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ARTIFACTS IN ISOELECTRIC FOCUSING

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SUMMARY

Isoelectric focusing of an acidic protein from wool in acrylamide gels has shown that artifacts may occur possibly due to complexes of the protein with ampholines. This phenomenon has also been demonstrated for some basic proteins. Methods for demonstrating the presence of artifacts involve varying the ratio of ampholine to protein, re-running of discrete bands, or applying the protein sample at different positions on the gel.

INTRODUCTION

Although formation of complexes between proteins and ampholytes during isoelectric focusing is possible, it has been assumed that such a complex will have the same isoelectric value as the free protein[1]. During the course of experiments aiming to demonstrate micro heterogeneity in an alkylated wool protein by isoelectric focusing in acrylamide gels, serious variations in the number of zones obtained and their apparent isoelectric points were found according to the position of sample application on the gel. This raised the suspicion that the different zones observed might be due either to formation of a number of specific complexes with a single protein rather than to the separation of closely related proteins, or to the formation of multiple boundaries[2]. Experiments were therefore designed to investigate this behaviour more fully.

MATERIALS

The protein used in these experiments, component 8, is a major low-sulphur fraction prepared from reduced and S-carboxymethylated wool[3]. It is an acidic protein with a molecular weight of 45,000 and is homogeneous as determined by gel filtration on Sephadex G-200 and by starch gel electrophoresis in 8 *M* urea buffer[4]. However, recent evidence, both immunological[5] and chemical[6], indicates that the component is a mixture of closely related proteins.

Cyanogum was purchased from Kodak and ampholine from L.K.B. All other reagents were of analytical grade and were used without further purification with

the exception that urea solutions were deionised by passage through a mixed-bed ion-exchange resin and subsequently stored at 5°.

METHODS

Electrofocusing

The experiments were carried out in 5% acrylamide gels cast into trays 15 × 5 cm and 3 mm deep.

To make the acrylamide gel, the required amount of cyanogum was dissolved in 28 ml 8 *M* urea, and 2 ml of ampholine (3–10 range) and 0.04 ml of TMED* were added. Immediately before pouring the solution into the tray, 0.5 ml of 4% ammonium persulphate was added. The tray was then covered with a perspex sheet and left for 1 h.

Samples of protein (2% in 8 *M* urea) were applied on small pieces of filter paper pushed into slots cut into the gel.

Voltage was applied to the gel via two cellulose wicks dipping into 2.5% ethanolamine (cathode) and 2.5% phosphoric acid (anode). Focusing experiments were generally run at 150 V for 5 h, then at 200 V for a further 20 h.

For determination of the pH gradient along the gel, small discs were cut out with a cork borer and placed into 1 ml of water. The pH of the solution was measured after 3 h.

Staining of gels

Generally staining of acrylamide gels containing ampholine is very time consuming as the ampholytes must be first washed from the gel, and this may take up to several days. Two dyes, Light Green SF and Fast Green FCF, have been used[7] which do not form insoluble complexes with ampholine, but were considered inferior to Comassie Blue.

During the course of this work, a range of dyes was discovered which can be used directly to stain the gel, and which can be washed from the gel in a few hours. The dyes are characterised by being amphoteric, having generally two sulphonic acid groups and one or two charged nitrogens. In addition, the presence of bulky unsubstituted aromatic rings appears to favour the formation of insoluble complexes with ampholine.

The dyes investigated were Kiton Rhodamine B (C.I. 45100), Disulphine Blue VN (C.I. 42045), Fast Acid Blue B (C.I. 44035) and Coomassie Violet R (C.I. 42650). Although Kiton Rhodamine B is probably too weak to be used by itself, it is useful when mixed with other dyes (particulary Disulphide Blue VN and Fast Acid Blue B) as the contrast between the stained protein and the background is greatly enhanced. Although Fast Acid Blue B and Coomassie Violet R do form precipitates with ampholine, this only occurs in the staining liquid, and does not occur within the gel itself.

The dyes have satisfactory fastness properties provided that the pH of the staining or destaining solutions is not below 2.5. For this reason, trichloroacetic acid

* TMED = N,N,N'N'-tetramethylethylenediamine.

cannot be used, and satisfactory results were obtained by the use of aqueous 5% acetic acid both in the staining and destaining solution.

For staining the protein used in these experiments a mixture of 0.05% Fast Acid Blue B and 0.05% Coomassie Violet R in 5% acetic acid was used, followed by destaining in 5% acetic acid. After 4 or 5 h destaining, the background was sufficiently reduced for the gel to be photographed.

For prolonged storage of the gels it is recommended that they be kept in the original destaining solution and no attempt made to completely clear the gel. Even though the background is still somewhat coloured, the contrast obtained is very strong and remains so even after several weeks.

This dye mixture may not be satisfactory for all proteins, and some experimentation with different dyes may be necessary to obtain maximum sensitivity.

RESULTS

In the first experiment samples were applied to the gel at three positons corresponding to the alkaline, neutral and acid regions after the gradient had been

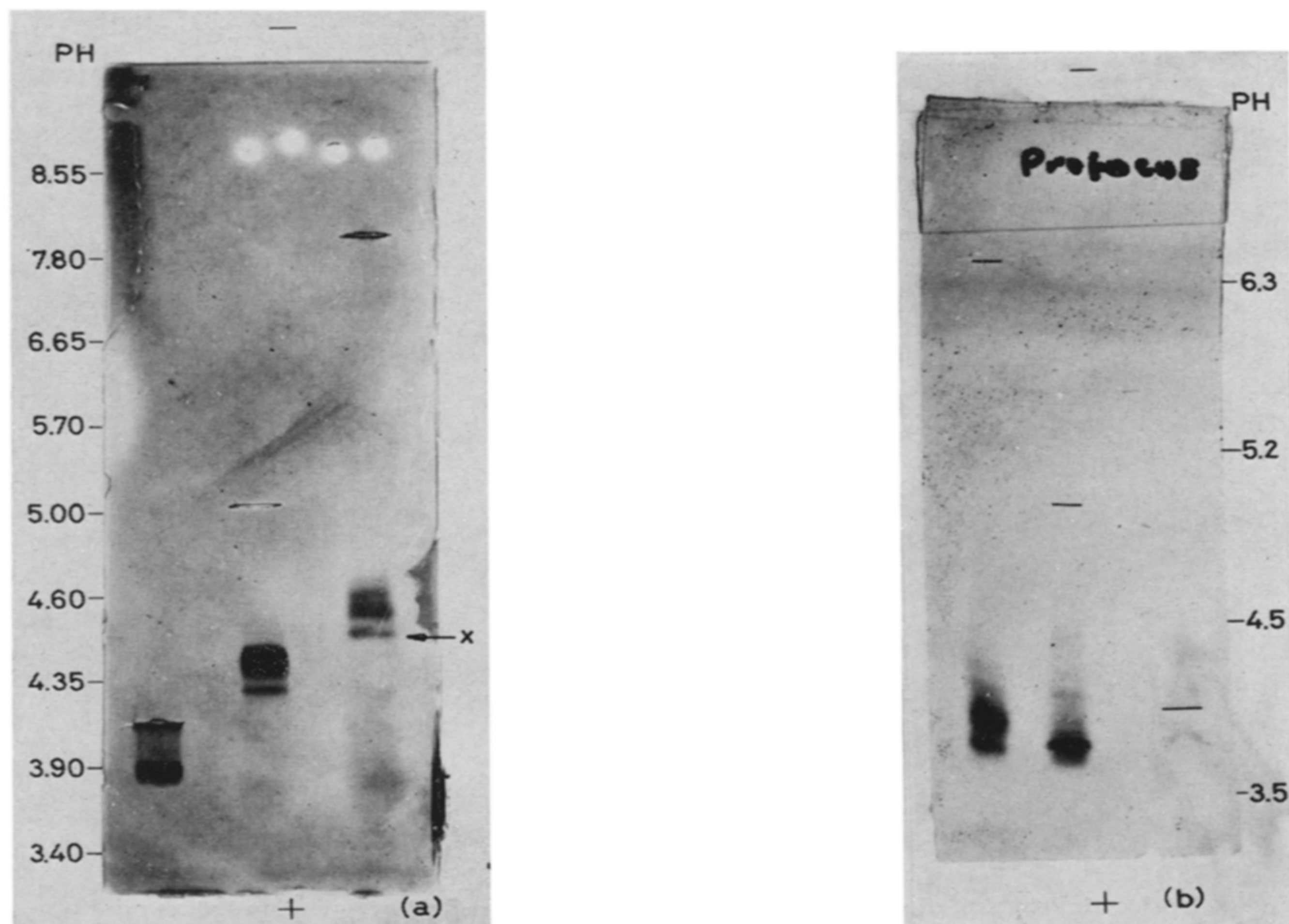
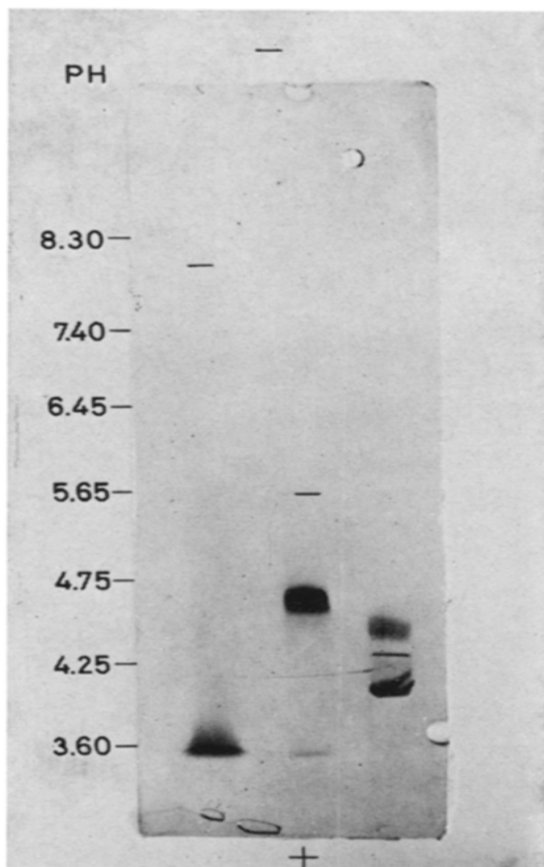


Fig. 1. Isoelectric focusing of a low-sulphur wool protein on acrylamide gel in the presence of 8 *M* urea. The gel contained 2 ml of 3–10 ampholine per 30 ml. (a) No prefocus; 25-h run. (b) 5 h prefocusing, then 20 h with protein; protein loaded at marked slot positions.

established. As can be seen from Fig. 1a the number of protein bands observed and their apparent isoelectric points vary according to the initial position on the gel.

If complex formation were responsible for this result, then it seemed reasonable that the differences seen were due to exposure of the protein to acidic and basic ampholines for different periods of time. Thus the sample loaded near the basic end would be exposed to more basic ampholines while the protein migrated through the establishing gradient than proteins loaded near the acidic end of the gel. If this were the case, then application of the protein after the gradient had been established should give different apparent isoelectric points. A gel was prepared in the normal manner and a potential of 150 V applied for 5 h after which time the current had dropped to a tenth of its initial value and the gradient was assumed to have been established. Samples of protein were then applied at the same three positions on the gel and electrofocusing continued for a further 20 h at 200 V.

The results of this experiment are seen in Fig. 1b and demonstrate that indeed prefocusing does affect the results. Because of the increased number of bands seen and the slightly higher apparent isoelectric points in protein exposed to more basic ampholines, it is likely that complexes are forming predominantly with basic



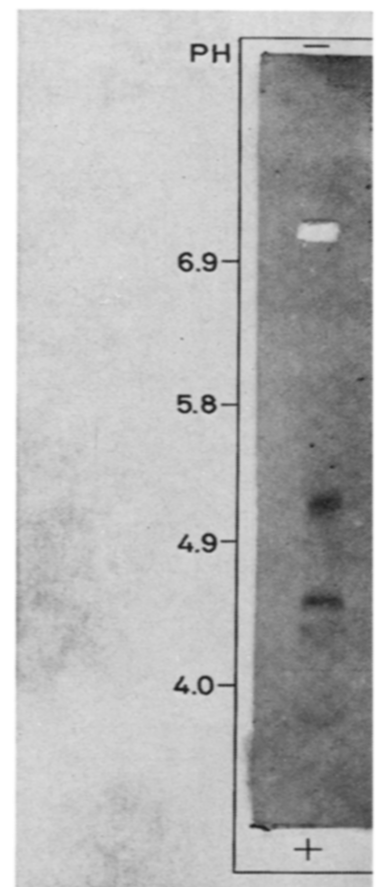


Fig. 2. Isoelectric focusing of wool protein. The gel contained 0.5 ml of 3–10 ampholine per 30 ml. No prefocus, focusing run for 25 h at 200 V.

Fig. 3. Repeated electrofocusing of band taken at point "X" in Fig. 1a. The gel contained 2 ml of 3–10 ampholine per 30 ml. No prefocus.

ampholines. An interesting observation here is that staining of the protein exposed only to acidic ampholines is very much weaker (Fig. 1b) than that exposed to basic ampholines, indicating that protein–ampholine complexes take up more of the acid dye than does the protein alone.

The last two experiments suggest that the results could be explained by complex formation. According to CANN[8], a characteristic of multiple protein zones caused by reversible buffer–protein interactions is that this may be modified by alteration in concentration of one of the constituents. In a demonstration of this phenomenon, the concentration of ampholine was decreased to 0.5 ml/30 ml gel, and samples of protein were run in three positions (no prefocusing). The results (Fig. 2) show that this modification causes marked alterations in the patterns of zones obtained.

Further evidence was obtained by re-electrofocusing one of the protein bands formed during the electrophoresis. To do this it was necessary to locate the protein in the gel without staining, and fortunately this could be done by simply soaking the gel in water to precipitate the protein. Thus the band corresponding to the one marked X in Fig. 1a was cut from the gel and inserted into a trough cut into the basic end of a fresh gel. Electrofocusing was then carried out in the usual manner, and, as can be seen in Fig. 3, multiple zones again reappear.

DISCUSSION

That artifacts may occur during electrofocusing experiments is amply demonstrated by the results presented. The artifacts may be due either to complex formation, or to the formation of multiple boundaries[2]. It should be noted that the positions of bands found are equilibrium positions and that alteration of the conditions of electrophoresis will alter this equilibrium.

As demonstrated for the acidic protein from wool it seems that basic ampholines are involved more than acidic ones and this is also supported by the fact that attempts to electrofocus the protein using a narrow-range ampholine (3–6) did not produce comparable results. In this case, no discrete bands were observed, but a broad, poorly staining smear extending from approx. pH 4.5 to 5.2 was seen.

Since these experiments were performed with only one acidic protein it is important to know if artifacts occur with other proteins. In a limited number of experiments with some basic proteins (myoglobin and haemoglobin) artifacts were not observed. However, in some experiments performed by Dr. J. R. YATES of this Division artifacts were observed in electrofocusing on acrylamide gels of some basic microbial proteases (pronase, alcalase, and serratia protease).

Thus it appears that artifacts may occur in the electrofocusing of both acidic and basic proteins, and this possibility should be considered when using the technique for any protein.

The use of acrylamide gels for these experiments greatly facilitates the observation of artifacts, and is a recommended preliminary before proceeding to larger-scale separations on columns.

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REFERENCES

1 O. VESTERBERG AND H. SVENSSON, *Acta Chem. Scand.*, 20 (1966) 820.
2 M. BIER, *Electrophoresis*, Academic Press, New York, 1959.
3 E. O. P. THOMPSON AND I. J. O'DONNELL, *Australian J. Biol. Sci.*, 17 (1964) 973.
4 E. O. P. THOMPSON AND I. J. O'DONNELL, *Australian J. Biol. Sci.*, 18 (1965) 1207.
5 R. FRATER, *Australian J. Biol. Sci.*, 21 (1968) 815.
6 I. J. O'DONNELL, R. FRATER AND E. O. P. THOMPSON, in W. G. CREWTHER (Editor), *Symposium on Fibrous Proteins, Australia, 1967*, Butterworths (Australia), Sydney, 1968, p. 315.
7 R. F. RILEY AND M. K. COLEMAN, *J. Lab. Clin. Med.*, 72 (1968) 714.
8 J. R. CANN, *Biochemistry*, 5 (1966) 1108.