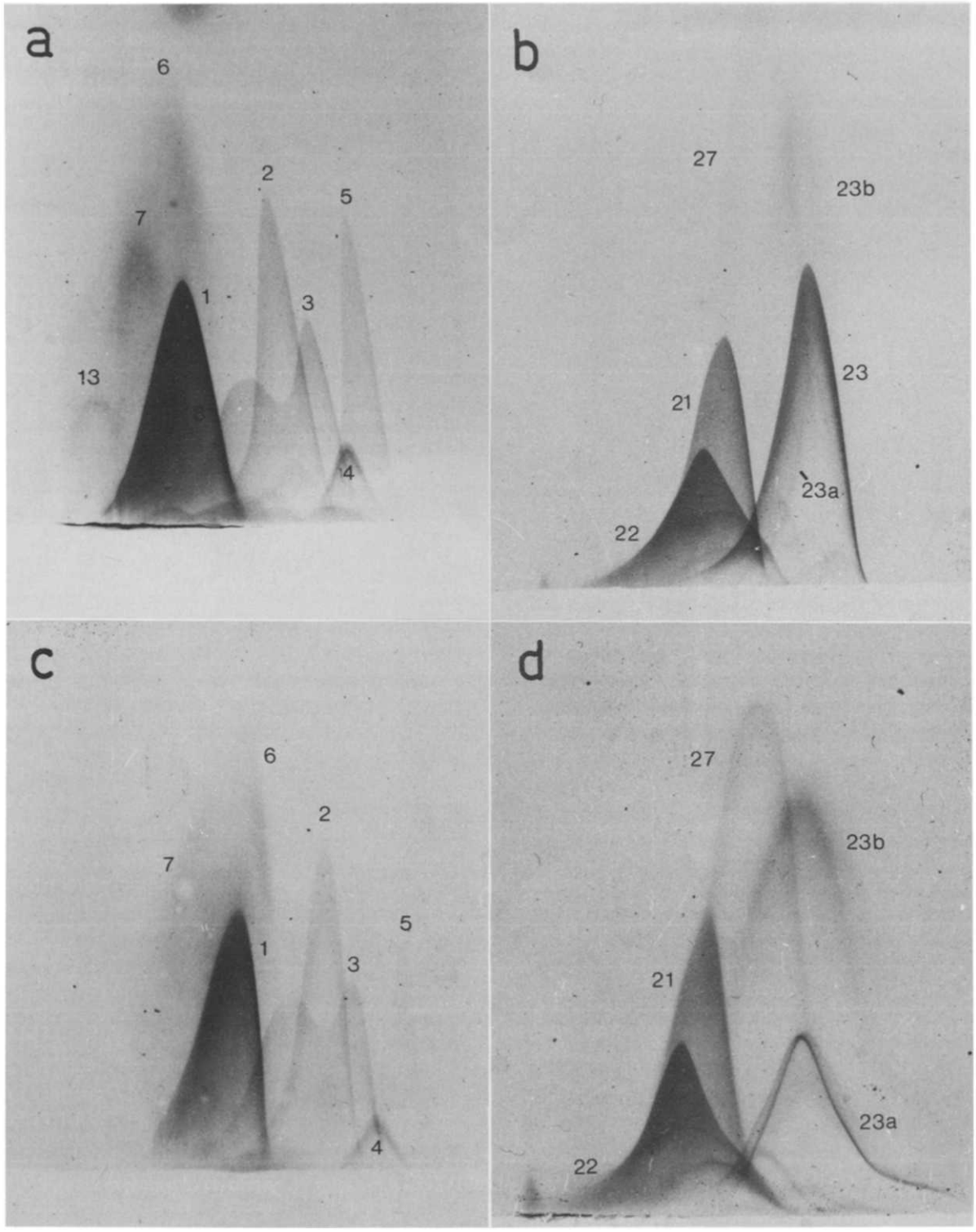


Fig. 3. Comparison of crossed immunoelectrophoresis using goat antiserum raised against membranes prepared from bromelain-modified erythrocytes. In (a) 200 μg of normal membrane proteins and in (b) 100 μg of membrane proteins from bromelain-modified erythrocytes were electrophoresed into the antiserum against membranes from bromelain-modified erythrocytes, used at a final dilution of 1/20. Electrophoresis was performed as in Fig. 1.

(compare Figs. 2 and 3). Changing the antiserum led to alterations in the shape and relative heights of precipitates 21 and 22 in the patterns incorporating membrane proteins from bromelain-modified erythrocytes. Further, precipitates 2 and 26, from the patterns obtained with membrane proteins from normal erythrocytes and bromelain-modified erythrocytes, respectively, were only present when the goat antiserum to normal erythrocyte membranes was used.

Further evidence of the antigenic similarity of the membranes from normal erythrocytes and bromelain-modified erythrocytes was obtained by adsorbing the goat sera with either type of membrane preparation. The preadsorptions completely removed the ability of the sera to form precipitates with solubilized membrane proteins, when used in crossed immunoelectrophoresis (results not shown).

The goat antiserum to membranes from normal erythrocytes was adsorbed with intact cells to determine which precipitates represented surface antigens. Adsorptions with either normal erythrocytes (Fig. 4c and d) or bromelain-modified erythrocytes (results not shown) produced the same effects. Precipitates 8 and 13 were not formed and the staining intensity of precipitate 1 was reduced when adsorbed sera were used in the crossed immunoelectrophoresis of membrane proteins from normal erythrocytes. The effect on precipitate 1 was more readily apparent in the precipitation patterns of membrane proteins from bromelain-modified erythrocytes. Precipitate 23, which is antigenically related to precipitate 1, was not formed with the adsorbed sera (Fig. 4d). However,


a
6
2
5
7
1
3
13
4
b
27
23b
23
21
23a
22
c
6
2
7
5
1
3
4
d
27
23b
21
23a
22

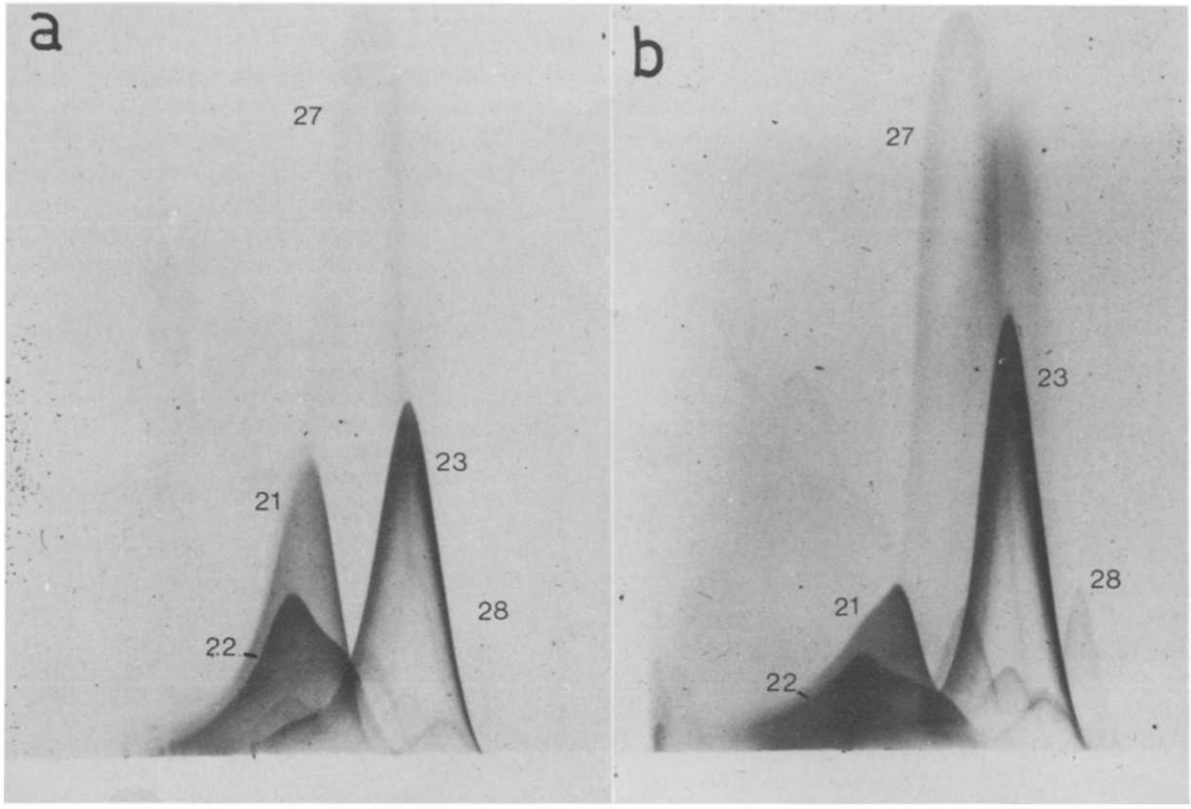


Fig. 5. Crossed immunoelectrophoresis of membrane proteins following treatment of erythrocytes with neuraminidase. In (a) 100 μg membrane proteins from bromelain-modified erythrocytes, and (b) 100 μg membrane proteins from bromelain-modified erythrocytes pretreated with neuraminidase, were electrophoresed. Other details as in Fig. 1.

precipitates 23a and 23b, which probably represent different antigens on the same protein, were still formed.

Intact erythrocytes and solubilized membranes were treated with neuraminidase to identify which precipitates represented sialoglycoproteins. The enzyme treatment of whole cells changed the mobility of the components represented by precipitates 21 and 22 from the crossed immunoelectrophoresis pattern of solubilized membranes from bromelain-modified erythrocytes (Fig. 5a and 5b). Treatment of the membranes after solubilization led to a more severe digestion of the antigens represented by precipitates 21 and 22 but did not lead to the digestion of any other antigens (results not shown).

Discussion

Serologically it has been shown that the treatment of mouse erythrocytes with bromelain leads to changes in the antigenicity of the cells [1,2]. We have used the technique of crossed immunoelectrophoresis to examine the effects of bromelain on the antigens contained within the erythrocyte membrane.

Treatment of mouse erythrocytes with bromelain led to the partial digestion of at least one protein.

Fig. 4. Identification of immunoprecipitates representing surface antigens. The crossed immunoelectrophoresis was performed using goat antiserum to membranes from normal erythrocytes that was either unadsorbed (a and b) or preadsorbed with normal erythrocytes (c and d). In (a) and (c) 200 μg of membrane proteins from normal erythrocytes and in (b) and (d) 100 μg of membrane proteins from bromelain-modified erythrocytes were electrophoresed. Other details as in Fig. 1.

The protein is represented by precipitates 1 and 6 in the crossed immunoelectrophoresis patterns obtained with membrane proteins from normal erythrocytes and 23, 23a and 23b in the patterns incorporating membrane proteins from bromelain-modified cells. Specific adsorptions of the goat antisera with intact erythrocytes indicates that parts of this protein were exposed on the surface of both normal cells and bromelain-modified cells. Bromelain-modification also led to changes in the solubility of the erythrocyte membranes in Triton X-100. Precipitates 21 and 22 represent glycoproteins that are only soluble, in Triton X-100 at 20°C, after bromelain-modification. The neuraminidase digestion of bromelain-modified erythrocytes indicates that parts of the glycoproteins represented by precipitates 21 and 22 are exposed on the outer cell surface. However, adsorptions of the goat antisera with intact erythrocytes did not affect the formation of these two precipitates.

The specific adsorptions of the goat serum with intact erythrocytes did not provide any evidence of antigens unique to the surface of either normal erythrocytes or bromelain-modified erythrocytes. One possible explanation for this is that the unique antigens may be masked in precipitates that represent several antigenic structures [6]. It has been shown in human erythrocytes that the predominant surface components are parts of molecules that span the membrane and presumably contain many antigenic determinants [6,11,12].

The bromelain-modification does not appear to induce any major changes in the antigenicity of solubilized erythrocyte membranes. The crossed immunoelectrophoresis patterns were similar using serum from goats injected with membranes prepared from either normal erythrocytes of bromelain-modified erythrocytes. Further, the ability of the sera to form precipitates with membrane proteins from either normal erythrocytes or bromelain-modified erythrocytes could be totally abrogated by adsorptions of the sera with membranes prepared from either cell type. These results support the view that bromelain-treatment does not lead to the creation of any new antigens [1,3].

Acknowledgements

This work was supported by grants from the National Health & Medical Research Council of Australia and from the Flinders University Research Committee.

References

1 Cunningham, A.J. (1976) Transplant. Rev. 31, 23–43
2 Cox, K.O., Baddams, H. and Evans, A. (1977) Aust. J. Exp. Biol. Med. Sci. 55, 27–37
3 Cunliffe, D.A. and Cox, K.O. (1979) Mol. Immunol. 16, 427–433
4 Bjerrum, O.J. and Bog-Hansen, T.C. (1976) in Membrane Methods (Maddy, A.H., ed.), pp. 378–426, Chapman and Hall, London
5 Bjerrum, O.J. (1977) Biochim. Biophys. Acta 472, 135–195
6 Bjerrum, O.J. and Bog-Hansen, T.C. (1976) Biochim. Biophys. Acta 455, 66–89
7 Bjerrum, O.J. and Lundahl, P. (1974) Biochim. Biophys. Acta 342, 69–80
8 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
10 Markwell, M.A.K., Haas, S.M. Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210
11 Steck, T.L., Fairbanks, G. and Wallach, D.F.H. (1971) Biochemistry 10, 2617–2624
12 Steck, T.L. (1974) J. Cell Biol. 62, 1–19