

The chemical polymerization reaction is effected by using ammonium persulphate together with dimethylaminopropionitrile. Potassium ferricyanide slows the polymerization reaction. Atmospheric oxygen strongly inhibits polymerization whereas ultra-violet light increases the polymerization reaction.

Further versatility of the acrylamide gel can be obtained by gel buffers containing various components such as urea (Bloemendal *et al*, 1962; Cleland, 1964; Cohen & Porter, 1964; Bauer & Thomasson, 1966; Florini & Brivio, 1969; Berkman *et al*, 1969), phenol (Hermans *et al*, 1960; Cotman *et al*, 1968) and glycerol (Jarabak *et al*, 1966; Weinberg *et al*, 1967). Secondly polymers such as agarose (Loening, 1967; Peacock & Dingman, 1968; Dahlberg *et al*, 1969), hydrophilic groups and many more can be added to the gel itself to increase the mechanical strength of low concentration acrylamide gels as well as the resolution of the separating molecules (Gordon, 1975). Thirdly ionic detergents such as sodium dodecyl sulphate and sarkosyl can be introduced in the electrophoretic system (Shapiro *et al*, 1966; Virella & Parkhouse, 1971; Brimacombe *et al*, 1971). Stabilizing agents and cofactors (Ferris *et al*, 1964), ligands (Nikolai & Seal, 1966) or reducing agents (Brewer, 1967) may also be added to the gel.

For these reasons electrophoresis has been widely used as an analytic tool in clinical and research laboratories. At present, one of its most wide-spread applications lies in the field of protein separation and it is indeed from this field that most of the major advances in recent years have derived (Gordon, 1975).

#### 1.2.2 Immunoelectrophoresis

Early in the 20th century Beckhold (1905) discovered that immunochemical reactions could be performed in gels (Clausen, 1971). In 1946 Oudin elaborated this idea by introducing

the diffusion in gels of a single antigen and antibody for quantitative purposes. The basis of his work was extended and modified by Elec (1948). In 1953 Grabar & Williams described immunoelectrophoresis to combine the electrophoretic separation of proteins in an agar gel with a subsequent antigen-antibody reaction performed by immunodiffusion in the same agar gel. It may be performed on a macro-scale (Grabar & Williams, 1953; Poulik, 1958), or as a micromethod (Scheidegger, 1955).

In 1965 Laurell suggested that the resolving power of immunoelectrophoresis (Grabar & Williams, 1953) could be considerably increased by substituting the immunodiffusion step by electrophoresis of the antigens into an antibody-containing agarose gel with the electric field turned  $90^\circ$  to the direction of migration used in the first separation. It also requires only a few hours and is semiquantitative (Laurell, 1965; Ganrot, 1972). The resolving power of this technique is dependent on the resolving power of the first step. Johansson and Stenflow (1971) believed that electrophoresis on polyacrylamide gels, with its high resolving power and excellent reproducibility, would be a powerful tool in the first separation step when high resolution is required. According to Skude and Jeppsson (1972) and Lundahl and Liljas (1975) it is extremely difficult to obtain precipitation patterns which are distinct and reproducible as suggested by Johansson and Stenflow (1971) because of electroendosmosis (to be discussed later). It was also found essential that the thickness of the polyacrylamide gel should be the same as that of the agarose gel used in the second step (Johansson & Stenflow, 1971). The use of polyacrylamide for the second run was not recommended, probably because the molecular sieving effects inhibit the growth of the antigen antibody complexes (Johansson & Stenflow, 1971). The removal of excess antibodies from acrylamide slabs was also found to be difficult (Lundahl & Liljas, 1975). In addition immunoprecipitates develop slowly in acrylamide gels.

The process where different gel media is used for the two

electrophoretic processes has also been described by Ressler (1960a, b), Laurell (1965, 1966), Johansson and Stenflow (1971), Virella and Parkhouse (1971), Grubb (1973), Axelsen and Svendsen (1973), Bjerrum and Lundahl (1974), Lundahl and Liljas (1975) and Sweet and Elvins (1976).

The main obstacle in the use of an acrylamide gel for the first electrophoretic separation, is the difference in electroendosmosis between the agarose gel, containing the antibodies, and the polyacrylamide gel containing the separated antigens (Johansson & Stenflow, 1971; Skude & Jeppsson, 1972; Grubb, 1973; Johansson & Hjertén, 1974). This obstacle can be overcome by using an agarose gel which, like an acrylamide gel, has very little or no electroendosmosis. It is the negatively charged groups, such as sulphate in agarose, which cause distinct electroendosmotic flow and sometimes also adsorption of protein (Johansson & Hjertén, 1974). This may be reduced by reaction of the negatively charged groups with the introduction of positive charges (Grubb, 1973), by incorporation of methylcellulose (Johansson & Stenflow, 1971), or by linear acrylamide polymers (Johansson & Hjertén, 1974; Lundahl & Liljas, 1975). Other workers (Rotbol, 1970; Dames *et al*, 1972) more or less successfully utilized the acrylamide-agarose combination of gels without reducing the electroendosmosis of the agarose.

Although the rates of diffusion of some proteins in acrylamide gels are extremely low (O'ordon, 1975), proteins do diffuse relatively rapidly from zones in the polyacrylamide gel into the agarose gel since the gradient in protein concentration at the surface between the gels is initially high and remains high due to faster spreading of protein in 1% agarose gel (Lundahl & Liljas, 1975).

The choice of buffers is important seeing that if two different buffers are used during crossed immunoelectrophoresis, the current decreases during the second electrophoretic step by 30-50% (Lundahl & Hjertén, 1973). According to Lundahl



and Liljas (1975) the polyacrylamide gel can be soaked in the appropriate buffer.

There are many difficulties and artifacts associated with immunoelectrophoresis which may be the cause of nonreproducible results. Such difficulties are e.g. the antibody-antigen ratio, the concentration of the reactants, the variation in different batches of agarose, antibodies, etc. During melting of the agarose, the temperature should not exceed  $100^{\circ}\text{C}$  or be maintained for too long at this temperature as prolonged heating causes partial destruction of gel structures and the appearance of brownish decomposition products. Artefacts could also possibly be due to interaction of antigens with non-antibody substances e.g. enzymes to form a precipitate, or the duplication of immunoprecipitates, for instance by using horse antibody (Crowle, 1961). In addition artefacts may arise due to immunodiffusion not being performed at constant temperature (Crowle, 1961; see also antiserum preparation). It is possible that, in a slow precipitating system, a soluble complex of the two reactants might diffuse beyond the barrier of the immunoprecipitate to result in a second line of precipitation (Korngold & Van Leeuwen, 1957). Finally, duplication of precipitation lines may be caused by the presence of different determinants attached to the same antigen molecule (Clausen, 1971).

Bispecific antibodies, which are capable of precipitating either of two unrelated antigens, can produce spurs (Ouchterlony, 1962, 1967; Berg, 1964). Antigens may also abnormally bind to the papain fragment Fc of the antibody, whereas the normal site of antigen-antibody binding is the H- and L-chains (Jensen, 1959; Forsgren & Sjöquist, 1966; Gustavson et al, 1967).

There are so many factors which determine the reproducibility of immunoelectrophoretic results that the quality of the results obtained depend primarily on the technical skills of the person performing the experiments (Clausen, 1971).



#### 1.2.2.1 Antisera Preparation

Because of the unavailability of commercially prepared antisera to the plasmas of some of the animals studied, relevant antisera had to be prepared. It is therefore appropriate to review different methods of antisera preparation.

Numerous methods have been proposed for the immunization of various species of animals. Many different species of animals may be used for antibody production, for instance, horse, goat, sheep, pig, duck, hen, rabbit and guinea pig. Experience has shown that the same immunization procedure, and the same antigen dose per unit body mass, may produce variable ratios between the titres and the individual antigens in an antibody-antigen mixture (Clausen, 1971; Harboe & Ingild, 1973).

The horse type of antibody is characterized by forming well defined precipitation arcs because its antigen-antibody complex is soluble in excess of both antigen and antibody (Clausen, 1971). The disadvantages of using horses are that they are expensive and that the horse antibody may easily give rise to multiple immunoprecipitates under unbalanced or unstable conditions. For instance, an unbalanced system may produce migrating precipitates and eventually multiplication of lines. Even established precipitates may dissolve in an excess antigen or antibody (Clausen, 1971).

In order to avoid these effects of multiple immunoprecipitates, rabbit antisera may be preferred. Rabbits are inexpensive, but it has been found that there are large individual variations in the abilities of rabbits to produce antibodies and similarly the antibody titre from the individual rabbit can vary from bleeding to bleeding (Harboe & Ingild, 1973). It is possible to use large groups of animals from which those producing only the specific antibody at optimal titre, may be selected (Clausen, 1971). With rabbit-type antisera the zone of immunoprecipitate shows a sharp contour only at the side

facing the antigen well. Facing the antibody well, the precipitate is blurred because the precipitate in that case is insoluble in excess of antibody. Because the rabbit-type antibody does not dissolve precipitates already formed, the antisera may be used in search of minor impurities in certain preparations of protein and in the study of antigens only present in trace amounts in a sample (Clausen, 1971).

Although the antibodies produced in birds, especially hens, are good, their antisera are not directly applicable in immunochemical reactions, not only because the sera is hyperlipaemic, but also because their antibodies are different to those from mammalian sources (Crowle, 1961).

Many different schedules for antigen injection have been suggested (Gallien et al, 1970; Higgins & Rand, 1974; Sweet & Elvins, 1976). These include daily intravenous injections of antigens, but this consumes a large amount of antigen which is undesirable. It must also be realised that a foreign protein is not a single antigen, but a whole mosaic of antigenic determinants (Schultze & Heremans, 1966), and thus the resistance of the animal may also decrease.

Long term immunization has been found to be more effective. Intradermal or subcutaneous immunization is the most convenient method. The antigen is generally mixed with various local irritants or other adjuvants to overcome the difficulty that some macromolecules are not effective antigens and do not elicit any significant antibody response when the pure antigen is injected (Clausen, 1971). The exact mechanism of operation of adjuvants is still not fully understood. In all probability the adjuvants induce local inflammation with proliferation of macrophages and causing immediate contact with the antigen (Clausen, 1971).

Freund's adjuvant is most widely used and is commercially available in two forms, complete and incomplete. The complete type is the incomplete plus myobacteria, Myobacterium byturicum or M. smegmatus (Crowle, 1961). Both these are tubercle bacilli.

In many cases it is advisable to use the isolated immunoglobulins (antibodies) instead of a total antiserum. For example, immunotechniques, where staining of immunoprecipitates is necessary, demand complete removal of the surplus protein after the antigen-antibody reaction is completed. This washing process is simple if pure immunoglobulins are used. The lipoproteins, especially, give rise to background staining (Harboe & Ingild, 1973). The specific isolation and purification of antibody from antiserum is fundamentally quite simple. The antibody can be precipitated from serum by the addition of a specific soluble precipitating agent. The precipitated complex can be dissolved by adjusting the pH to 3.0. After dissociation the antigens and antibodies can be separated by differential precipitation or centrifugation (Singer & Campbell, 1954). Non-specific methods of antibody purification include isolation of the  $\gamma$ -globulin. This can be done by alcohol precipitation, ammonium sulphate precipitation, by starch-block electrophoresis of the serum, etc. (Campbell *et al*, 1963).

An important factor in the characterization of an antibody is the titre. This can be expressed as the maximal amount of antigen precipitated by 1ml of antibody. The antibody titres can only be compared if the titration methods are identical (Harboe & Ingild, 1973).

### 1.3 PHYSICAL EXERCISE AND HEAT STRESS

Special interest in the biological and physiological changes of body fluids during stress has been shown during the past few years. Heat and physical stress have been receiving a great deal of attention and especially the changes in haematocrit (Hct), plasma volumes, protein concentrations, protein movements, free fatty acids etc., have been some of the main parameters considered.

A wide variety of exercise formats have been used to induce changes in the haematological and haemostatic systems. Pro-



longed physical exercise such as long distance running (Haralambie, 1969; Noakes & Carter, 1976), swimming (Johnson & Wong, 1961; Haralambie et al, 1976) and skiing (Astrand & Saltin, 1964) are mainly used, although short exhaustive physical exercise (De Lanne et al, 1958; Poortmans, 1971; McAlpine et al, 1971; Prentice et al, 1972) is more convenient. Billimoria et al (1959) and Bennett et al (1968), walked their subjects for 2 miles at 4 mph, while Cash and Allen (1967) and McAlpine et al (1971) walked their subjects on a treadmill at 5° elevation for 8 minutes. Biggs et al (1947) and Egeberg (1963), used subjects running up and down a spiral staircase and Menon et al (1969) used a Master's two-step test. A bicycle ergometer is also commonly used (Ikkala et al, 1963; Tibes et al, 1974; Senay et al, 1976).

Plasma protein concentrations and Hct values for untrained and physically trained subjects, before and after an exercise period, were reported by McAlpine et al (1971), Tibes et al (1974) and Haralambie et al (1976). At rest, physically trained subjects had elevated serum levels of  $\alpha_2$ -macroglobulin, transferrin and copper as compared to untrained subjects (Haralambie et al, 1976). On the other hand Tibes et al (1974) reported significantly lower plasma protein values in physically trained as compared to untrained subjects. The values reported were  $68,7 \pm 2,5$  g/l and  $72,2 \pm 1,4$  g/l (mean  $\pm$ SD) respectively. A more significant rise in plasma protein concentrations in untrained than in physically trained subjects after the same working period, was also noted (Tibes et al, 1974). Several of the untrained group showed a much greater maximum effect than their physically trained counterparts, and all untrained subjects showed the effect at much lower levels of exercise (McAlpine et al, 1971). After the first 120 min exercise test of untrained subjects, a significant rise in serum  $\alpha_1$ -acid glycoprotein,  $\alpha_1$ -antitrypsin, haemopexin,  $\alpha_2$ -macroglobulin, ceruloplasmin, transferrin, iron, copper and  $\alpha_2$ -HS-glycoprotein was noted. After a training period of regular physical exercise, the same (now physically trained) subjects only showed small changes following the 120 min exercise test, especially regarding the parameters of iron metabolism (Haralambie et al, 1976). Mc-

Alpine et al (1971) only reported a bond change in the region of the  $\beta$ -globulins following exercise, and this only occurs if fibrinogen is present.

Tibes et al (1974) found an increase in Hct during physical exercise of both physically trained and untrained subjects. Because there is no change in the mean red blood cell mass and in the mean haemoglobin concentration (Nylin, 1947; Böning et al, 1972), the Hct can be used to estimate the changes in plasma protein concentration to be related to changes in plasma volume (Tibes et al, 1974). Thus the increased Hct indicates a relative decrease in plasma volume. If the increase in protein concentration is correlated with the decrease in plasma volume, it follows that the protein increase is fractionally greater than the haemoconcentration (De Lanne et al, 1959; Poortmans, 1971).

Most reports stated no change in red cell count of physically trained subjects following exercise (Kaltreider & Meneely, 1940; Ebert & Steed, 1941; Nylin, 1947; Uehlinger & Bühlman, 1961; Astrand & Saltin, 1964; Ekelund, 1967b). An average decrease in red cell volume was reported by Wennesland et al (1962), but it was within the normal experimental error, while Barbour et al (1925) found addition of erythrocytes from various reservoirs. It has often been stated that under conditions of a constant red cell volume, an increase in Hct after exercise (Senay & Fortney, 1975; Noakes & Carter, 1976), is associated with a decrease in plasma volume (Bazett et al, 1940; Stein et al, 1948; Best & Taylor, 1955; Senay & Fortney, 1975). Over several decades these changes in Hct and plasma volumes have been found to be of equal magnitude (Bazett et al, 1940; Stein et al, 1948; Gregersen, 1959; Saltin, 1964; Bullard et al, 1970; Poortmans, 1971). Mathematical calculation proves that the changes in Hct cannot be numerically equal to a change in plasma volume when the red cell volume is constant (van Beaumont, 1972). Mechanical destruction of the red cell may also occur during prolonged strenuous exercise as reported by Gilligan et al (1943) and Uehlinger and Bühlman (1961).

Senay (1972) suggested that the changes in plasma protein concentration must also be considered when haematocrit ratios are used to interpret plasma volumes.

Authors only experimenting with physically trained subjects and who have found only an increase in plasma protein concentration after muscular activity are Bojanovsky and Filip (1956), De Lanne et al (1958), Tiblin et al (1966), Haralambe (1969), Poortmans (1971) and Noakes and Carter (1976). Haemoconcentration occurring during physical exercise, could be due to a filtration of water out of the vascular bed (Barcroft & Kato, 1915; Landis, 1931; Kaltreider & Meneely, 1940; Ebert & Steed, 1941; Nylin, 1947; Adolph et al, 1947; Holmgren, 1956; De Lanne et al, 1958; Saltin, 1964; Kozlowski & Saltin, 1964; Ekelund, 1967a). During physical activity, muscle metabolites are produced and because of their lower molecular mass they have a greater osmotic pressure and result in a movement of water from the capillary blood into the interstitial fluid (Barcroft & Kato, 1915; Landis, 1931; White et al, 1933). Water is also lost from the body surface in the form of sweat and it is accepted that plasma and interstitial fluid are the main contributors (Adolf et al, 1947; Bass & Henschel, 1956; Giec, 1960; Saltin, 1964). Sweat is hypotonic to body fluids, therefore sweating causes an increase in osmolarity of the body fluids (Kozlowski & Saltin, 1964). Haemoconcentration has also been reported as a result of a higher permeability (Poortmans, 1971), but according to Kjellner (1965), it is clear that it is not the permeability that changes, but that the precapillary sphincters open, which result in an increased surface area available for the exchange of water as well as protein between the blood and tissues. At the same time, the cardiovascular adaptations during exercise probably result in a greater capillary blood pressure (De Lanne et al, 1958) which accelerates this fluid transfer.

Several experiments have been done with subjects exposed to a hot environment with or without mild exercise (Kozlowski & Saltin, 1964; Senay & Christensen, 1965; Senay, 1973; Senay & Fortney, 1975; Noble et al, 1976; Röcker et al,



1976; Senay et al, 1976).

The dehydration of cats (Schultze et al, 1972), mice and rats (Horowitz & Borut, 1975) revealed different results, possibly because dehydration of cats was done by deprivation of water for 48 hours and the rats were kept at an ambient temperature of  $37 \pm 1^{\circ}\text{C}$ . Schultze et al (1972) found that the plasma protein concentration and albumin/globulin ratio remained essentially constant, although the plasma volume of the cats decreased. It was also found that lymph showed a slight albumin increase and plasma a concomitant decrease. During dehydration of mice and rats, it was found that the plasma colloid osmotic pressure (COP) increased and the permeability of the blood capillaries to serum albumin decreased (Borut et al, 1972; Horowitz & Borut, 1973). Schultze et al (1972), working with cats, reported a decrease in plasma volume, while the heat exposed rats maintained their plasma volume (Horowitz & Borut, 1975). The latter authors suggested that the unchanged plasma volume is mainly due to the increase in COP. Thus it seems that heat affects the capillaries and is indeed important for the maintenance of plasma protein concentration. This is also stressed by other authors such as Schultze and Heremans (1966), Reeve and Chen (1970) and Wraight (1974). During water deprivation the extracellular fluid volume was also found to decrease.

Haemodilution occurs in resting subjects exposed to hot environments when water balance is maintained (Bass & Henschel, 1956; Senay & Christensen, 1965; O'Kelly, 1973; Noble et al, 1976). If the balance is not maintained, and the subjects are allowed to dehydrate, haemoconcentration occurs (Kozlowski & Saltin, 1964; Saltin, 1964; Senay, 1975).

A difference in reaction of human males and females to heat exposure has been noticed (Wyndham et al, 1965; Morimoto et al, 1967; Fox et al, 1969; Senay, 1973). These authors also reported females not to haemodilute significantly. Senay (1973) ascribes this response mainly to differences in

the skin surface to blood volume ratio.

The Hct of humans was found not to change much throughout the initial hour of heat exposure (Senay, 1973), while the Hct of bovine blood rose during short periods of heat stress (Noble et al, 1976). The Hct tended to decrease during prolonged heat exposure of cattle, although the effect was not significant (Walshe & Gilles, 1962; Whittow, 1965). Working on cattle, Noble et al (1976) concluded that the increase in Hct is largely a result of an increase in the number of circulating erythrocytes.

There was an increased leaking of proteins, especially albumin, from the vascular bed of pre-acclimatized subjects during dehydration in heat (Glickman et al, 1941; Ladell, 1948; Röcker et al, 1976). Senay (1975), reported that the proteins do not enter the interstitial fluid until the subjects have been exposed to the heat for more than one hour. Acclimatization to heat clearly affected the net transfer of protein to the vascular system during the first hour of heat exposure. Furthermore, an increase in plasma protein concentration, resulting from heat exposure, was also reported by Senay and Christensen (1965), Senay (1970), Röcker et al (1976) and Senay et al (1976). The increase in total circulating protein was significantly greater for acclimatized subjects. If the subjects were not heat acclimatized, the protein concentration decreased (Senay, 1972). The increase in protein concentration during heat exposure was due to the addition of proteins to the vascular system. The increase of the globulins exceeded that of albumin, thus the albumin/globulin ratio decreased during the dehydration period of heat exposure (Glickman et al, 1941; Senay & Christensen, 1965).

Much data has been published on the effect of heat on exercising subjects. The albumin/globulin ratio has been found to increase after an exercise period of heat exposed men (De Lanne et al, 1958; Senay et al, 1976). The increase in con-

centration of albumin at the beginning of activity presumably is the result of a rapid return of protein via the lymph (De Lanne *et al*, 1958; Senay, 1970), which is greater than the increased leaking and filtration of proteins, especially albumin, from the vascular bed, as reported by Ladell (1948). During physical exercise and heat stress vasodilation occurs which could possibly lead to a decrease in blood pressure with a resulting influx of water from the interstitial spaces into the vascular system. Proteins enter the vascular system to rectify the haemodilution caused by the water influx.

Equally important to protein concentration is the total amount of circulating protein within the vascular volume. Concentration and content cannot be compared, since these two parameters can move in the same direction, or they can move in opposite directions. In this study only protein concentration was determined and not total circulating protein.

To summarise, it seems that in most cases physically trained subjects have an elevated protein concentration as compared to untrained subjects. It also seems that similar changes in Hct and protein concentration occur in both physically trained and untrained subjects, with the exception that the changes were found to be more drastic in the untrained subjects. It would appear that the changes in protein concentration and Hct during physical stress can be related to a decrease in plasma volume since there is no change in the red blood cell count. Similar changes in plasma protein concentration and Hct has been found during heat and fever stress, although the cause(s) for the increase may differ between the different types of stress. Where the red blood cell count remained constant during physical exercise, the red blood cell volume has been reported to increase during heat exposure.



## II METHODS

### 2.1 ELECTROPHORETIC METHODS

#### 2.1.1 Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was performed according to the method of Davis (1964) at room temperature in a Shandon electrophoretic chamber, with the exception that a stacked gel was not used. On the grounds of the various properties of the acrylamide gel, protein separation was performed in a 5% homogeneous gel in 0,05 M Tris-glycine buffer at pH 8,6.

The apparatus used and the basic preparation of the gels will be discussed under Sephadex-acrylamide gel electrophoresis. The staining and destaining will also be discussed later. The electrophoretic separation times varied between the two types of gels and also depended on the animal and the type of body fluid.

The solutions used to make the gels were modified from those described by Davis (1964) as follows:

Solution A: 20% Acrylamide  
0,8% NN -methylene-bis-acrylamide

Solution B: 1,6g Dimethylaminopropionitrile  
40ml 0,5M Tris-glycine buffer  
Total volume 100ml (distilled water)

Solution C: 0,48% Ammonium persulphate as catalyst-  
accelerator in distilled water

Tris-glycine Buffer Stock (0,5M):  
3g Tris (hydroxymethyl) methylamine  
14,4g glycine  
100ml distilled water

Running Buffer (0,05M)

10 times diluted Tris-glycine buffer stock.

No potassium ferricyanide was used. Diluted Tris-glycine buffer was used instead as fourth solution. Equal volumes of each of the four solutions were used during the preparation of the gel.

Distilled water was used for all the solutions prepared in this study and they were kept in the dark at 4°C for not more than two weeks. The ammonium persulphate solution was made up fresh every three days.

#### 2.1.2 Sephadex-Acrylamide Gel Electrophoresis (SAGE)

SAGE is a new technique which has been developed in our laboratory during the past few years for the separation of proteins (Hattingh *et al*, 1978). The method involves the preparation of a gel containing Sephadex beads suspended in a polyacrylamide gel and subsequent electrophoresis in this medium in an attempt to obtain better separation of the proteins.

Solution A: 24% Acrylamide  
0,09% NN -methylene-bis-acrylamide

Solution B: 2,0g dimethylaminopropionitrile  
40ml 0,5M Tris-glycine buffer  
Total volume 100ml (distilled water)

Solution C: 1,2% Ammonium persulphate

Tris-glycine Buffer Stock (0,5M):  
3g Tris (Hydroxymethyl) methylamine  
14,4g Glycine  
100ml distilled water

Running Buffer (0,05M)

10 times diluted Tris-glycine buffer stock.

All the solutions were kept in the dark at 4°C. As with PAGE no potassium ferricyanide was used to delay the gelling time. Sephadex G-25 (Superfine) was swollen in running buffer (1g/100ml). This was allowed to mix for 30 minutes with a magnetic stirrer and then left for more than 12 hours to settle at 4°C.

#### Preparation of the gels:

A minimum of one hour before gel pouring, the excess buffer was removed from the swollen Sephadex beads leaving approximately 0,5ml buffer in the beaker. This 0,5ml buffer was mixed with the Sephadex granules to obtain a thick Sephadex suspension. Approximately twice the amount of Sephadex needed (10ml) was drawn up into a 10ml syringe without using a needle. The syringe was left standing on its plunger for 60 to 90 minutes to let the Sephadex and buffer separate. A very thin needle was used to prevent buffer from being forced out. Approximately one hour before gel pouring, all the solutions, as well as the glass tubes were placed in a waterbath at 24,5°C.

In this study glass tubes with a total volume of 1,8ml were used for both PAGE and SAGE. These were 80mm in length and had inside diameters of 0,5mm. Seeing that polymerization of acrylamide only takes place in the absence of O<sub>2</sub>, a space on top of the gel solutions was left for 25 to 50  $\mu$ l distilled water and also for the protein sample after gelling.

The buffer and top few ml of packed Sephadex in the syringe, standing on its plunger, were pushed out (without a needle) until exactly 5,6ml of packed Sephadex was left in the syringe.

As in the case of PAGE, diluted buffer replaced potassium ferricyanide. Equal volumes of the four stock solutions were used, and to obtain a ratio of acrylamide stock solutions to Sephadex of 1: 1,4, exactly 1ml of each stock solution was used. (This ratio was obtained by experimentation).



After the solutions had been mixed in a beaker in the following order, solution A, buffer, solution C and then solution B, the 5,6ml of packed Sephadex was also added.

The resultant mixture was mixed for 30 seconds in the beaker and then drawn into the same syringe (no needle) in which the Sephadex was allowed to settle. An air bubble (0,5ml) was used to obtain a homogeneous solution by tilting the syringe repeatedly and slowly for approximately one minute.

Without delay, the homogeneous solution thus obtained was transferred to the vertically positioned glass tubes with rubber bungs at the bottom. A 10cm long (19G) needle was found to be the most convenient for this purpose in order to reduce pressure during pouring. The mixed solution was poured from the bottom end of the glass tubes. Care was taken that no air bubbles were trapped along the sides.

Immediately after the first Sephadex-acrylamide gel had been poured, 25 to 50  $\mu$ / distilled water was placed very carefully on top of the gel solution in the glass tube without disturbing the surface. For this addition a small hypodermic needle, 23G, attached to a syringe was used. This method undoubtedly provided the most convenient means for a neat and flat gel surface for sample application and the subsequent formation of regular bands.

In the meantime the gel solution in the syringe was kept homogeneous by tilting the syringe regularly.

The pouring process was repeated for all the gels. The reason for pouring one gel at a time was to prevent too much settling of the Sephadex before gelation.

The Sephadex beads settled out until gelling occurred. The acrylamide column forming the top few mm of the gel was found to be no longer than 5 to 6mm (total gel length is 75mm).

The solution prepared as above was enough for only six Sephadex-acrylamide gels. If more gels were required a separate six had to be poured independently. Gelling time was approximately 6 minutes at  $24,5^{\circ}\text{C}$ . Before using the gels a period of 30 to 45 minutes was allowed for complete gelation.

Both PAGE and SAGE was carried out in a Shandon electrophoretic chamber powered by a Shandon-Southern-Vokam Power Unit. Constant voltage and direct current were applied unless mentioned otherwise. At pH 8,6 most proteins are negatively charged and therefore the positive electrode (anode) of the power unit was attached to the bottom electrode of the electrophoretic chamber. The usual voltage and time period was 90 volts for 20 minutes and then 160 volts for a further 20 minutes (this varied for different animal species).

#### Sample Solution:

All protein samples contained 7,5mg/ml of sucrose to increase specific gravity. As a result of the added sucrose the density of this solution was sufficient to prevent mixing with the buffer in the top chamber. The samples were placed on top of the gels after the chambers had been filled with buffer. Sample application was by microsyringe. Volume varied depending on the type of body fluid and the species of animal.

#### Removal of Gels:

Seeing that the Sephadex-acrylamide gel was extremely difficult to work with, special care was taken when handling the gel. For the removal of the acrylamide and Sephadex-acrylamide gel a hypodermic syringe with a long 26G needle was used to inject distilled water between the gel and glass tube. For the more adherent gels this procedure was repeated several times while the tube was gently rotated so that the whole surface of the gel was freed.

### Staining and Destaining:

Because of the absence of reactive groups in the acrylamide gels, a wide variety of staining methods could be applied. Staining these gels to reveal the presence of proteins in particular areas is most commonly carried out either with Coomassie Brilliant Blue, Amido Black or Bromophenol Blue (Gordon, 1975). The proteins in this study were mainly stained with a saturated solution of Amido Black dissolved in 5% aqueous acetic acid for a period of 30 minutes for the acrylamide gels and 45 minutes for the sephadex-acrylamide gels. Electrophoretic destaining was done in a Transverse gel-rod Destainer with 5% acetic acid. Thirty minutes were allowed for the acrylamide gels and a minimum of 45 minutes for the sephadex-acrylamide gels. This electrophoretic destaining method has been described by Richards and Gratzer (1967). The gels were stored in 5% acetic acid.

### Gel Scanning:

The acrylamide gels were scanned using a Vitatron MPS modular photometer system with attached photometric range selector and equipped with a Vitatron integrator. The sephadex-acrylamide gels are opaque and therefore could not be scanned. Photographic representation of the gels was therefore decided upon.

## 2.2 IMMUNOCHEMICAL METHODS

### 2.2.1 Crossed Immunoelectrophoresis

Crossed Immunoelectrophoresis was done with the first zone electrophoretic separation in an acrylamide or Sephadex-acrylamide gel followed by a second electrophoresis of the separated antigens into an agarose gel containing antibodies, with the electrical field perpendicular to the one used in the first separation. This electric field induces migration of the antigens which react with the antibodies to form precipitation zones resembling ascending rockets (Laurell, 1966).



Glass slides ( 8 x 5cm) were degreased by washing them with warm water and soap and thereafter in 95% alcohol before finally rinsing them with distilled water. After drying, the slides were stored at 70°C prior to being coated with melted agarose (1%) to form a very thin film (Wieme, 1959). The newly coated slides were oven dried to ensure that the antibody-containing agarose gel would adhere to the slide.

To cover the glass slide with a 1% agarose solution, 1mm thick, 0,085g of commercial agarose was melted in 8,5ml barbitone buffer. During melting, care was exercised not to exceed 100°C and was not maintained at this temperature for too long since prolonged heating caused partial destruction of gel structures and the appearance of brownish decomposition products (Clausen, 1971). The melted agarose was then covered and oven stored at 70°C until use. A maximum storage period of 90 minutes was permitted.

After gel electrophoresis the gels were removed from the glass tubes as already described. The gels were then placed along the length of the coated slides which in turn were placed horizontally in a perspex mold which prevented overflowing of the agarose.

Before pouring the agarose plate, the agarose was cooled down to 52°C and only then was the desired amount of antibodies added. This was carefully mixed with the liquid agarose, taking care to avoid bubbles. The agarose mixture was then poured onto the glass slide. The agarose-antibody mixture was poured so that the agarose ran against the gel on the side of the slide. Bubble formation in the agarose was avoided. The slide was left for approximately 6 minutes to gel. It was especially important that the agarose

was of equal depth throughout the slide, otherwise, during the crossed immunoelectrophoretic procedure, irregularities would cause asymmetrical migrations. Furthermore, the thickness of the agarose was not to exceed 1mm. A thicker agarose plate could be the cause of difficulties in washing the unreacted antigen and antibody out and also peeling of the agarose from the slide after drying (Clausen, 1971).

The slides were placed on the cold surface of the electrophoretic apparatus which was constantly cooled with circulating water at 4°C from a waterbath. Since some of the plates were placed adjacent to each other, connecting bridges of 4 layers of Whatman no 1 filter paper, soaked with the buffer, were established in order to assure current passage.

At pH 8,6 (Barbitone buffer) most serum proteins migrate to the positive electrode (anode). The second electrophoresis was run at a constant current of 48 mA for 4 plates (3mA/cm). The plates were run for 2,5 hours depending on the amount of antigen applied in relation to antibody concentration.

#### Washing, Staining and Destaining:

The washing, staining and destaining of the agarose plates was done according to the procedures described by Clausen (1971).

After crossed immunoelectrophoresis the plates were placed on filter paper. The gel (acrylamide or Sephadex-acrylamide) was carefully removed. The agarose plate was then covered with a wet Whatman No 50 filter paper. A 4cm thick layer of soft tissue paper was placed on the slide and a pressure of 40g/cm<sup>2</sup> was applied for 15 minutes. During this procedure the gel was squeezed in a very effective manner and the liquid phase of the gel containing most of the non-precipitated proteins was eliminated.

Thereafter the agarose plates were respectively washed in 0,1M NaCl and distilled water to remove the remaining unreacted proteins (antigens and antibodies). The agarose plates were desalted in distilled water. The washing process was followed by a second pressing and then the plates were dried under a current of warm air.

The agarose plates were stained in a Coomassie solution and destained until the background was clear.

Barbitone Buffer: pH8,6

13,14g Barbitone-Na  
2,07g Barbitone  
0,40g Calcium lactate  
1l distilled water

Coomassie Stainer:

5g Coomassie Brilliant Blue R  
450ml Ethanol 96%  
100ml Acetic acid  
450ml Distilled water

Destainer:

250ml Ethanol 96%  
100ml Acetic acid  
450ml Distilled water

### 2.2.2 Immuno-electrophoresis

In this study 1% agarose was used for microimmuno-electrophoresis of normal animal sera against corresponding freshly prepared anti-whole sera. It was thus attempted to determine the effectiveness of the antisera prepared in our laboratory.

The antigen (protein) samples were applied to two suitable wells in the buffered agarose on a microscope slide. The two wells were placed one on each side of a central longitudinal trough which was cut parallel to the long edge of the



plate with a scalpel blade. This method is according to Clausen (1971).

Electrophoresis was carried out ( $2,5\mu$ /per sample) along the length of the microscope slide, parallel to the trough to separate the protein mixture into various zones. A constant current of 5mA per plate was applied. Bromo-phenol Blue was used as tracking dye to determine the time of separation. Constant cooling with water was applied. After electrophoresis the trough was filled with the corresponding antibody (approximately 0,5ml). The plate was then left for 72 h to allow diffusion of the antibody and antigen towards one another. The plates were kept at  $4^{\circ}\text{C}$  to prevent growth of microorganisms.

During the 72 h period, double diffusion took place. Because of the electrophoretic separation, precipitates were dispersed in a characteristic array of areas with precipitated lines located alongside the electrophoretic zones. The curve of the precipitates in immunoelectrophoresis was influenced both by the electrophoretic mobility of the antigens (Hirschfeld, 1960) and by the fact that double diffusion in two dimensions had taken place.

### 2.2.3 Preparation of Antiserum

Polyspecific antisera were required for studies of the proteins of various animals such as cats, baboons, pigs, etc. Seeing that these are not commercially available, the antibodies were prepared in our laboratory. Antiserum to whole serum was prepared in rabbits according to the method of Clausen (1971). The sera of several animals of the same species were pooled. Eleven subcutaneous injections of 0,25 ml serum in 0,25ml complete Freund's adjuvant (Difco) were administered over a 16 week period. A 26G hypodermic needle was used. This adjuvant is known as an immunological adjuvant with a non-specific enhancing effect upon immune responses (Clausen, 1971). The subcutaneous route of injection is reported to be the most effective (Wilkie, 1974). The first eight injections were two weeks

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