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An electrophoretic investigation of rat interstitial fluid proteins

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2015-02-09
http://hdl.handle.net/10539/16943

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jection of rat plasma as before. Another 50 mℓ of blood was again withdrawn from the rabbit seven days after the booster injection. The blood was allowed to clot and the serum carefully removed. The serum from three rabbits were pooled and immunoglobulins isolated according to the method of Harboe and Ingild (1973). The purified immunoglobulins were stored in the refrigerator at 4°C for subsequent use.

3.6.2 Immunoglobulins against interstitial fluid

The same procedure as for rat plasma was used, except that interstitial fluid replaced the rat plasma.

3.7 Electrophoresis

3.7.1 Disc electrophoresis

3.7.1.1 Preparation of mixed Sephadex G-25 polyacrylamide gels (Sephadex gels)

This was done according to the method of Hattingh et al (1978). Sephadex G-25 was swollen overnight in Tris-glycine buffer, pH 8,5 (0,3 g Tris and 1,44 g glycine per 100 mℓ). Equal volumes of 24 per cent polyacrylamide (m/v) containing 0,9 per cent methylenebisacrylamide (m/v), Tris-glycine buffer (as above), 1,2 per cent ammonium persulphate (m/v) and 2 per cent 3-dimethylaminopropionitrile (v/v) in Tris-glycine buffer, pH 8,5 (3 g Tris and 14,4 g glycine per 100 mℓ) were mixed in the above order. The Sephadex sus-

pension, which had been allowed to settle in an inverted syringe for 1 hour, was then immediately added to this mixture in a volume ratio of 1,4 packed Sephadex to 1 of polyacrylamide mixture. The resulting mixture was transferred to a clean syringe and gently mixed for 1 minute. The mixture was poured into glass tubes, 76 mm in length and 5 mm in internal diameter, and distilled water was very carefully layered on top. The whole procedure was performed in a water bath at 24°C.

3.7.1.2 Preparation of polyacrylamide gels (Davies, 1964)

The gels consisted of equal volumes of 20 per cent polyacrylamide (m/v) containing 0,8 per cent methylenebisacrylamide (m/v), Tris glycine buffer (0,3 g Tris and 1,44 g glycine per 100 mℓ), 0,48 per cent ammoniumpersulphate (m/v) and 1,6 per cent 3-dimethylaminopropionitrile (m/v) carefully mixed in this order. This was also poured in 76 mm long glass tubes with an inner diameter of 5 mm. Distilled water was again carefully layered on top of the gels.

3.7.1.3 Electrophoretic separation of plasma and interstitial fluid proteins

After the polyacrylamide gels were allowed to

stand for half an hour and the Sephadex gels for one hour, they were inserted into a Shandon electrophoresis chamber with Tris-glycine buffer, pH 8,5 (0,3 g Tris and 14,4 g glycine) in both compartments and pre-run for 5 minutes at 90 volts.

A 2,5 µl sample of interstitial fluid and a 1,25 µl sample of plasma, each containing about 5 per cent sucrose, were then layered on separate gels. The samples were then subjected to electrophoresis at 90 volts for 20 minutes and at 160 volts for a further 20 minutes in the case of the Sephadex gels and at 160 volts for 30 minutes in the case of acrylamide gels.

After completion of the experiment the gels were removed from the glass tubes in the convential way and stained with amido black (a saturated solution in 5 per cent acetic acid) for 45 minutes or longer. Destaining was done electrophoretically (transversely) in 5 per cent acetic acid in a Shandon destaining apparatus. The destained gels were stored in 5 per cent acetic acid.

3.7.2 Crossed immunoelectrophoresis (Axelsen et al, 1973; Laurell, 1965; Weeke, 1970)

A gel, removed from the glass tube after electrophoretic seperations of the proteins, was placed unstained along the one edge of a clean, degreased glass plate, 50 x 75 x 1,5 mm. One per cent (m/v) agarose in barbital buffer, pH 8,6 (13,1 g barbital-sodium, 2,07 g barbital and 0,4 g calcium lactate per 1 ℓ of solution) was heated to 51°C and antiserum added to this solution. The warm mixture was then carefully poured onto the glass plate containing the Sephadex or acrylamide gel until the thickness of the agarose gel was 1,5 mm. The amount of antiserum added to the plate was such that each plate had 0,3 mℓ antiserum mixed with the agarose.

The plate was placed horizontally on the cooling unit of an electrophoretic developing tank containing barbital buffer, pH 8,6 (see above), in each of the two troughs. A current of 25 mA was applied for 2½ hours while the plates were being constantly cooled by pumping water at 5°C through the cooling unit.

Upon completion the polyacrylamide or Sephadex gel was carefully removed and the agarose layer on the plate was washed, dried and stained with Coomassie Brilliant Blue R stain (0,5 g Coomassie Brilliant Blue R, 45 mℓ 96 per cent ethanol, 10 mℓ concentrated acetic acid and 45 mℓ water). Destaining was done in a mixture consisting of 45 mℓ 96 per cent ethanol, 10 mℓ concentrated acetic acid and 45 mℓ water.

3.7.3 Immunoelectrophoresis

A heated 1 per cent agarose in barbital buffer, pH 8,6 (see section 3.7.2) was carefully poured onto a microscope slide, 2,5 x 75 x 1 mm, until a gel thickness of 1,5 mm was obtained. After setting and cooling of the agarose layer, two small circular holes were made, one on each side of the central longitudinal trough which was cut parallel to the long edge of the plate.

Approximately 2,5 µl sample was placed into each of the two holes and a constant current of 5 mA was applied across the length of each microscope slide, parallel to the trough, to separate the protein mixture into various zones. Constant cooling of the plates with water at 5°C took place. After electrophoresis the trough was filled with the corresponding antibody and the plates were left for 72 hours at 4°C for diffusion to take place. Afterwards the plates were washed, stained and dried in a manner similar to that for crossed immunoelectrophoresis.

3.8 Recordings

3.8.1 Disc electrophoresis

After separation and consequent staining, the protein fractions appeared as bands, varying in width and intensity, at various intervals along the length of the gels. Separation in the case of the acrylamide gel is thought to have been achieved according to both the size and charge of the molecules.

Sephadex gels appeared to separate primarily according to charge with very little interference from molecular size (Hattingh et al, 1978).

Only the polyacrylamide gels were scanned due to the opaqueness of the Sephadex gels. The scanning was done on a Gelman ACD-18 Automatic Computing Densitometer at a wavelength of 550 mm and a slit width of 0,5 x 0,3 mm.

The electrophoretic fractions were assigned numbers from 1 to 9 in order of decreasing mobility starting with the fraction immediately next to albumin. Each fraction was carefully cut out and weighed on a Mettler analytical balance. The concentration of each protein fraction was then calculated from the total protein concentration of that specific sample. In certain cases fractions 2 and 3 as well as fractions 8 and 9 could not be clearly identified and had to be considered as single fractions.

3.8.2 Crossed immunoelectrophoresis

The combination of electrophoretic separation of proteins in polyacrylamide and Sephadex gels followed by electrophoresis perpendicular to these in an antibody-containing gel makes this method superior to the immunoelectrophoretic technique according to Grabar and Williams (1953). (Sections 3.7.3 and 3.8.3.) This method is based upon the electrophoretic migration of

antigens (proteins) in an antibody-containing agarose gel and a specific immunoprecipitation of the antigens by means of the corresponding precipitating antibodies. Individual precipitates are formed for each antigen/antibody system present. The area enclosed by these precipitates is proportional to the antigen/antibody ratio (Axelsen et al, 1973).

3.8.3 Immunoelectrophoresis

This method is also based on an antigen/antibody reaction (section 3.8.2). Because of the electrophoretic separation, the precipitates were dispersed in a characteristic system of arcs with precipitin lines located alongside the electrophoretic zones. The shape of the precipitates is influenced both by the electrophoretic mobility of the antigens (proteins) and by the fact that double diffusion in two dimensions had taken place (Hirschfeld, 1960).

3.9 Cold and heat exposures and exercise

3.9.1 Normal

White rats of both sexes were used for all the different tests. They were housed in individual cages and were given sufficient food and water. The environmental temperature was 20° ± 1°C. Whenever it was necessary to obtain a second sample from the same rat, a recovery period of at least one week was allowed. Each of the standard values were obtained from a different rat. (For sampling procedure see section 3.2.)

3.9.2 Exposure to cold

Rats of both sexes were housed in individual cages in a cold room at 4° ± 1°C for three weeks. They were given sufficient food and water. As it was found that a rat died very easily after blood was withdrawn for the third time by cardiac puncture, it was decided to obtain blood samples only after the termination of the exposure period. Interstitial fluid samples were obtained in the usual way (section 3.2) before commencements of the tests and thereafter at weekly intervals.

3.9.3 Exposure to heat

White rats of both sexes were housed individually and kept in a hot air oven at 32° ± 1°C for a period of three weeks. They had sufficient food and water. Blood samples were also obtained after the termination of the exposure period, but interstitial fluid was sampled before commencement of the test and thereafter at weekly intervals.

3.9.4 Exercise

White rats of both sexes were trained on a variable speed treadmill as follows:

DAY	TIME DURATION (min.)	SPEED (km/h)
1	5	0,5
2	5	0,5
3	5	1,0
4	10	1,0

5	15	1,0
6	15	1,5

On the seventh day (test day) the rats ran for 25 minutes at 1,5 km/h. Interstitial fluid samples were taken immediately before and approximately ten minutes after the final exercise. Blood samples were taken within ten minutes after exercise.

3.10 Chemicals

All the chemicals used were analytically pure (A.R.) and were obtained from Merck and B.D.H. The anti-rat serum protein (produced in the rabbit) was obtained from Dako-immunochemicals. Freund's complete adjuvant from Difco was used. Rat cubes obtained from Epol were fed to the rats. Intraval Sodium (thiopentane sodium) was obtained from Maybaker.

3.11 Statistics

Where necessary results were compared using a paired Students' t-test. The levels of significance are indicated in the various tables.

4. RESULTS

4.1 Disc electrophoresis

4.1.1 Polyacrylamide gel electrophoresis

The samples on these gels were originally [illegible] volts for 20 minutes and then at 160 volts for a further 20 minutes (similar to the Sephadex gels). Due to incomplete separation of the fractions, the latter time was increased to 30 minutes (section 3.7.1.3). After scanning of the gels, the concentrations of the different fractions were then calculated (section 3.8.1). It was then decided to number only those plasma fractions which showed a definite resemblance to the corresponding interstitial fluid fractions. Increasing the number of fractions would have served no purpose as this would have resulted in some fractions having even lower concentrations. This would then have made the comparisons between fractions of the samples more difficult.

The globulin concentration of each sample was taken as being the difference between the total protein and albumin concentrations. Strictly speaking this was incorrect as the fibrinogen concentration was taken as being part of the globulin concentration. As the different fractions were not identified, the one representing fibrinogen could not be taken into isolation. The fibrinogen concentration however, will not have a marked influence on the globulin concentration as it

represents a small portion of the plasma protein (in the human it is about 6,5 per cent according to Harper, 1971 or 0,25 mg per 100 mℓ, according to Wright, 1956). If the fibrinogen value were to be excluded from the results, the globulin concentration would possibly be slightly lower, while the albumin-globulin ratio would be slightly higher. This might effect the comparisons drawn between data obtained from this research work and that obtained by other investigators. It will have a minimal, if any, effect on the conclusions drawn between the normal values and those obtained under the various conditions.

4.1.2 Sephadex gel electrophoresis (Refer to section 3.7.1.1)

Previous investigational work of Hattingh et al (1978) indicated the possibility of obtaining a better separation of certain protein fractions, especially of the albumin fractions, by using these gels. They found peaks in human plasma which implied that circulating plasma albumin consists of several forms with different isoelectric points. (For possible mode of separation see section 3.8.1.)

Comparison between the different disc electrophoretic methods (polyacrylamide and Sephadex) shows a different mode of separation (plates 10 to 13). This is in accordance with Hattingh et al (1978). For a discussion of the results obtained under various conditions see the relevant sections dealing with these.

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Name of thesis An electrophoretic investigation of rat interstitial fluid proteins 1981

PUBLISHER:
University of the Witwatersrand, Johannesburg
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