DEMONSTRATION OF BINDING OF TRITON X-100 TO AMPHIPHILIC PROTEINS IN CROSSED IMMUNOELECTROPHORESIS

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1. Introduction

Binding of non-ionic detergents is a characteristic property of 'intrinsic', amphiphilic membrane proteins [1,2] and there is currently an increasing interest in methods which permit detection of such detergent-binding. In this paper it will be shown how incorporation of ^{14}C- or ^{125}I-labelled Triton X-100 in agarose gels renders detection of amphiphilic proteins possible in crossed immunoelectrophoresis of complex protein mixtures. Due to binding of Triton X-100, such proteins give rise to radioactive precipitates which may be selectively visualized by autoradiography.

2. Materials and methods

2.1. *Detergents*

Triton X-100 (polyoxethylene-*p*-*tert*-octylphenol) (scintillation grade) was obtained from BDH Chemicals, Pool, England. ^{14}C-labelled Triton X-100 (0.461 μCi/mg) was a generous gift from A. M. Rothman, Rhom and Haas, Philadelphia. Berol EMU-043 (polyoxyethylene alkohol) and Tween-20 (polyoxyethylene sorbitol ester) were purchased from MoDoKemi, Stenungsund, Sweden and Atlas Chemie, Essen, FRG, respectively.

2.2. *Proteins*

Human erythrocyte membranes prepared as in [3] were solubilized in 1% (v/v) Triton X-100, Berol EMU-43, Tween-20, or a combination of 1% Triton X-100/0.1% radioactive Triton X-100. EDTA-extractable protein, M,N glycoprotein and the major 'intrinsic' protein of human erythrocyte membranes were prepared as described in ref. [4–6], respectively. Human serum from a healthy donor was stored at −20°C and was in some experiments preequilibrated with radioactive Triton (0.4–10%) for 1 h at 25°C.

2.3. *Antibody sources*

Antibody sources were as given in ref. [7].

2.4. *^{125}I-Labelling of Triton X-100*

^{125}I-Labelling of Triton X-100 was performed using the procedure of Fischetti et al. [8] and employing $Na^{125}I$ from Behringwerke, Marbrug, FRG (spec. act. 8–16 Ci ^{125}I/mg I). Labelling of 200 μl aliquots of 10% Triton X-100 resulted in 4 ml final product with an activity of about $2 \cdot 10^5$ cpm/ml.

2.5. *Crossed immunoelectrophoresis*

Crossed immunoelectrophoresis was performed as in [3] in 1% (w/v) agarose gels (code HSA, Litex, Glostrup, Denmark) containing 0.038 M Tris and 0.10 M glycine, pH 8.9 (16°C). In initial experiments, the Triton X-100 concentration in the gels was 0.25%, but in later experiments the concentration was lowered to 0.1% with no change in results. When radioactive Triton X-100 was utilized, it constituted 10% (^{125}I-labelled Triton X-100) or 25% (^{14}C-labelled Triton X-100) of the cold Triton concentration. The concentration of radioactive Triton could be lowered but the exposure time necessary for autoradiography accordingly increased. In certain experiments detergent-free gels were used.

After immunoelectrophoresis, plates were pressed under filter paper and fanned dry at 25°C without

washing. The plates were then placed in X-ray cassettes, RPX-omat films exposed 1–3 weeks and then processed in an RPX-omat processor (Kodak). Before final staining for protein with Coomassie Brilliant Blue [9], the plates were washed for 3 h in 0.1 M NaCl, pressed and dried.

3. Results and discussion

Figures 1A and 1C are autoradiographs of crossed immunoelectrophoreses of human erythrocyte membrane proteins electrophoresed in the presence of ^{14}C- and ^{125}I-labelled Triton, respectively. Three precipitates selectively bind Triton. By comparison with the protein-stained precipitation pattern (fig.1B and 1D (earlier characterized and numbered in ref. [3,7]) they were identified as the M,N glycoprotein (No. 21) and the major 'intrinsic' protein which is present in two different immunochemical states (No. 16 and 18) [3,10]. These are the two major amphiphilic proteins of human erythrocyte membranes. By contrast, spectrin (No. 6) and haemoglobin (No. 15) do not significantly bind Triton. Crossed immunoelectrophoresis of isolated membrane proteins, including the major 'intrinsic' protein, which had been exposed to sodium dodecyl sulphate during the isolation procedure [6] gave comparable results in the same electrophoresis system (fig.2).

The results obtained for serum proteins electrophoresed in ^{125}I-labelled Triton-containing gels are depicted in fig.3. Only a single, heavily-stained precipitate identified with specific antibodies as prealbumin is seen on the autoradiograph (fig.3A). Albumin also took up small amounts of radioactivity (visible only on the original film). With ^{14}C-labelled Triton similar results were found, but the staining of prealbumin was much fainter. The observed Triton-binding could be explained by the presence of discrete high-affinity binding sites for detergent molecules on albumin [11] and the known transport function of prealbumin for tyroxine [12], the structure of which is similar to iodinated Triton [8]. The lack of bidirectional shift in charge-shift electrophoresis [7] indicates a different nature of Triton binding to prealbumin compared to micellar binding to amphiphilic proteins [1,13].

Three different variations in the experimental procedure were tested:

(i) Membrane proteins equilibrated with hot or cold detergent and analysed in gels containing radioactive Triton. Radioactive precipitates were observed regardless of the detergent initially used for membrane solubilization, and also when radioactive Triton was not present in the applied protein sample.

(ii) Membrane proteins were equilibrated with radioactive Triton but electrophoresed in gels containing cold Triton only. This system consistenly yielded negative results and binding of Triton could not be detected. This shows that exchange of detergent molecules bound to membrane proteins occurs rapidly in detergent solution. For this reason, radioactive Triton could also be omitted from membrane protein samples when electrophoresis was performed in gels containing radioactive Triton.

(iii) Samples equilibrated with radioactive Triton, but electrophoresed in detergent-free gels. This was used to analyse serum lipoproteins, because they do not form immunoprecipitates in presence of Triton [14]. Figure 4 shows that detergent-binding to lipoproteins can be demonstrated under these conditions. The results may indicate that exchange of Triton molecules occurs at a slower rate in detergent-free environment.

With the exception of prealbumin, the results obtained with ^{125}I-labelled Triton and ^{14}C-labelled Triton were comparable. ^{125}I-labelled Triton can therefore be used in such experiments instead of ^{14}C-labelled Triton which is not commercially available.

The selective uptake of radioactive Triton in the immunoprecipitates corresponding to the known amphiphilic membrane and serum proteins indicates that the method reliably permits differentiation between hydrophilic and amphiphilic proteins using quantitative immunoelectrophoresis. Other systems which have been analysed including bovine milk fat globule membranes [15], rat brain synaptosomes [16] (supplied by E. Bock) and brush border aminopeptidase (supplied by H. Sjöström and O. Norén) have yielded further consistent results, and the amphiphilic proteins present in these systems were readily detected. The only other methods which currently permit detection of amphiphilic proteins in complex

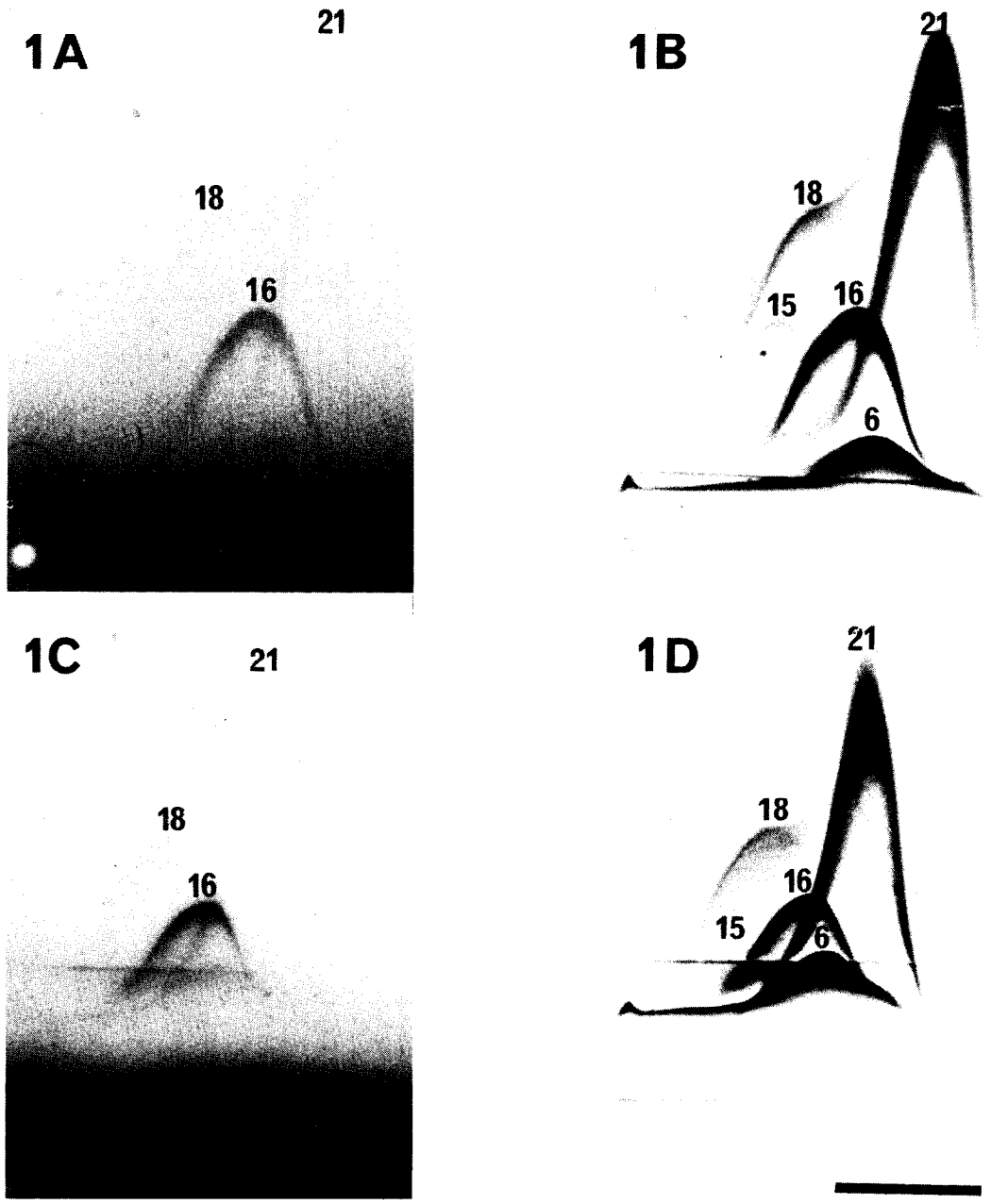

Fig.1. Crossed immunoelectrophoresis of 12 μg Triton-solubilized human erythrocyte membrane proteins electrophoresed in presence of radioactive Triton X-100. (A and C) Autoradiographs of ^{14}C- and ^{125}I-labelled Triton, respectively. (B and D) The same plates after Coomassie Brilliant Blue staining. The radioactive precipitates correspond to the M,N glycoprotein (No. 21) and the major 'intrinsic' protein (No. 16 and 18). First-dimension electrophoresis was performed for 45 min at 10 V/cm. Anode to the right. Second-dimension electrophoresis was performed at 2 V/cm for 18 h. Anode at the top. For experiment C–D an antibody-free intermediate gel has been used, so the precipitates appear above the more stained background of the thicker, first-dimension gel [9]. Antibody content of the gels: 10 μl/cm^2. Exposure time for autoradiographs: 21 and 10 days for A and C, respectively. The bar represents 1 cm.

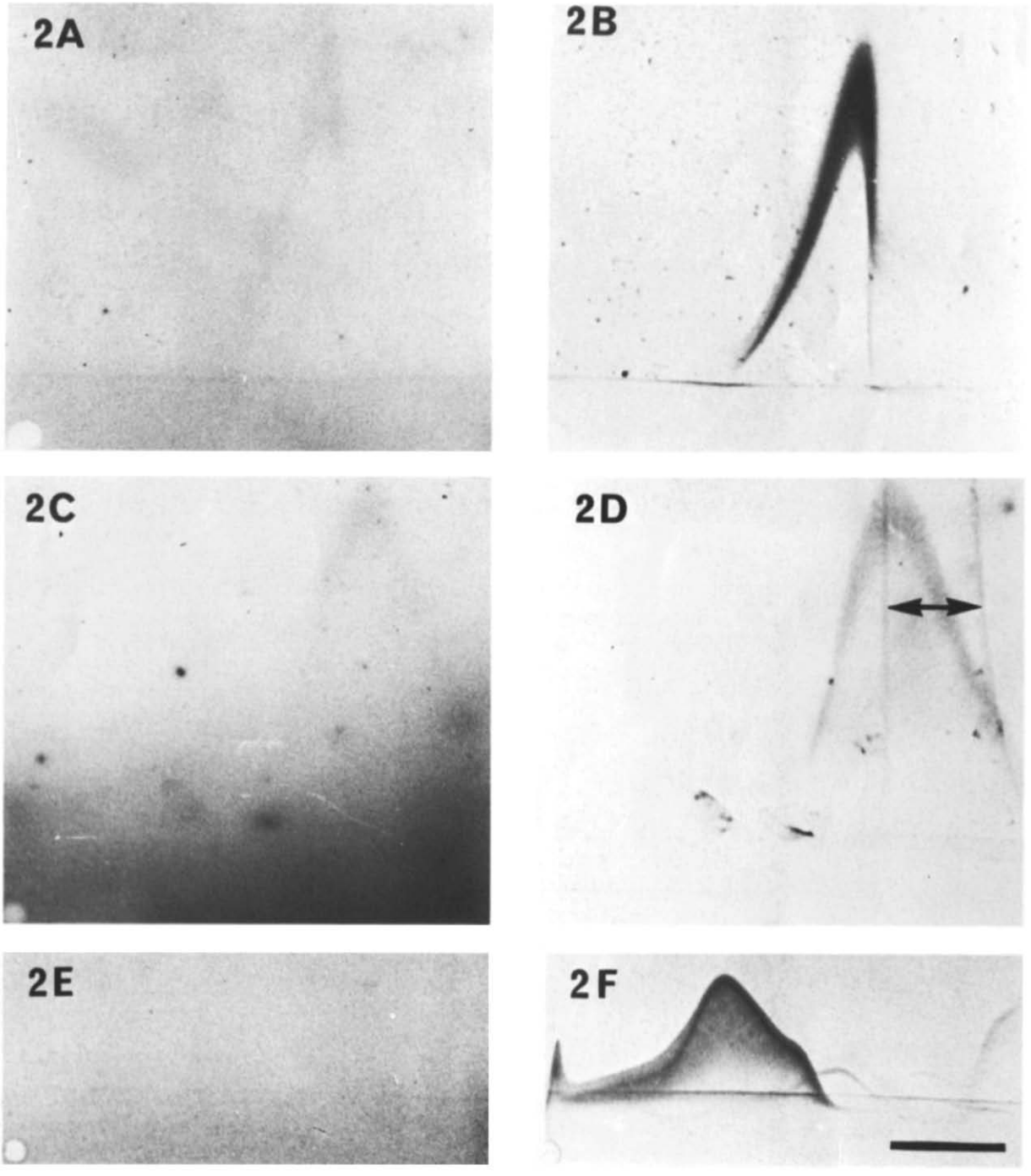

Fig.2. Crossed immunoelectrophoresis of 6 μg isolated M,N glycoprotein (B), 12 μg major intrinsic protein (D) and 5 μg EDTA extract (spectrin) (F) of human erythrocyte membranes electrophoresed in presence of ^{125}I-Triton X-100. (A, C and E) Autoradiographs of the same plates. Arrows indicate a protein impurity from the buffer vessel. Antibody contents of the gels were 16 μl/cm^2, 16 μl/cm^2 and 8 μl/cm^2, respectively. Experimental conditions were as for the experiment of fig.3.

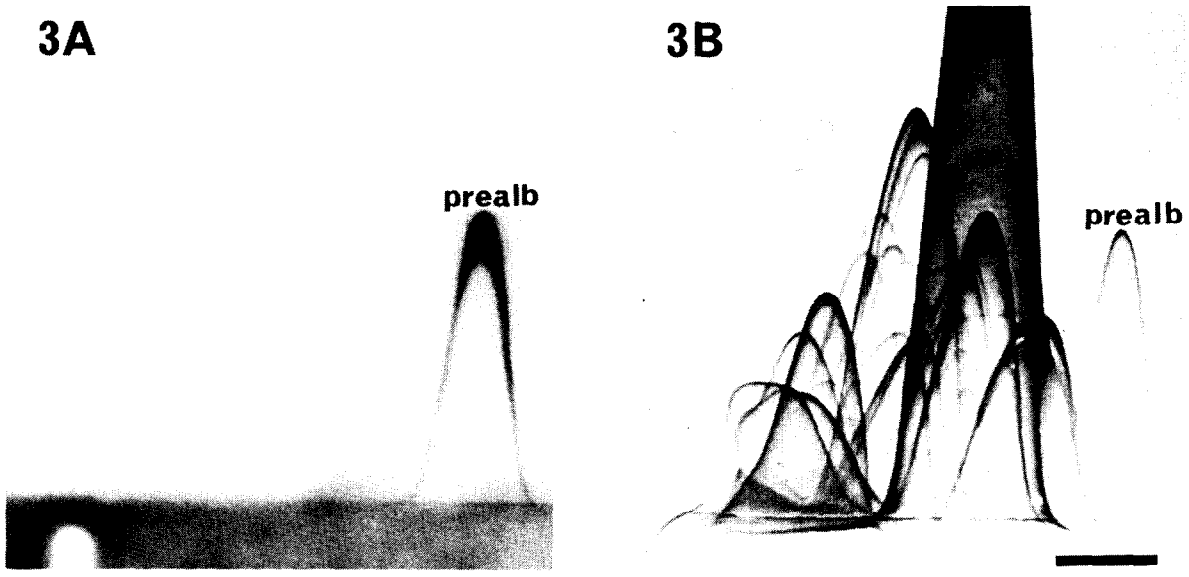

Fig.3. Crossed immunoelectrophoresis of 1.5 μl human serum electrophoresed in presence of ^{125}I-labelled Triton X-100. (A) Autoradiograph. (B) The same plate after Coomassie Brilliant Blue staining. Prealbumin (prealb) selectively demonstrates radioactivity. First and second dimension electrophoresis: 1 h at 10 V/cm and 18 h at 2 V/cm, respectively. Antibody content of the gel: 13 μl/cm^2. The bar represents 1 cm.

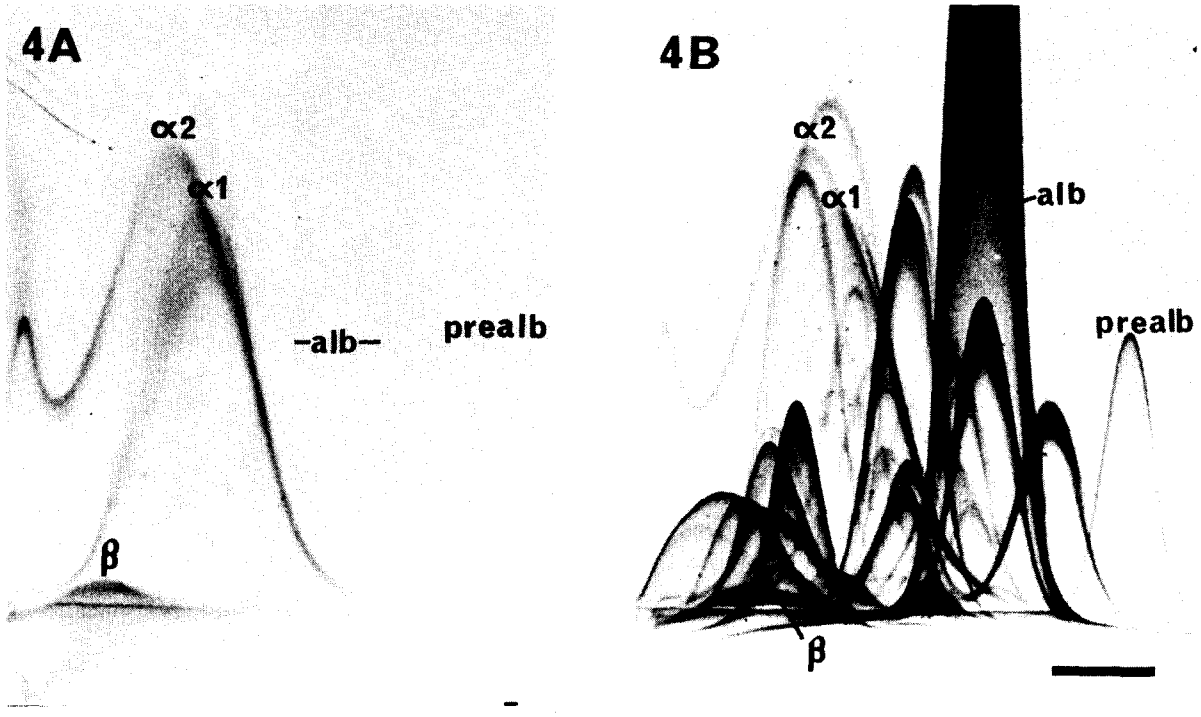

Fig.4. Crossed immunoelectrophoresis of 1.5 μl human serum mixed with 10 μl 10% ^{14}C-labelled Triton X-100 electrophoresed in detergent-free gels. (A) Autoradiograph. (B) The same plate after Coomassie Brilliant Blue staining. Five precipitates bind radioactive Triton and were with specific antibodies identified as: Prealbumin (prealb), albumin (alb), β-lipoprotein (β) and α-lipoprotein (α1 and α2). Note the changed position, splitting and doubling of the precipitates of α-lipoprotein. Experimental conditions were as for the experiment of fig.3.

mixtures are those involving charge-shift electrophoresis [7,13]. The present method is less sensitive than charge-shift crossed immunoelectrophoresis for detection of very minor amphiphilic proteins. Also it cannot be used to detect non-precipitating amphiphilic peptides as in two dimensional charge-shift electrophoresis. The advantage lies in the direct demonstration of Triton-binding, as opposed to the more indirect principle of charge-shift electrophoresis.

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References

[1] Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79.
[2] Tanford, C. and Reynolds, J. (1976) Biochim. Biophys. Acta 457, 133–170.
[3] Bjerrum, O. J. and Bøg-Hansen, T. C. (1976) Biochim. Biophys. Acta 455, 66–89.
[4] Bhakdi, S., Knüfermann, H. and Wallach, D. F. H. (1975) Biochim. Biophys. Acta 394, 550–557.
[5] Marchesi, V. T. and Andrews, E. P. (1971) Science 174, 1247–1248.
[6] Bhakdi, S., Bjerrum, O. J. and Knüfermann, H. (1976) Biochim. Biophys. Acta 446, 419–431.
[7] Bhakdi, S., Bhakdi-Lehnen, B. and Bjerrum, O. J. (1977) Biochim. Biophys. Acta in press.
[8] Fischetti, V. A., Siviglia, G., Zabriskie, J. B. and Gotschlich, E. C. (1976) Anal. Biochem. 73, 65–69.
[9] Axelsen, N. H., Krøll, J. and Weeke, B. (1973) in: A Manual of Quantitative Immunoelectrophoresis. Methods and Applications. Universitetsforlaget, Oslo; alias Scand. J. Immunol. 2, suppl. 1.
[10] Norrild, B., Bjerrum, O. J. and Vestergaard, B. F. (1977) Anal. Biochem. in press.
[11] Makino, S., Reynolds, J. A. and Tanford, C. (1973) J. Biol. Chem. 218, 4926–4932.
[12] Pages, R. A., Robbins, J. and Edelhoch, H. (1973) Biochemistry 12, 2773–2779.
[13] Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. USA 74, 529–532.
[14] Nielsen, C. S. and Gjerrum, O. J. (1975) Scand. J. Immunol. 4, suppl. 2, 73–80.
[15] Nielsen, C. S. and Bjerrum, O. J. (1977) Biochim. Biophys. Acta 466, 496–509.
[16] Bock, E., Jørgensen, O. S. and Morris, S. J. (1974) J. Neurochem. 22, 1013–1017.