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ISOELECTRIC FOCUSING OF *TRYPANOSOMA BRUCEI* SUBGROUP ANTIGENS IN POLYACRYLAMIDE GEL THIN LAYERS

A METHOD FOR RESOLVING AND CHARACTERISING PROTEIN–CARBOHYDRATE COMPLEXES OF AN ENZYMIC AND IMMUNOLOGICAL NATURE

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SUMMARY

The high resolution afforded by isoelectric focusing in thin layers of polyacrylamide gel has been applied to the separation of trypanosome antigens that previously could not be resolved either by column chromatography or by starch-gel electrophoresis. The above method, in combination with characterisation reactions, showed that a group of precipitating antigens were protein–carbohydrate complexes which appeared to have enzymic activity.

INTRODUCTION

Previous studies of proteins of the *Trypanosoma brucei** subgroup organisms have revealed a group of soluble antigens (4S) which appeared to be heterogeneous by immunodiffusion analysis but could not be separated by starch-gel electrophoresis or column chromatography[1]. In order to resolve and identify the antigens further, a method incorporating isoelectric focusing in polyacrylamide gel thin layers followed by characterisation reactions for proteins, carbohydrates, enzymes and immunological activity has been developed.

The principle of isoelectric focusing as first reported by IKEDA AND SUZUKI[2] had little practical application. It was the work of SVENSSON[3] followed by that of VESTERBERG AND SVENSSON[4] which led to the commercial availability of 'Ampholine' carrier electrolytes (LKB Produkter-AB, Sweden), which are a mixture of relatively low-molecular weight ampholytes. Subsequently, there have been reports of iso-

* The *Trypanosoma brucei* subgroup contains the pathogens causing sleeping sickness in man and nagana in cattle, which are widespread in tropical Africa. The organisms have a single flagellum and are classified with the Kinetoplastida, Zoomastigophorea, Protozoa. (HONIGBERG *et al.*, *J. Protozool.*, 11 (1964) 7).

electric focusing in sucrose density gradients[5–10], including a review of the applications by HAGLAND[11], but only a few reports of isoelectric focusing in polyacrylamide gel. Of these, two were based on the disc electrophoresis method[12,13] using the apparatus described by DAVIS[14], and one a polyacrylamide gel thin-layer method[15]. The applications of the latter method have been widened by using it together with the aforementioned characterisation reactions. This method, as reported in detail below, has been applied to the study of *Trypanosoma brucei* subgroup organisms but further valuable information may be obtained when it is applied to other trypanosome groups or biological material.

MATERIALS AND METHODS

Trypanosoma brucei homogenate

Infected rats were bled at peak parasitaemia, using EDTA as an anticoagulant, and the trypanosomes harvested by differential centrifugation[16] and the use of DEAE-cellulose[17]. The packed trypanosomes so obtained were mixed with an equal volume of glass distilled water and homogenised in an all glass tissue grinder. The protein concentration was estimated from the absorption at 280/260 nm of the clear supernatant obtained after centrifuging for 1 h at 4° and 120,000 × *g* in an MSE 'Superspeed 50'. This material was stored, in capillary tubes, at —20° until required.

Preparation of the gel

A 5% polyacrylamide gel containing 2% carrier ampholytes was prepared from the following solutions.

Catalyst stock solution. N,N,N′,N′-tetramethylethylenediamine, 1.4 ml; riboflavin, 14.0 mg; and water to 100.0 ml.

Acrylamide stock solution. N,N′-methylenebisacrylamide, 0.8 g; acrylamide, 30.0 g; and water to 100.0 ml.

When kept at 4° and in the dark both solutions could be stored for at least one month.

Gel mixture. The quantities are sufficient for a plate 20 × 10 cm. 6 ml acrylamide solution were mixed with 1.6 ml of catalyst solution and 0.7 ml of carrier ampholytes, water was then added to make 36.0 ml.

The gel mixture was prepared immediately before use by mixing the stock solutions as shown above. The chemicals for the stock solutions were purchased from Koch–Light, U.K. and the carrier ampholytes of either pH range 3–10 or 5–7 were purchased as 40% solutions from LKB Produkter-AB, Sweden. A gel mould was prepared on a level table using grease-free TLC plates (20 × 10 cm). The lower plate was covered by a sheet of Melinex (200 gauge, 'S' type, ICI Products, Great Britain) overlapping at the sides; the Melinex being held in close contact with the plate by a water-film. The upper plate was raised 1 mm above the lower plate by plastic spacer strips. The overlapping edges of Melinex were raised by means of a horizontal glass rod along the length of the plates in such a way that the Melinex inclined downwards and inwards towards the plates. Using a syringe with an 18 gauge needle attached, gel mixture was carefully run down the inclined Melinex plane to replace the air space between the plates. Photopolymerisation was effected using two 10 Watt fluorescent strip lights placed 5 cm above the gel for 1 h. The whole preparation was inverted, the

top glass slid from the Melinex, and the remainder left to cool at 4° for a further hour before use. The Melinex was carefully peeled off to expose the gel surface and left for 5 min in ambient laboratory conditions, to ensure that the gel surface was suitable for sample application.

Isoelectric focusing

A template was placed under the supporting plate (top plate of mould) as a guide for the application of the samples. The samples were applied to the gel over a rectangle of 1.5 cm long by 1.0 cm wide, commencing 1.5 cm from the plate edge, with 1.0 cm left between applications. Most samples contained 100–400 μg protein in not more than 40 μl of fluid. For dilute samples, volumes of up to 75 μl were applied if they were first absorbed onto a 1.5 cm by 1.0 cm piece of cellulose acetate membrane (Schleicher and Schüll, G.F.R.). The volumes of the samples were adjusted to within 5 μl of each other with glass distilled water. To apply a single sample across the gel, either for a small scale preparation or for comparative analysis, a 1.5 cm strip of cellulose acetate membrane was used to ensure that the sample was applied uniformly to the gel. The rear edge of the rectangle of sample was 5 cm from the anode end of the gel when using pH 5–7 carrier ampholytes and 4 cm from the cathode end of the gel when using pH 3–10 carrier ampholytes. Electrical contact was made by inverting the plate so that the gel rested in a horizontal plane across two carbon rod electrodes 17.5 cm apart. Immediately before placing the gel on the electrodes the cathode was moistened with 5% (v/v) ethylenediamine, and the anode with 5% (v/v) phosphoric acid. Isoelectric focusing was performed in a humid chamber at 4° by applying 350 V (20 V/cm) from a constant voltage supply for 16 h. The initial current was 16 mA but dropped during the first hour to about 2 mA for the remainder of the run. At the completion of isoelectric focusing the cellulose acetate membrane, when used, was removed by moistening with a little glass distilled water before carefully peeling off. The pH gradient was determined by cutting 5 mm discs (No. 2 cork borer) at 5 mm intervals along the length of the gel. Each disc was added to 0.75 ml glass distilled water in a 75 × 10 mm test tube. The pH was measured using a 5 mm diameter glass electrode and a Pye 'Dynacap' pH meter, after equilibrating the samples for 3 h at room temperature. Readings were taken on the expanded scale to an accuracy of 0.02 pH unit. Discs of gel removed from between two samples served as ready reference marks.

Handling of the gel

It was not necessary to remove the gel from the glass plate when all the samples were to be examined in the same manner, but treatment of parts of the gel by different identification techniques was normally required. This necessitated the cutting of strips of gel and their removal from the glass plate. Strips of Melinex, 2 cm wide, were laid over the gel using the original template to indicate where the samples had been run, leaving a small gap, sufficient to admit a scalpel blade, between the strips of Melinex. The gel was cut and the strips, adhering to the Melinex, were cautiously peeled from the glass plate.

Protein staining

A method similar to that described by URIEL[18] for staining protein in agar gel

was found to be suitable for polyacrylamide gel using sulphosalicylic acid[19] as fixative.

Fixing solution. 5% w/v sulphosalicylic acid

Staining solution. Ponceau S (G.T. Gurr Ltd.), 0.1 g; acetic acid, 1.0 *M*, 45 ml; sodium acetate, 0.1 *M*, 45 ml; glycerol, 10 ml.

Destaining solution. Acetic acid, glacial, 20 ml; glycerol, 125 ml; water to 1000 ml.

Except where indicated May and Baker Reagent Grade chemicals were used throughout.

The carrier ampholytes, being aminocarboxylic acids, normally interfered with the staining procedure for proteins and were removed in the present case during fixation of the gel. Sulphosalicylic acid was found to give fixation comparable to trichloroacetic acid and has the advantage of not being so corrosive to the skin. Each strip of gel was fixed for 48 h in four changes of 150 ml of sulphosalicylic acid and stained for 1 h in 0.1% Ponceau S. The gel was readily removed from the Melinex after it had been fixed for about 1 h. A near colourless background was obtained after a few changes of destaining solution.

Carbohydrate staining

The use of Schiff's reagent (sulphited fuchsin) with periodic acid as oxidant has been utilised by McMANNUS[20] and HOTCHKISS[21] for the histochemical characterisation of carbohydrate. This reaction has been used to demonstrate the presence of carbohydrate following the isoelectric focusing of trypanosome homogenate in polyacrylamide gel.

Fixing solution. Acetic acid, glacial, 2 ml; ethyl alcohol, 50 ml; water to 100 ml.

Schiff's reagent. This was prepared according to DE TOMASI's method[22].

Periodic acid solution. 1% w/v periodic acid in 0.2 *M* sodium acetate.

Sulphurous acid solution. 10% sodium metabisulphite, 25 ml; hydrochloric acid, 2 *N*, 25 ml; water to 500 ml.

Glycerinated sulphurous acid solution. 400 ml sulphurous acid solution and 100 ml glycerol.

The strip of gel, attached to the Melinex, was fixed for 3–6 h, during which time it shrank to about half-size and freed itself from the Melinex. Following fixation the gel was rinsed briefly in 0.2 *M* sodium acetate and oxidised in the periodic acid solution for 15 min. Excess periodic acid was removed by washing in running water for 30 min after which time the gel had returned to its original size. Schiff's reagent was diluted with an equal volume of glass distilled water and applied for 10 min. This was followed by three washes of 3 min each in freshly prepared sulphurous acid solution and a final wash in glycerinated sulphurous acid solution for 1 h after which the gel could be stored in the same solution. The results, particularly if there were any faint bands present, were photographed within 24 h. There was no apparent further loss in depth of colour after the first 48 h, and in most instances, after several weeks. As an alternative after photographing, the gels were washed overnight in one change of 5% sulphosalicylic acid and then counterstained for protein. Although a satisfactory result was obtained there was a general loss in intensity of staining compared with the direct method.

Immunological analysis

The gel was transferred to another supporting plate and a frame formed around it so that an area of 17.5 × 2.5 cm was left on one long side of the gel while the other sides were in contact with the frame. The frame was formed from glass strips 3 mm thick cemented into position with a 1.5% agar solution in normal saline containing 0.02% sodium azide. The same agar solution, at 50°, was poured into the enclosed area to a depth of approximately 1.5 cm. When the agar had set a trough was cut, 2.5 mm wide, parallel to and 1.5 cm from the gel strip. The trough was filled with rabbit anti-trypanosome serum and allowed to develop in a humid chamber at room temperature. When the antiserum had diffused into the agar the trough was filled with normal saline containing 0.02% sodium azide and the system left for seven days. The soluble protein was then removed in three changes of normal saline over 48 h and the gels allowed to stain overnight in 0.01% Ponceau S. The slight background colour was removed with protein destaining solution.

Enzyme location

The positions of trypanosome enzymes have been located histochemically as formazan deposits following starch-gel electrophoresis[23]. The same principle has been applied following isoelectric focusing in polyacrylamide gel.

Substrate solution. Phenazine methosulphate (PMS), 1.0 mg; tetrazolium salt, (MTT), 1.5 mg; NAD/NADP, 1.0 mg; magnesium chloride, 0.1 *M*, 0.75 ml; substrate, 0.2 *M*, pH 7.0–7.2, 0.05 ml.

Agar solution. Agar, 78.0 mg; Tris buffer, 0.2 *M* (pH 8.0), 7.0 ml.

Stop bath. Methyl alcohol, 45 ml; acetic acid, glacial, 10 ml; water, 45 ml.

For this procedure, which was completed as soon after the run as possible, the gel was not removed from the glass plate. Strips of glass, 3 mm thick, were used to form a frame around the gel and cemented in position as before. The agar solution was cooled to 50°, mixed with the substrate solution and immediately poured evenly over the gel. Once the agar had set the overlayered gel was incubated in the dark at 37° for 30 min. The enzyme appeared as a blue band. The reaction was stopped, to control nonspecific background colour, by immersing in the stop bath solution for 1 h in the dark.

Photography of results

All results were photographed on Kodak VP 120 film using transmitted light and a good diffusing screen. A green filter enhanced the contrast.

RESULTS AND DISCUSSION

The total soluble trypanosome proteins were resolved within the pH range 4.0–8.6 (Fig. 1a). A group of bands within the pH range 5.5–6.1, which stained for protein, also stained for carbohydrate (Fig. 1b). Similar p*I* values were obtained for this group after isoelectric focusing using narrow range, pH 5–7, carrier ampholytes (Figs. 2a and 2b). Immunodiffusion analysis, using homologous antiserum, gave a common precipitin line covering all components of the group within the range pH 5.5–6.1 (Fig. 3). This suggests that some, or all, of the antigens of this group may have a common antigenic determinant. None of the NAD/NADP specific dehydro-

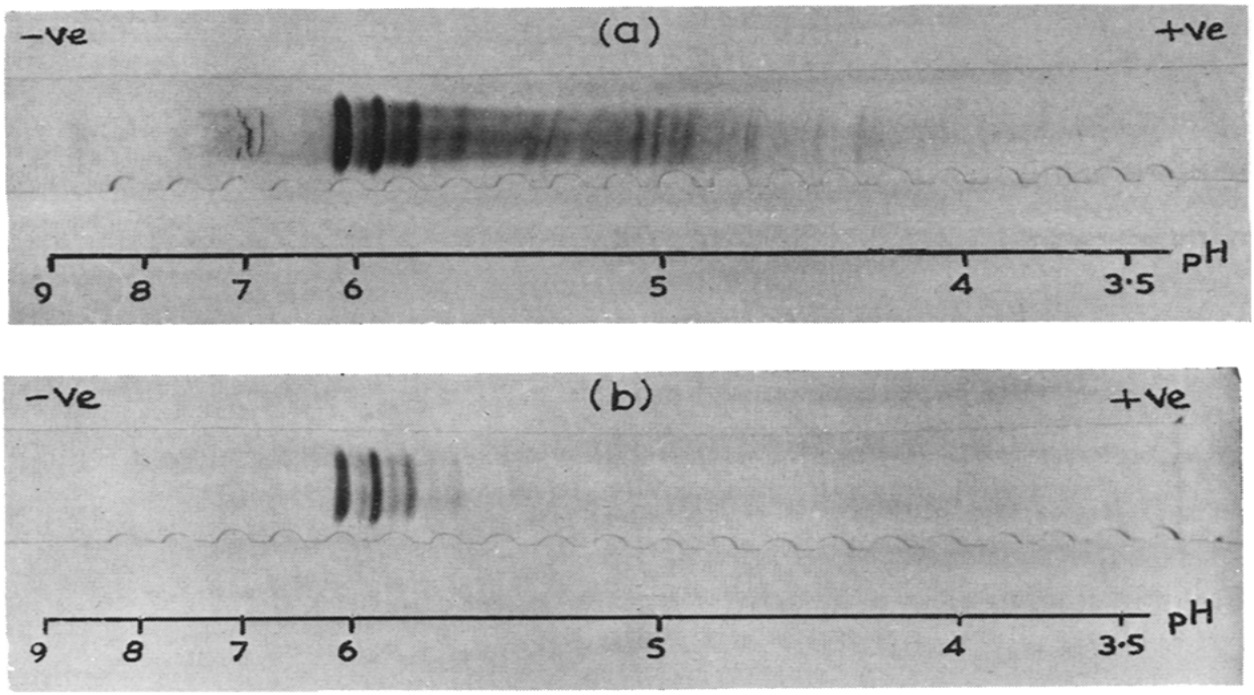

Fig. 1. Isoelectric focusing of trypanosome homogenate using pH 3–10 carrier ampholytes. (a) Stained for protein with Ponceau S; (b) stained for carbohydrate by periodic acid—Schiffs' reaction.

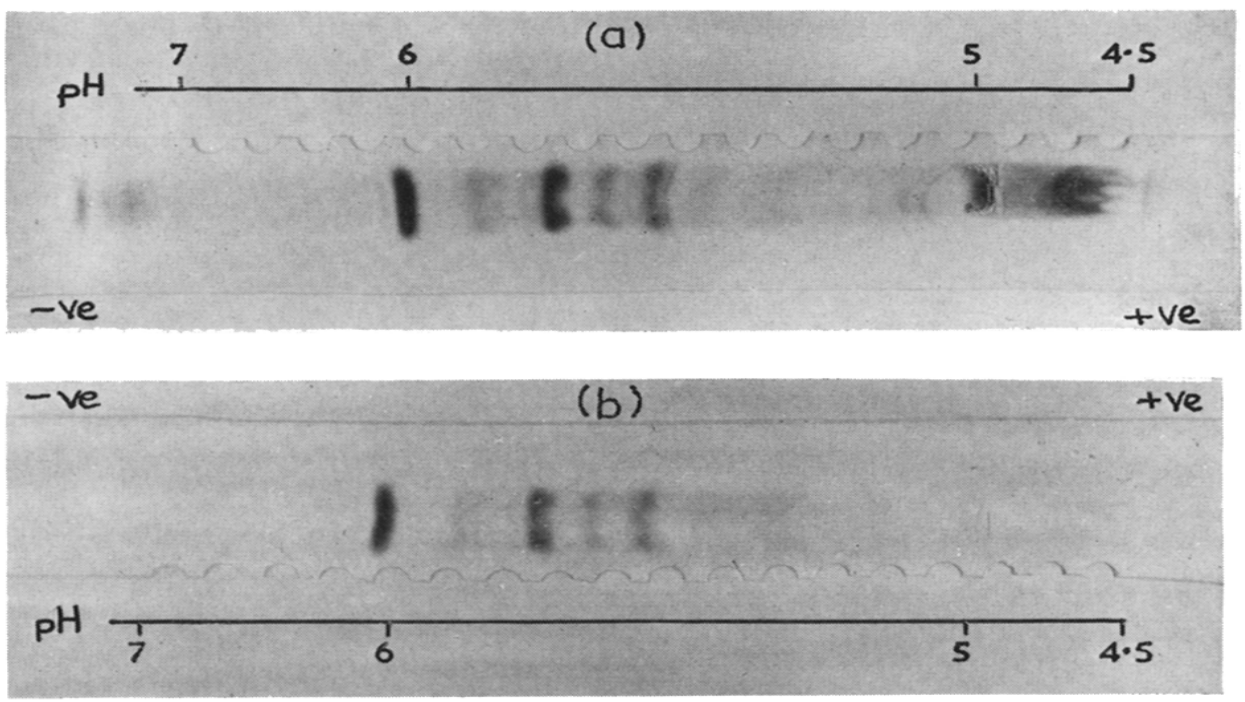

Fig. 2. Isoelectric focusing of trypanosome homogenate using pH 5–7 carrier ampholytes. (a) Stained for protein with Ponceau S; (b) stained for carbohydrate by periodic acid—Schiffs' reaction.

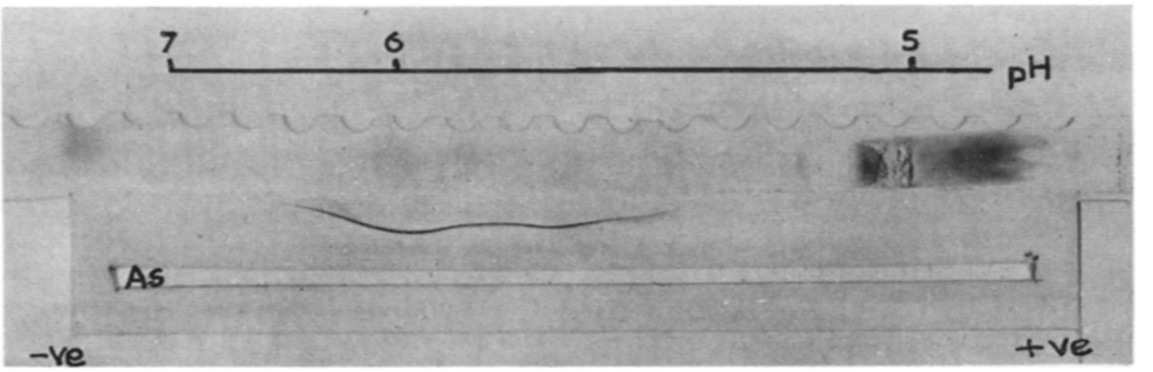

Fig. 3. Immunodiffusion pattern from trypanosome homogenate *vs.* rabbit anti-trypanosome serum (As) after isoelectric focusing of the homogenate using pH 5–7 carrier ampholytes.

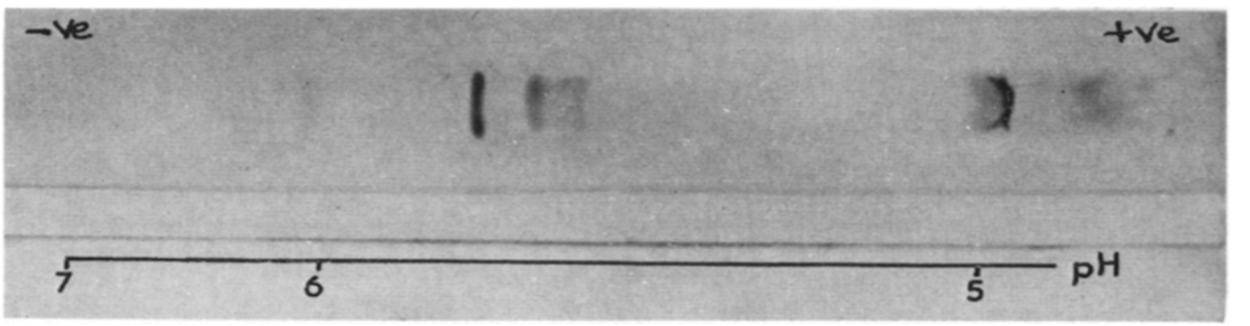

Fig. 4 Location of Ochoa's enzyme in the gel as a formazan deposit following isoelectric focusing of the trypanosome homogenate using pH 5–7 carrier ampholytes.

genases of the glycolytic pathway, known to be present in *Trypanosoma brucei* organisms[24], were located within the range of these protein–carbohydrate complexes. However, not only could Ochoa's malate (decarboxylating) enzyme be located within this group but it would appear to be an isoenzyme (Fig. 4). It was possible to stain for nucleoprotein and lipoprotein following isoelectric focusing but neither were detected in this sample.

Isoelectric focusing in polyacrylamide gel thin layers provides a method of separating complex mixtures containing as little as 100 μg protein, with high sensitivity and high resolution. This method is very favourable as samples, which are incorporated in the gel for the disc method, are not exposed to adverse conditions during polymerisation nor are they present to inhibit polymerisation. Any hydrostatic or electro-osmotic effects are eliminated as the gel is run in a horizontal plane and in direct contact with the electrodes.

The results provided by this method have given a new perspective to the study of trypanosome antigens and antigenic variations and will be discussed elsewhere.

NOTE ADDED IN PROOF

The above method has been applied on a micro-scale to study samples of tsetse fly haemolymph. The gel, 0.75 mm thick, was supported on a 7.5 × 2.5 cm microscope slide and a 10 μl sample containing about 100 μl protein applied to an area 1.0 cm long by 0.5 cm wide. Isoelectric focusing was performed using similar conditions to

those described above after which the pH gradient was determined by eluting 2.5 mm discs, cut at 5.0 mm between centres, in 0.5 ml glass distilled water.

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