MODIFICATIONS TO THE TECHNIQUE OF TWO-DIMENSIONAL IMMUNOELECTROPHORESIS

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SUMMARY

1. An examination of the technique of two-dimensional electrophoresis as developed by Minchin Clarke & Freeman (1968) has resulted in improvements for its routine use.

2. These include the use of smaller plates, a re-designed modular electrophoresis tank and modifications in the method of preparing the plates and in the process of running the electrophoresis.

In recent years a number of sensitive immunological techniques have been developed to study proteins in biological fluids. The most recently described is that of Minchin Clarke & Freeman (1966) which is a variant of an immunoelectrophoretic method originally described by Ressler (1960) and given the name of crossed immunoelectrophoresis by Laurell (1966). It is highly sensitive and by using a combination of gel fractionation and the Minchin Clarke & Freeman (1966) technique, Freeman & Smith (1970) claim that more than sixty immunologically distinct proteins can be recognized. With undiluted serum the present authors can recognize as a routine more than thirty individual proteins.

The method consists of an initial electrophoretic separation on a narrow agarose strip; this is then transferred to a second plate, the remainder of which is filled with agarose containing antiserum. The current is next directed at right angles to the initial direction and the partially separated proteins are driven into the bed of antiserum. Each protein appears as a precipitin curve, the area under which is proportional to the concentration of that protein provided the concentration of the antiserum present is constant.

Because this technique is so expensive the procedure has been re-examined and a number of modifications have been introduced which make it more suitable for routine use.

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MATERIALS AND METHODS

The procedure of Minchin Clarke & Freeman (1968) for producing electrophoretic patterns on large (e.g. 10 cm × 10 cm) glass plates was the basis against which subsequent modifications were assessed. The detailed procedure as described by these authors was followed in the production of such plates, except as described. The final concentration of barbitone buffer pH 8.6 in the electrophoresis tank and in the agarose was 0.03 M. Agarose was obtained from L'Industrie Biologique Française S.A., 35 à 39, Quai du Moulin de Cage, Gennevilliers (Seine). Sheep anti-whole human serum was obtained from Paines & Byrne Ltd, Greenford, Middx, U.K. Acetylated albumin which was used as a reference was also obtained from Paines & Byrne Ltd. Except where otherwise stated, all electrophoresis runs were carried out in an immunoelectrophoresis tank type 260 in conjunction with a power supply type 264 both manufactured by Medical and Biological Instrumentation Ltd, Kingsnorth Industrial Estate, Ashford, Kent, U.K.

After the electrophoretic run the plates were washed for 24-48 h in 0.15 M-NaCl with added NaN₃ (1 : 10 000), dried and stained with Amido Black. The serum was obtained from one individual. It was divided into portions which were stored at -10°C. When required a sample was removed from the refrigerator, thawed, used once and the surplus discarded.

EXPERIMENTAL

The effect of plate size on the electrophoretic pattern of human serum

Two sizes of plate were compared: the large plate (10 cm × 10 cm) as used by Minchin Clarke & Freeman (1968), and slide cover glasses (5 cm × 5 cm) obtained from Kodak, the small size plate.

For the small plate, 1 μl of serum was used and the potential gradient in the first dimension was decreased to 8 V/cm and the run was for 35 min. In the second dimension, the potential gradient was 1 V/cm and the run was for 17-19 h, i.e. overnight.

In Fig. 2 (upper) photographs are shown of the two sizes of plate after the development of the protein patterns from the same serum. It can be seen that the pattern on the small plate is identical with that on the large plate. That there is no loss in resolution, both in respect of the number of curves shown and their relative positions, can be seen from Fig. 2 (lower) in which an area of the small plate has been enlarged to the same size as an equivalent area on the large plate.

Because the use of the smaller plates results in a considerable saving in antiserum these have been used exclusively throughout the remainder of the investigation.

Superimposition of plates

During the course of the experiments described a series of small-sized plates were placed on top of one another in the M.B.I. tank during the second stage of the electrophoresis. To run the second dimension in this manner the lint wicks were folded as shown in Fig. 1(a) and the voltage was adjusted to ensure a potential difference of 1.0-1.5 V/cm across each plate. The plates were run overnight. Several experiments have been carried out with the plates arranged in this way and in every case a satisfactory result was obtained. The protein patterns were
closely similar and clearly formed (Fig. 3). As many as six plates can be superimposed but as the number of plates increases, adjustments to the voltage must be made. In addition it becomes increasingly difficult to attach the wicks.

For routine purposes three plates superimposed in this way would seem to be the optimum. Each M.B.I. tank will take four such piles. With a larger number of plates the electric current passing through the plates becomes so great that the pH of the tank buffer changes and when the buffer in this state passes on to the plates it causes the antigen–antibody complex to break down with resultant loss of peaks.

A semi-automated system for the production of plates

Modular tank system. The dimensions of the large plate determined the size of the electrophoretic tank. For the small plates a smaller tank was clearly desirable. In addition, by pre-pouring the plate (see next section) the manipulative work between the first and second dimensions could be greatly decreased. As a result a small modular system has been developed which gives considerable flexibility with economical use of buffer in relation to the number of plates to be run. The basic module (20 cm × 20 cm × 5 cm) is divided into four compartments each 10 cm × 10 cm × 5 cm (Fig. 1b). Each compartment is connected to its diagonal neighbour through the buffered plate and wicks. The interchange of connections as shown enables the current to be run at right angles to the previous direction. The size of the tank can be effectively increased by the use of half modules. Thus by the addition of a half module consisting of two compartments, two carriers can be used and the number of plates doubled. The addition of half modules permits the build up of a very large tank if required. This design of tank makes it possible to simplify the actual manipulations and if both dimensions of the agarose plate are poured before the addition of the sample, subsequent manipulations consist only of changing over the electrical connections.
Preparation of the pre-poured plate. The small plate is initially poured with 2.8 ml of 2% agarose solution mixed with 2.8 ml of barbitone buffer and left for at least 1 h. A strip of agarose gel 1 cm in width is removed and placed on a new plate the rest of which is filled with 2.25 ml of 2% agarose mixed with 2.25 ml of barbitone buffer containing 0.25 ml of anti-whole human serum. Pre-poured plates in this form may be prepared up to 4 h before use.

Fig. 1(b). Module arrangement for one slide showing attachment of modules. The arrows show the direction of current during the first-dimension run.

Carriers and wicks. The Perspex carrier of the Minchin Clarke & Freeman (1968) system was designed to hold the plate in the inverted position in the second dimension only. Since pre-poured plates must be used inverted for both dimensions a new design of carrier was necessary (Fig. 1c). The lint wicks butt up against marker strips on the carrier and support the plate, supplying an even current flow to the agarose gel and allowing a limited air circulation over the surface of the agarose to prevent the build up of excess of moisture. This circulation must be sufficiently large to prevent any condensation formed from coming into contact with the agarose surface, since this causes 'spurring'; nor should it be too great since there is a tendency for the agarose to dry out.

The first-dimension lint wicks are cut so that they are only in contact with the first-dimension strip of agarose. In this way the current is prevented from driving the antigens into the antiserum bed, and neither is the antiserum itself subjected to any voltage that would tend to cause concentration of the antibodies at the cathode and so produce distortion of the final protein pattern. The remaining length of the wick is cut wider so that the potential difference between slide and buffer compartment is kept small. In this manner only a small initial voltage need be maintained across the first dimension buffer compartments, for a fixed voltage of 40 V to be applied across the plate. The wick patterns are shown diagrammatically in Fig. 1(d).

Operation of the technique. To carry out a run the sample well is cut into the first-dimension strip on the pre-poured plate and 1 µl of serum added. The plate is placed inverted on the carrier and the first-dimension connections linked up. The potential gradient is adjusted to
Fig. 2. Upper: protein patterns of the same serum developed on two different sized plates. Lower: equivalent area of plate B magnified to the same size as plate A to show comparable resolution on each size of plate.
Fig. 3. Four plates developed simultaneously by superimposition.
Fig. 4. Comparison of protein patterns with plates prepared according to Minchin Clarke & Freeman (upper) and those prepared by ‘pre-pouring’ (lower).
8 V/cm and the current run for 35–40 min. At the end of this time the electrical connections are switched and the second-dimension voltage, adjusted to 1–2 V/cm and the current run overnight. If the voltage in the first dimension is kept at a total of 40 V across the plates no evidence of excessive heating is encountered and hence no cooling is necessary.

**Fig. 1(c).** Perspex carrier for use with 'pre-poured' plates.

**Fig. 1(d).** Wick system for use with 'pre-poured' plates.

*Results with the modular system.* A comparison of the patterns produced on the pre-poured plates and plates produced with the standard Minchin Clarke & Freeman technique is shown in Fig. 4. As can be seen, there is no loss in detail or clarity. No current leakage occurs from the first-dimension wicks to the second provided that the wicks are not saturated. This has
been shown by measuring the current passing through the plate when 170 V is applied across the buffer compartments in the first dimension, initially when the second-dimension wick is present, and then after its removal. If the wicks previously immersed in buffer are squeezed out before use, the current drops from 4.6 to 4.5 mA when the second-dimension wick is removed. This is a fall of 2% or less. However, if the wicks are saturated, the current drops from 4.9 to 4.5 mA, a fall of approx. 8%. There is no distortion of the first-dimension run unless buffer seeps on to the carrier and comes in contact with the agarose surface. This usually only occurs if the first-dimension voltage is too high, resulting in over-heating of the agarose.

The volume of buffer can be decreased to very small amounts but the optimum volume is about 150 ml/compartment. During the running of the second dimension the pH changes markedly in these compartments, but this is unimportant provided the wick length from slide to buffer is kept sufficiently long to prevent any altered buffer solution from reaching the slide during the second-dimension run. The minimum length required is 8 cm. The maximum length is governed by the initial voltage supplied. If the wicks are too long, a very large voltage is required across the compartments to maintain a potential difference of 40 V across the plate in the first dimension.

**DISCUSSION**

It has been shown in this investigation that by using plates one-quarter the size of those recommended by Minchin Clarke & Freeman (1968) two-dimensional immunoelectrophoretic patterns of human sera can be produced that are closely comparable to those obtained with the larger plates. Two important advantages stem from the use of the small plates.

1. There is a fourfold decrease in the volume of antiserum required.

2. The small plate (5 cm x 5 cm) is a standard cover glass for 35 mm slides, hence it can be directly projected on to a large screen without further processing. This enables individual curves to be more easily identified and greatly facilitates the presentation of the electrophoretic patterns for group discussion.

Two additional modifications in technique have been developed as a result of using the small plates; the use of superimposed plates and also of a semi-automatic modular system. Superimposition enables a threefold increase in the number of plates which may be produced by using the existing equipment. The semi-automatic system is more flexible than the original, since the size of the equipment and the volume of buffer can be graded to the number of plates to be produced. If for example one plate is required, only 600 ml of barbitone buffer would be needed, compared with 3 litres with the original method.

The semi-automatic system requires a plate on which both dimensions have been poured. Since the antiserum diffuses on storage, even at 4°C, into the first-dimension strip, such plates cannot be kept longer than 4 h.

The use of two-dimensional immunoelectrophoresis in clinical medicine has been well demonstrated by many workers. The modifications described here result in a cheaper and more easily produced plate, making the method even more suitable for routine use.

The present authors have used these modifications extensively during the last year in the study of protein changes in the sera of burnt patients and also in the examination of complement concentrations in immune-complex diseases, e.g. rheumatoid arthritis and systemic lupus erythematosus.
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REFERENCES


