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# A comparative study of the proteins in the body fluids of animals

**Coetzee, Naudine**

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apart and the last three inoculations were only one week apart. After seven days the rabbits were bled by cardiac puncture (40 to 50ml/rabbit). No anticoagulant was used. The blood was immediately transferred to a 250ml Erlenmeyer flask and then covered with parafilm. The blood was kept at 4°C for 48 hours. Most of the serum was clear and was separated from the clot before centrifuging the clot for 15 minutes at 9 000 rpm.

The immunoglobulins were isolated from the serum by salt precipitation with ammonium sulphate, according to the method of Harboe and Ingild (1973). The immunoglobulins were purified by dialysing them against distilled water and acetate buffer (0,05 M NaAc, 0,021 M HAc) pH 5,0 followed by chromatography on DEAE Sephadex A50 equilibrated with the acetate buffer. The antibody solution was concentrated by freeze drying and was then dissolved in 5ml distilled water. Finally the immunoglobulins were dialyzed against 0,1M NaCl with 15mM $NaN_3$ (sodium azide) which prevented bacterial growth. The final antibodies were kept in sterile tubes with screw tops at 4°C.

## 2.3 BODY FLUID SAMPLING

### 2.3.1 Blood

Human blood samples were collected from healthy adult volunteers by finger prick under sterile conditions immediately before use. The prick was deep enough to prevent the necessity for any pressure on the finger to obtain blood. Plasma was obtained by centrifuging the blood for three minutes at 3 000 rpm in heparinised microhaematocrit tubes. The microhaematocrit method was used for the determination of the haematocrit (Hct) as described by Korzhuev (1964). To obtain plasma from larger blood samples, the blood was centrifuged at 5 000 rpm for 15 minutes.

Blood samples from laboratory animals used in this study were collected in different ways. Blood from cats and dogs

known that the dye binds to albumin molecules and changes the albumin's relative mobility as shall be discussed later).

The lymph vessel was punctured by a pointed 5cm polyethylene cannula. The external tip diameter was approximately 0,3 to 0,5mm. This length of cannula was used to reduce the resistance for lymph flow. As the lymph reached the other end of the cannula, it was allowed to flow into a heparinised haematocrit tube. No form of heat, nerve stimulation or any other type of stimulation was used to enhance the lymph flow. Passive movements of the limbs were used to facilitate the collection of lymph trapped in the vessels, as described by Lewis and Westcott (1968).

Thoracic duct lymph was also collected in the same manner as femoral lymph. The thoracic duct was cannulated high up in the abdomen. Generally the lymph samples were immediately electrophoresed and analysed for protein content.
If the samples had to be kept until the following day, they were kept at 4°C.

2.3.3 Interstitial Fluid

Experiments were conducted on male and female adult albino rats weighing between 190 and 350g. Interstitial fluid was collected from unperforated cylindrical hollow polypropylene capsules 0,5cm in diameter and 1,5cm in length (developed by Mitchell, D.). This is a modified method of the one described by Guyton et al (1975).
Each animal was anaesthetized with ether and two capsules were implanted subcutaneously on the back with the use of an appropriate sterile surgical technique. Antibiotics were administered for the first week post-operative to prevent infection.

Capsules left in the animal for over one month became partially filled with a fibrous and gelatineous material at the inner circumference of the cylindrical capsule. A small a-

mount of clear fluid was collected from the center (approximately 0,25ml per capsule). It should be emphasized that the results from this study are for subcutaneous tissue only and may not be valid for the interstitial fluid of the whole body.

Blood samples (from the caudal vein) as well as tissue fluid samples were collected from the same animal without anaesthetization. Analysis on the samples were done immediately.

### 2.3.4 Cerebrospinal Fluid (CSF)

Cerebrospinal fluid was collected from anaesthetized New Zealand rabbits and from baboons. The rabbits' heads were fixed in an appropriate position so that the CSF could be directly drawn from the cisterna magna under sterile conditions. After experimentation the animals were kept in the laboratory until complete recovery. The baboons were placed on their sides with their heads bent towards their chests. CSF was then directly collected from the cisterna magna. Approximately 0,5ml CSF was sampled from the rabbits and 0,75ml CSF from the baboons.

### 2.3.5 Aqueous Humor

Aqueous humor was sampled from anaesthetized rabbits and baboons. Bovine and sheep aqueous humor was collected directly after the animal was killed at the abattoir. Aqueous humor sampled from both eyes of the same animal was mixed. Rabbits yielded a total volume of approximately 0,25ml to 0,3ml and baboons 0,1ml to 0,2ml. Sheep yielded aqueous humor volumes of 1,0ml to 1,5ml per animal and samples collected from the cattle varied between 3,0ml to 4,0ml.

Aqueous humor and CSF samples which had to be concentrated, were lyophilized at -70 °C.

## 2.4 BIOCHEMICAL ANALYSES

### 2.4.1 Protein determination

2.4.1.1 Lowry-Folin method

This procedure is for the measurement of protein with the Folin-phenol reagent after alkaline copper treatment (Lowry et al, 1951). It is sensitive to the tyrosine content of the protein. In this study the Folin-phenol reaction used for the protein determination, was done according to the method of Lowry et al (1951) using BSA as a standard.

2.4.1.2 Biuret method

Although the Biuret method is not as sensitive as the Lowry for the determination of protein concentration, it was used for the determination of the protein content of the purified lower albumin sample since the Lowry-Folin method includes free tyrosine as protein. The presence of at least three peptide bonds (tetrapeptide) is required for the characteristic colour to appear. The method was performed according to that described by Gornall et al (1949).

2.4.2 Kjeldahl method for Nitrogen

The Kjeldahl method is a classical method used for the estimation of total nitrogen on a micro scale (Kjeldahl, 1883). It is a time-consuming method and nucleic acids and other non-proteins are also estimated. The nitrogen value was multiplied by a factor 6,25 in order to yield the amount of protein, or rather of polypeptide chains present (West et al, 1966).

2.4.3 Bromo-Cresol Green method for Albumin

The Bromo-Cresol Green method (Bartholomew & Delaney, 1966) is for the determination of albumin as it measures albumin concentration quickly and accurately. To identify albumin

on the gels after electrophoresis, the gels were submerged in a Bromo-Cresol Green solution. The albumins appeared as yellow-green bands.

### 2.4.4 Nitroso R-Salt method for Transferrin

This method as described by Mueller et al (1962), is used for the detection of iron-bound transferrins, which appear as faint distinct green bands after the proteins have been separated by electrophoresis. Occasionally, the transferrins were saturated by addition of equal volumes of plasma and a mixture of 0,005N HCl and 6$\mu$g/ml ferrichloride. The unbound iron was removed after 15 minutes with $MgCO_3$, Mueller et al (1962).

### 2.4.5 Cyanmethaemoglobin method for Haemoglobin

The haemoglobin (Hb)concentration was determined as cyanide methaemoglobin according to themethod described by Kleihauer and Betke (1957).

## 2.5 ESTIMATION OF RADIOACTIVITY IN GELS

The most suitable method for the estimation of radioactivity attached to certain individual plasma proteins, is to slice the gels after electrophoretic separation of the radio-labelled proteins. Electrophoresis of radio-labelled plasma proteins was done to determine the ability of plasma proteins to transport substances such as tryptophan, thyroxine, fats and iron. This method was also used to ascertain the effects of physiological stress conditions on the ability of plasma proteins to transport the above mentioned substances.

The rubber-like texture of acrylamide gels makes them very easy to handle, but unfortunately this elasticity has the disadvantage that cutting, either with a taught wire or a scalpel blade, is difficult. Similarly, the Sephadex-acrylamide gels are also difficult to cut since they have a very soft and sticky texture. The gels could, however, be

sliced very conveniently by either one of the above mentioned methods when preceeded by freezing.

The gels were placed lengthwise in a gutter of a special gel holder suitable for gel slicing. The evenly spaced grooves of the gel holder resulted in neat transverse disc sections after cutting. Because the Sephadex-acrylamide gels were very soft and adhered to any dry surface, the gel holder was submerged in 5% acetic acid when the gels were placed in the troughs of the holder. After straightening, the gels and holder were carefully rinsed with 96% alcohol which was at room temperature. Thereafter the gel and holder were immersed in a beaker containing alcohol at -70°C. Within 30 seconds the gels were frozen and ready to cut. A sharp scalpel blade was found to be the most effective. Each slice was 1,5mm thick.

The gel slices were then rendered soluble with 400$\mu l$ hydrogen peroxide ($H_2O_2$) at 50°C as described by Young and Fulhorst (1965). The acrylamide slices were left at 50°C for approximately 40 minutes and the Sephadex-acrylamide gel slices for about 80 minutes to dissolve. After the incubation period, 10ml of an appropriate scintillation fluid (Aquagel) were mixed with the $H_2O_2$ containing the radioactivity. Thereafter it was transferred to a "Tricarb" radioactive counter (Gordon, 1975).

## 2.6 PHYSICAL EXERCISE, HEAT STRESS AND FEVER CONDITIONS

### 2.6.1 Physical Exercise

#### 2.6.1.1 Controlled laboratory conditions

Seven subjects were used of whom two were white and the rest were black. Blood samples were taken from these subjects before and after a 5 hour treadmill exercise at approximately 45% $VO_2$ maximum. This short exhaustive exercise was repeated after six weeks of intensive physical training. Haematocrit, protein concentration and electrophoretic protein separations were performed on

each sample.

2.6.1.2 Field conditions

The same haematological analyses described above, were also performed on blood samples taken from trained athletes of all age groups before and after competing in long distance races, the distances of which varied between 16 and 96 km. Blood samples were also collected from trained subjects before and after competing in the "Veterans marathon" (distance 42 km, minimum age 45 y). In total, blood samples were collected from 53 athletes in this manner.

2.6.2 Fever Conditions

Fever was induced in six unanaesthetized white New Zealand rabbits by intravenous administration of 1ml (3$\mu$g/ml) bacterial endotoxin from Salmonella typhosa dissolved in saline. Blood samples were taken from the marginal ear vein before the onset of fever and at maximum body temperature and used immediately. Rectal temperatures were measured using indwelling thermometer probes (Yellow Spring Instruments) inserted to a depth of about 10cm. Haematocrit and plasma protein concentrations were determined and electrophoretic studies conducted. Plasma samples were labelled with radioactive tryptophan.

2.6.3 Heat Stress

Rats, in which capsules had been implanted as already described, were incubated at 37°C for up to three weeks. Interstitial fluid samples and blood samples were collected before incubation and every week after commencement of incubation. Interstitial fluid samples were taken alternatively from the two implanted capsules. During the incubation period the rats were on a balanced diet similar to that of the pre-incubation period and water was supplied ad libitum.

## 2.7 MATERIALS

### 2.7.1 Chemicals

Acrylamide, NN'-methylene-bis-acrylamide, 3-dimethylamino-propionitrile, ammonium persulphate, glycine, tris (hydroxymethyl) methylamine, glacial acetic acid, sucrose, barbitone sodium, sodium azide, Bromo-phenol Blue, Bromo-cresol Green, Folin, Coicalteu's Fenol reagent, bovine and human serum albumin, and Nitroso R-salt were all obtained from BDH Chemicals (AR grade).

Amidoblack 10B, Coomassie Brilliant Blue R, Evans Blue and Copper Sulphate were obtained from Merck.

Sephadex was obtained from Pharmacia Laboratories
Agarose from Sea Kem (ME)
Calcium lactate from Riedel-de Haën
Sodium chloride from Protea
Sodium hydroxide from Kanto Chemicals
Aquagel from Chemlab
Heparin B.P. 5 000 IU/ml from Evans Medical LTD.

### 2.7.2 Antibodies and Antisera

anti-bovine plasma proteins produced in rabbits from Miles
anti-$\alpha_1$antichymotrypsin produced in rabbits from ONTH (Hoechst)
anti-$\alpha_1$lipoprotein produced in rabbits from ORCC (Hoechst)
anti-human serum proteins produced in rabbits from Dako
anti-human prealbumin produced in rabbits from Dako
anti-human ceruloplasmin produced in rabbits from Dako
anti-human haemopexin produced in rabbits from Dako
anti-human $\alpha_2$-macroglobulin produced in rabbits from Dako
anti-human orosomucoid ($\alpha_1$-acid-glycoprotein) produced in rabbits from Dako
anti-human transferrin produced in rabbits from Dako
anti-rabbit serum produced in swine from Dako
anti-rat serum produced in rabbits from Dako.

# III RESULTS

## 3.1 Development of the Sephadex-Acrylamide Gel

Because of its versatility, polyacrylamide gel electrophoresis is extensively used in the separation of proteins. Combination of the electrophoretic technique with the porous character of acrylamide gels results in the separation of proteins based on the isoelectric point (pI) as well as molecular mass (m.m.) of each individual protein. The degree of porosity of the acrylamide gel is dependant upon total acrylamide concentration and the number of cross-linkages (Gordon, 1975). For the above reasons, polyacrylamide gel electrophoresis is suitable not only for simple protein separation, but also for determination of isoelectric points and molecular masses.

During the past few years, separation of plasma proteins was approached differently by the development of a new gel medium (Hattingh et al,1978) in our laboratory. The aim was to establish a gel medium with a large pore diameter, thereby reducing or eliminating retardation of protein molecules due to molecular size and conformation. A very low concentration acrylamide gel with relatively little cross-linking of the acrylamide polymers would ideally suit this purpose. However, the consistency and texture of pure low concentration acrylamide gels exclude the practical implementation of them in the identification and characterization of proteins separated electrophoretically. This problem was partly overcome by the incorporation of a stable, inert physical support (Sephadex) into the gel matrix of low concentration acrylamide gels. It was found that some plasma proteins (e.g. albumin and transferrin) could be separated into more components with the new sephadex-acrylamide gel than with the conventional polyacrylamide gels.

In order to establish a technique which would yield optimum results, several variations of the basic one, as described in the methods section, were investigated. These included variations in concentrations of components such as acrylamide and Sephadex, ommision of components e.g. ferricyanide, different buffer pH's and gel lengths. During this phase of the work, it was noticed that when Bromo-phenol Blue (BpB) was used as a tracking dye, the excess BpB migrated ahead of the albumin-BpB complex until the sephadex-acrylamide section of the gel was reached, whereupon the BpB (excess) was retarded and was bypassed by the albumin-BpB complex (see section 3.1.3).

Various Sephadex types were investigated with regard to suitability for incorporation as mechanical support in the low concentration acrylamide gels. These were G-10, G-15, G-25 Superfine, G-50 Superfine, G-75 Superfine, G-100 Superfine, G-150 Superfine and G-200 Superfine as well as DEAE and LH-20 Sephadex. Sephadex G-25 Superfine was found to be the most suitable because sedimentation of the beads during gel preparation and their exclusion limits were important criteria. Sedimentation of Sephadex G-10 and G-15 during gel preparation was relatively rapid, resulting in a relatively long upper gel section of clear polyacrylamide and consequent poor separation of proteins. Of the remaining Sephadex types, only Sephadex G-25 Superfine has a suitable exclusion limit (Above 5,000 daltons).

As described in the methods section, the Sephadex beads were swollen in buffer prior to gel preparation. The use of other gel stock solutions for this pretreatment of the Sephadex beads were also investigated. It was found that swelling of the Sephadex beads in either of these stock solutions (acrylamide, dimenthylaminopropionitrile and ammonium persulphate) resulted in a rigid gel adhering to the glass preparation tube. As a result removal of the gel without damage was impossible.

Because of its importance in determining the number of cross-linkages and also to some degree the acrylamide concentration of the gel (see p.29 ), various concentrations of bisacrylamide were also used. At low concentrations, sedimentation of the Sephadex beads during gel preparation was found to be undesirably high. Bisacrylamide concentrations higher than 0,9% resulted in a final acrylamide concentration which was too great for optimal separation conditions.

Although it was found that the sedimentation rate of Sephadex beads during gel preparation was a critical factor, it was equally important to ensure the presence of a short (approximately 6% of total gel length) upper gel section of clear polyacrylamide. For this reason ferricyanide, which retards gelation, was omitted and the ammonium persulphate concentration was increased. When ammonium persulphate concentration was above 1,2% (m/v), however, gelation occurred almost immediately, the resultant gel having too short a section of clear polyacrylamide.

### 3.1.1 Buffer pH

The buffer pH was also varied between 7 and 9,5. It was found that the plasma proteins separated much slower at pH7 than at pH9 as would be expected. Alpha$_2$-macroglobulin and transferrin separated much further from one another at a lower pH, presumably because of a difference in pI's. Albumin was found to separate into an upper and lower band at pH 8,95 as will be discussed in this study (see section 3.1.4). The upper albumin bands were identified by using BpB (and BcG) as tracking dyes on the control gels. By doing this the position of the upper albumin was located. At pH 8,0 no separation of the albumins was found whereas at pH 7,0 the positions of the two albumins on the gel were reversed. It appeared that the lower albumin migrated slower at pH 7,0 than the upper albumin.

Albumin, like most other plasma proteins, is negatively charged during electrophoresis at above mentioned pH values, and this state can be manipulated by the change in buffer pH (Rosenoer et al, 1977; p 10). This change in charged state of the proteins at different pH values could be the cause of the shift in the albumin positions noticed in this study.

### 3.1.2 Evaluation of results

A major disadvantage of the sephadex-acrylamide gel is the soft, opaque nature of the gel. Because it is soft and stretches easily, photographic presentation of results is made difficult and measurements of relative mobility have to be taken with care. The opaque nature of the gel in the Sephadex region results in poor visual resolution of separated proteins as compared to conventional polyacrylamide gels. For this reason quantification by conventional scanning methods is also impossible. Qualitative and quantitative evaluation of results can nevertheless be achieved by crossed immunoelectrophoresis and other techniques.

### 3.1.3 Tracking Dyes

During the development of the sephadex-acrylamide technique, tracking dyes were used to fascilitate observations on both acrylamide and sephadex-acrylamide gels. Dyes such as Bromo-phenol Blue (BpB), Bromo-cresol Green (BcG), Bromo-phenol Red (BpR) and Evans Blue were used. Erroneous densitometric estimation of albumin concentration was found when using BpB (Hattingh et al, 1977). It was observed that BpR did not bind to albumin and migrated fa r than unbound BpB on a polyacrylamide gel. Bromophenol Red was also found not to influence the densitometric estimation of albumin concentration. Evans Blue bound to some extent to albumin and had the same result as BpB. The excess Evans Blue hardly migrated into either the polyacrylamide or sephadex-acrylamide gel.

Erroneous densitometric estimation of albumin in the presence of BpB, was shown as follows. Bromo-phenol Blue was added to baboon plasma in a concentration of 1mg/ml and used as stock solution to prepare final BpB concentrations of 0,001, 0,01; 0,1 and 0,5 mg/ml. Albumin concentration was determined electrophoretically (see section 2). Both Coomassie Blue and Amido Black were used for gel staining. No statistical differences in electrophoretic banding and/or staining densities of the individual proteins were found. Amido Black was therefore used routinely. Albumin concentration of the plasma samples not pretreated with BpB was arbitrarily taken as 100%. In Table 1 it is shown that BpB resulted in relatively higher densitometric estimation of albumin concentration compared to homologous plasma samples lacking BpB ($P < 0,001$).

Table 1

Relative concentration (%) of plasma albumin prestained with BpB compared to untreated plasma albumin (100%).

| Stock solution | BpB (mg/ml) 0 | 0,001 | 0,01 | 0,1 | 0,5 |
|---|---|---|---|---|---|
| Plasma | 100 | 104 | 107 | 114 | 114 |
| | 100 | | 125 | 133 | 129 |
| | 100 | 105 | 116 | 129 | 129 |
| | 100 | 107 | 107 | 114 | 134 |
| $\bar{x}$ | 100 | 105,3 | 113,8 | 122,5 | 126,5 |
| SD | - | 1,5 | 8,6 | 1,0 | 8,7 |
| n | 4 | 3 | 4 | 4 | 4 |

Bromo-phenol Blue also influenced the area of the gel occupied by the albumin fraction, as shown in Fig. 1. This effect of BpB was restricted to the albumin fraction only. Prestaining of the plasma sample with BpB prior to electrophoresis resulted in the albumin fraction occupying a larger area of polyacrylamide gel (Fig. 1b) compared to plasma not prestained with BpB (Fig. 1a). In the sephadex-acrylamide gel, prestaining of the plasma with BpB resulted in a

decreased albumin width (Fig. 1c-1e). Furthermore, the gel area occupied by albumin prestained with a high concentration of BpB (0,5 mg/ml) corresponded to the area occupied by the lower albumin of the plasma not prestained with BpB. This would seem to indicate that the upper albumin when bound to BpB migrates along with the lower unbound albumin in the sephadex-acrylamide gel.

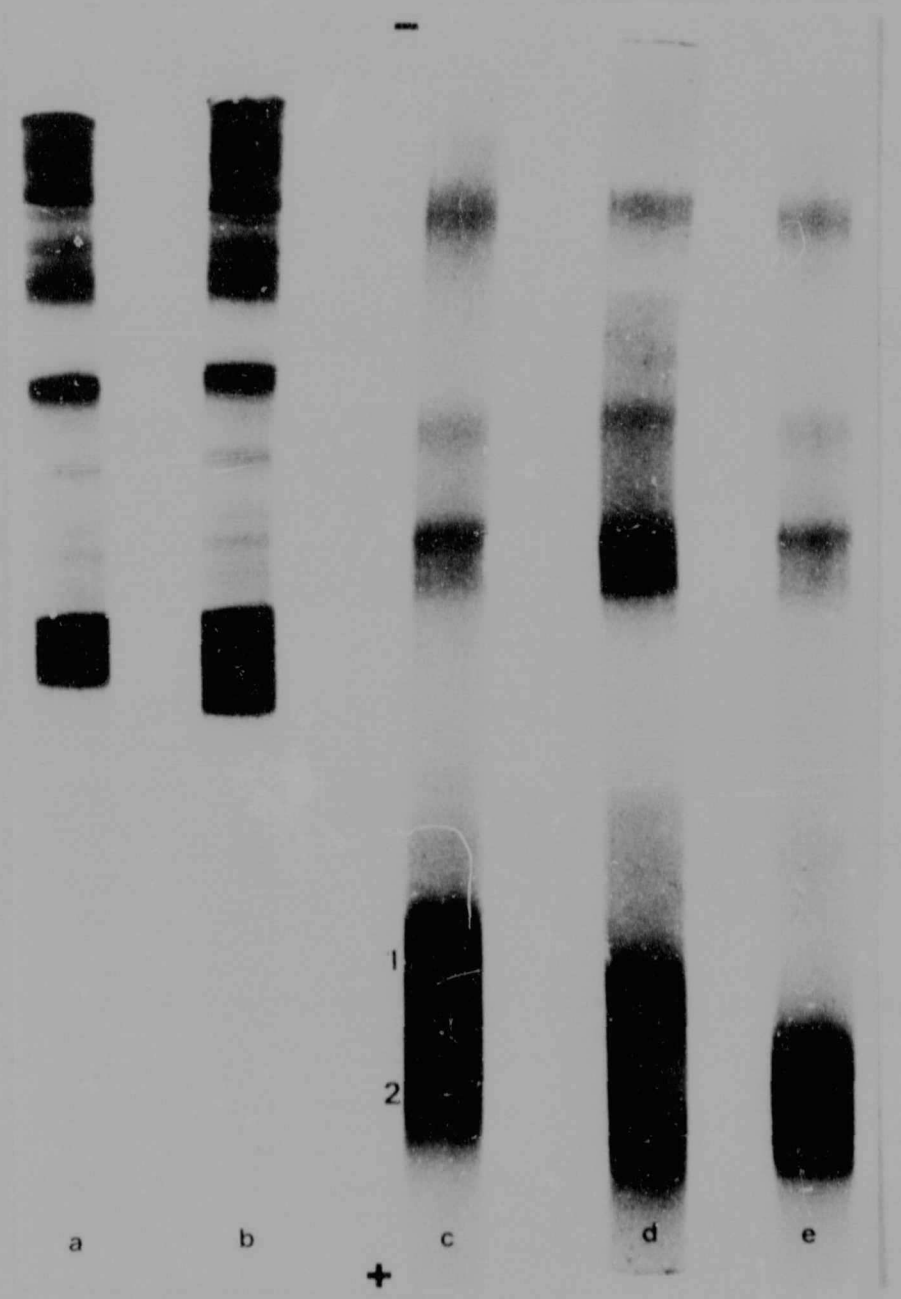



Figure 1
Effect of BpB on albumin during PAGE (a and b) and SAGE (c, d and e) a=no BpB; b=0,5mg/ml BpB; c=no BpB; d=0,01mg/ml BpB; e=0,5mg/ml BpB. (1) upper albumin (2) lower albumin.

3.1.4 Separation of Proteins

Because molecular sieving in the sephadex-acrylamide gel is minimized, the positions of some plasma proteins after electrophoresis on sephadex-acrylamide was found to be different to the positions of the same plasma proteins after electrophoresis on polyacrylamide, as shown in Fig. 2.

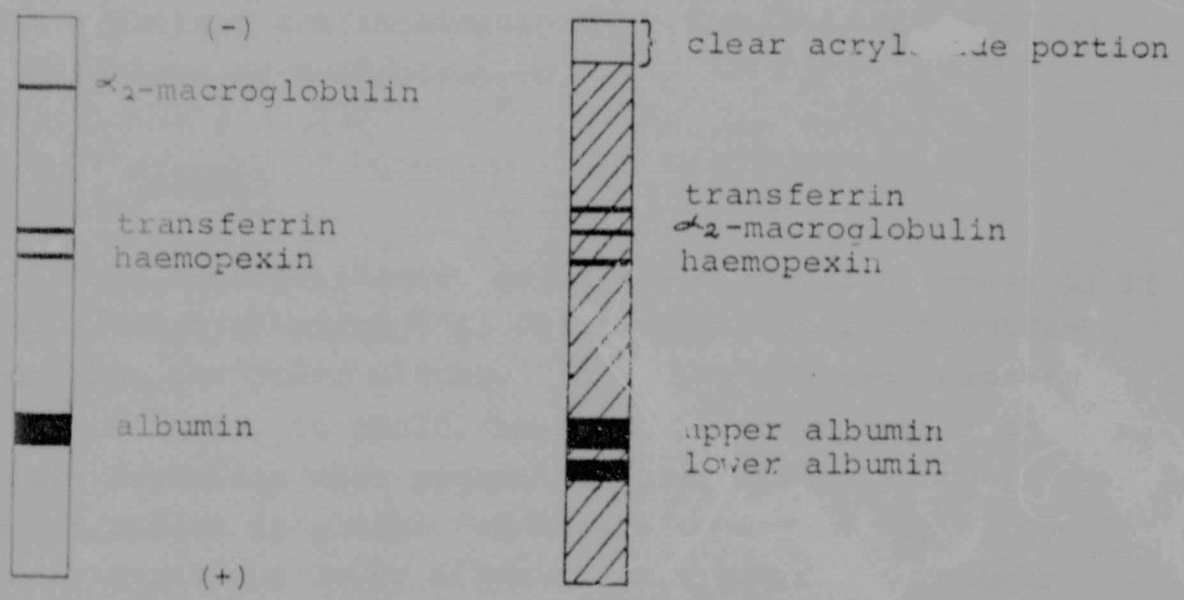



Figure 2

Characteristic positions of some human plasma proteins after electrophoresis in respectively polyacrylamide and sephadex-acrylamide gels.

It was also found that some plasma proteins e.g. albumin and transferrin could be separated into more components in the sephadex-acrylamide gel than in the conventional polyacrylamide gel. This was confirmed by doing CIE on the sephadex-acrylamide separated proteins using monospecific antibodies to these proteins (see Figs. 3 and 7). To eliminate the possibility of the separation of these proteins in sephadex-acrylamide being the artefactual result of the presence of relatively high concentrations of catalyst and/or accelerator, the gels were pre-run for times varying from 5 to 45 min at 40 V. No difference in separation patterns could be observed between pre-run gels and gels

not treated in this way. In order to determine whether the separation of some plasma proteins was not possibly due to a concentration factor, the following experiment was devised: After initial separation on sephadex-acrylamide, the albumin area was cut into four equal sections and each placed on top of a fresh sephadex-acrylamide gel. After electrophoresis of these preparations, it was found that the albumin in each individual section again migrated to its own unique position. It was thus shown that the differences in relative mobility of the different albumin fractions are inherently characteristic and not due to variations in concentration.

3.1.4.1 Albumin

On the sephadex-acrylamide gel it would visually appear as if upper albumin is present in relatively higher concentrations than the lower albumin. By doing crossed immunoelectrophoresis, it could, however, be shown that both albumin fractions were present in approximately equal concentrations in plasma, as can be seen from Fig. 3. To compare upper and lower albumin during CIE, the rockets were run to completion. See discussion p. 131.

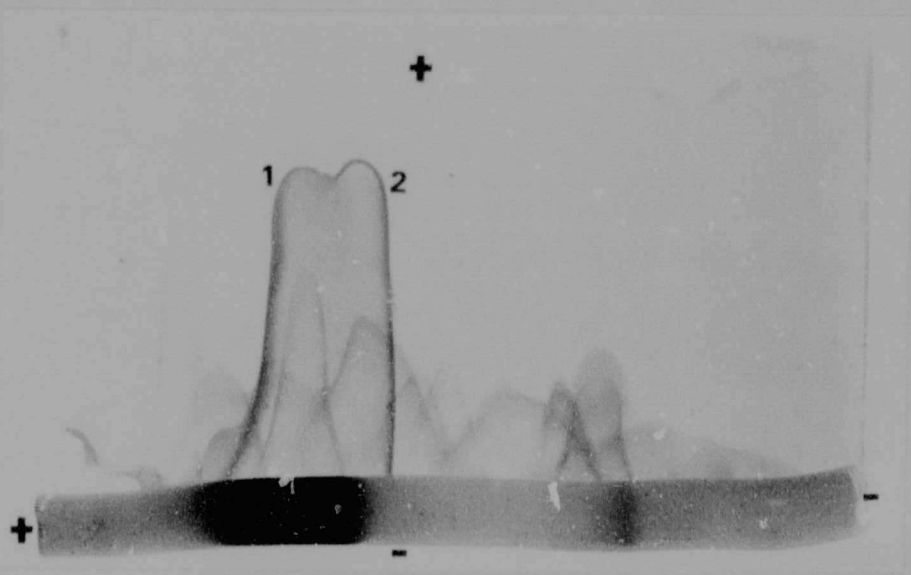



Figure 3

Crossed immunoelectrophoresis of human plasma separated on sephadex-acrylamide indicating the positions and relative concentrations of the albumin fractions.

(1) lower albumin
(2) upper albumin

For the investigation of the lower albumin, isolation of the lower albumin from a satisfactory source was necessary. It was noticed that the relative concentration of the lower albumin in plasma increased when blood samples were not centrifuged immediately after sampling. This indicated the blood corpuscles as a possible source of the lower albumin. This lower albumin was isolated from blood (human) by a washing process. Directly after a blood sample was taken, it was centrifuged. The plasma was then replaced by an equal volume of saline and mixed with the blood cells and centrifuged again. The washing process was repeated twice. After the third wash the sample was incubated for 1,5h at 38°C before the final centrifugation. The final supernatant was found to be precipitated immunochemically by specific albumin antibodies.

As shall be indicated in Table 2, BcG binds to the upper but not the lower albumin, a BcG test on the original plasma and all the supernatants was done to determine whether the final supernatant was free from the upper albumin. From the results in Fig. 4 it can be seen that the concentration of upper albumin in the supernatants decreased during the washing process as was expected. Upper albumin concentration in the final supernatant appeared to increase after incubation.

Due to haemolysis the haemoglobin concentration increased. Haemolysis could be the result of mechanical break-down of the R.B.S. during the centrifugation process, the incubation period and/or the saline. It was decided to determine whether BcG, which is regarded as an albumin-specific dye (Howorth, 1971), could erroneously indicate the presence of albumin in albumin-deficient solutions due to the presence of haemoglobin. A BcG test was done on a solution of commercially pure human haemoglobin in normal saline, yielding positive results indicating presence of albumin. It was therefore established that BcG is not strictly an albumin-specific dye, and could erroneously yield positive results in the presence of haemoglobin.

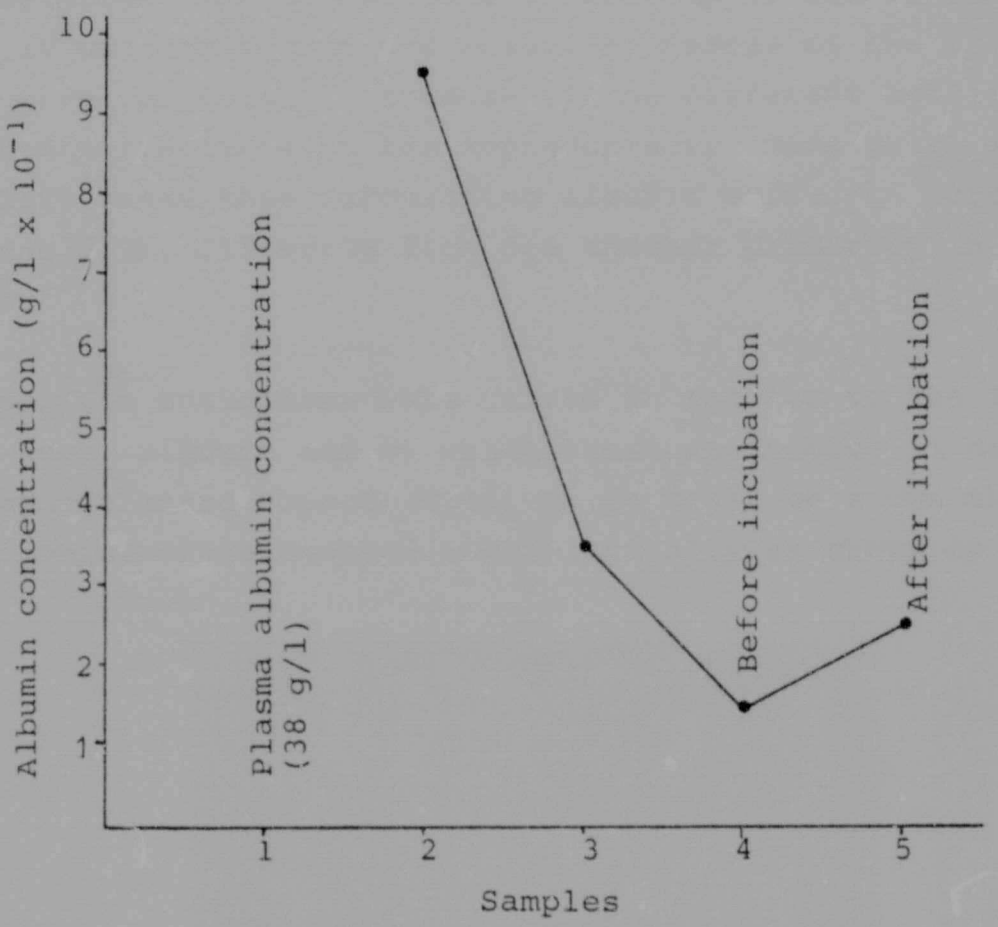



Figure 4

Concentration of upper albumin in supernatants during the washing process of blood.

The final supernatant was shown to contain the lower albumin as visualized by SAGE. By doing PAGE, haemoglobin could also be identified, but the concentration of other proteins (if present) was too low to allow identification. The supernatant was run through several Sephadex G-100 chromatography columns to isolate the lower albumin from the haemoglobin and traces of other plasma proteins that may have been present.

In general, it can be assumed that the behaviour of BSA can be extrapolated almost directly to human serum albumin (Rosenoer et al, 1977; p 54). For practical reasons bovine serum albumin was used as a source of upper albumin for comparison with the isolated lower albumin.

By doing SDS (sodium dodecyl sulphate) electrophoresis on the isolated lower albumin and on BSA (upper and lower albumin), it was found that the molecular masses of the fractions were identical. Because of the different mobilities of albumin fractions on the sephadex-acrylamide gels, it therefore seems that circulating albumin exists in more than one form, differing from one another primarily in charge.

Monospecific antibodies were raised in rabbits to the isolated lower albumin and by using these antibodies in crossed immunoelectrophoretic studies, it could be shown that the albumins cross-reacted immunologically as shown in Fig. 5.

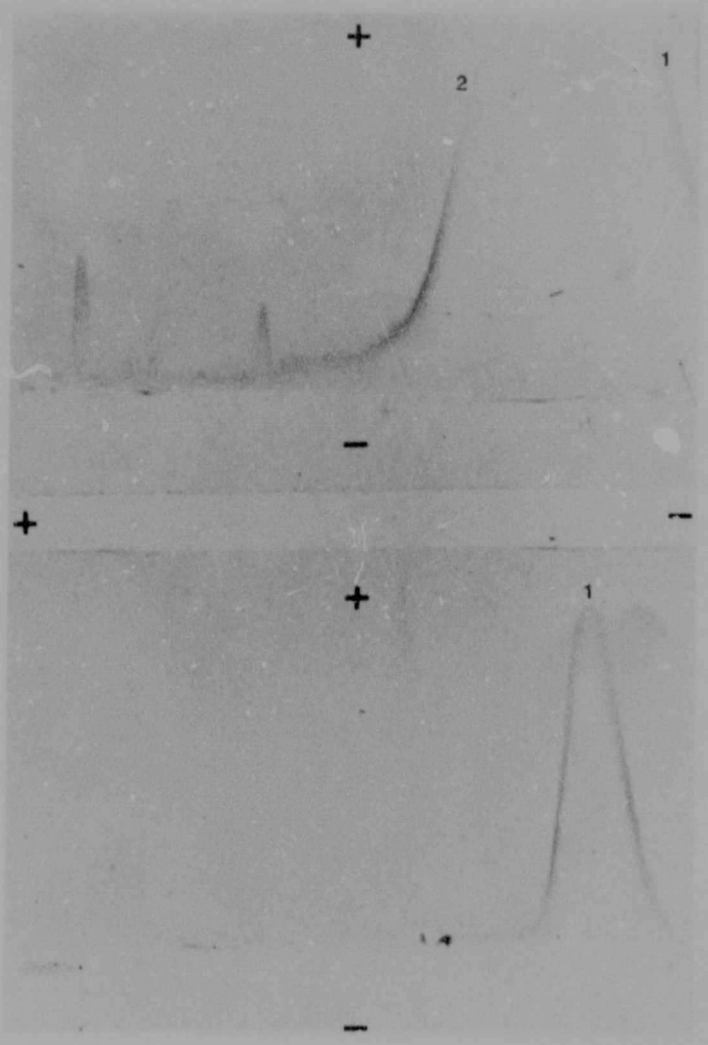



Figure 5

Crossed immunoelectrophoresis of BSA (top) and isolated lower albumin (bottom) using monospecific antibodies to the lower albumin. (1) lower albumin (2) upper albumin.

To further investigate some of the biochemical differences between the lower albumin and upper albumin, equimolar solutions of BSA and the isolated human lower albumin were made. The nitrogen content of the solutions were approximately the same as determined by the Kjeldahl method.

Table 2

Comparative biochemical analysis of isolated lower albumin and BSA.

| Method | Lower Albumin | BSA |
|---|---|---|
| Kjeldahl $N_2$ (g/l) | 1,31 | 1,22 |
| Lowry (g/l) | 0,40 | 1,20 |
| BcG (g/l) | 0 | 1,40 |

The results in Table 2 indicate that the lower albumin and BSA are biochemically, and therefore probably functionally as well, different from one another. Once again, it can be seen from Table 2 that, unlike the upper albumin, the lower albumin yielded negative results with BcG. Although it has to be considered that these results may indicate the possibility of the lower albumin not being an albumin, it was shown that the upper and lower albumins cross-reacted immunologically (see Figs. 5 and 10).

Finally, the BSA and lower albumin were labelled with radioactive tryptophan to investigate their respective tryptophan-binding abilities. After SAGE of the labelled BSA and lower albumin, it was found that tryptophan is mainly trans-

ported by the upper albumin and to a lesser extent by the lower albumin, as can be seen from Fig. 6.

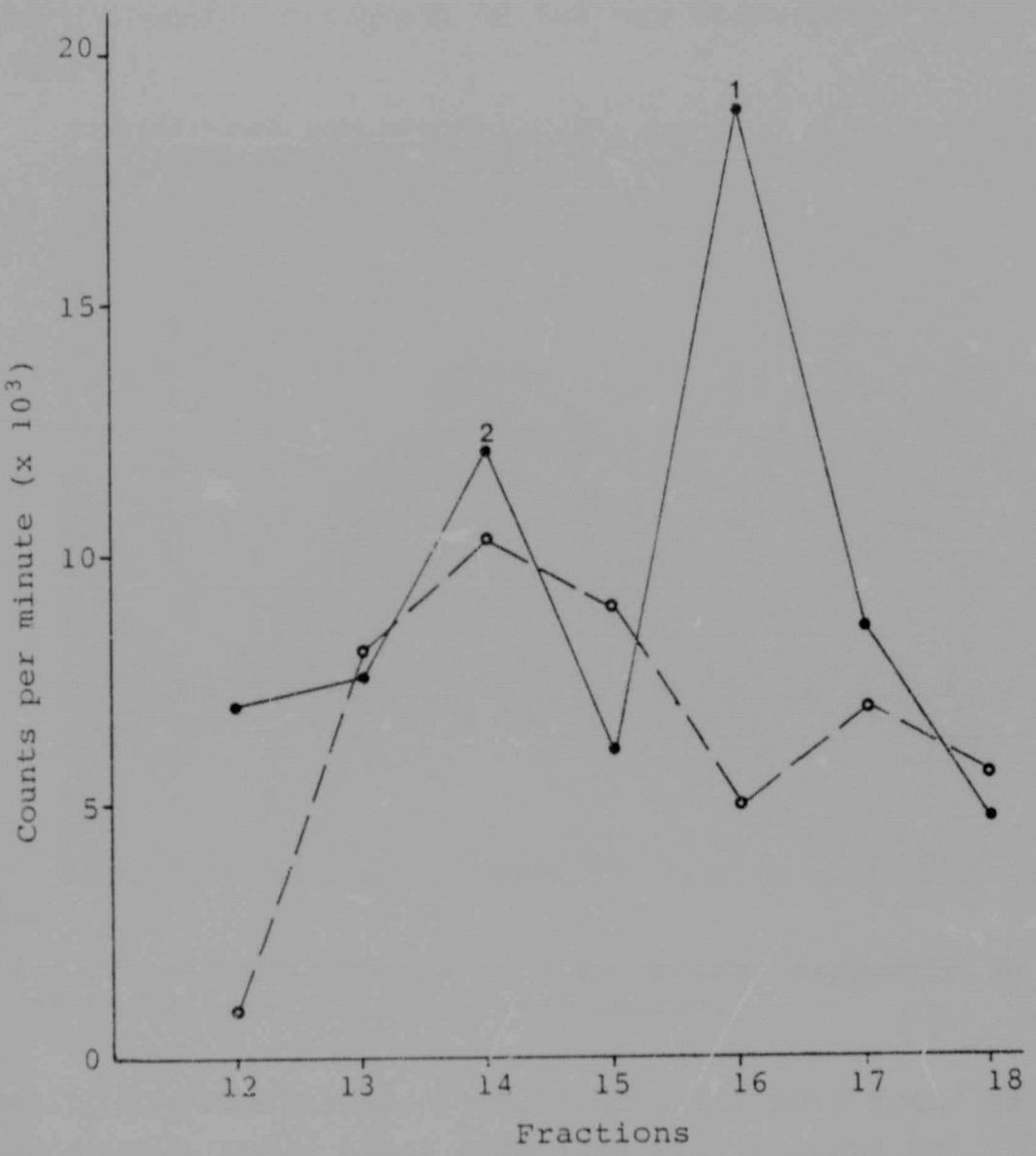



Figure 6

Binding of radioactive tryptophan to upper (1) and lower (2) albumin of BSA (——) and isolated human lower albumin (---).

The lower albumin was found to transport 25% of the total albumin bound tryptophan.

3.1.4.2 Transferrin

Although albumin was our main concern in this study, transferrin, which is also separated into more than one fraction on the sephadex-acrylamide gel, was also investigated with regard to its heterogeneity. CIE of human plasma separated

in SAGE against monospecific anti-transferrin produced a double-peaked rocket precipitate of continuous nature, indicating antigenic similarity of the two transferrin fractions (Fig. 7).

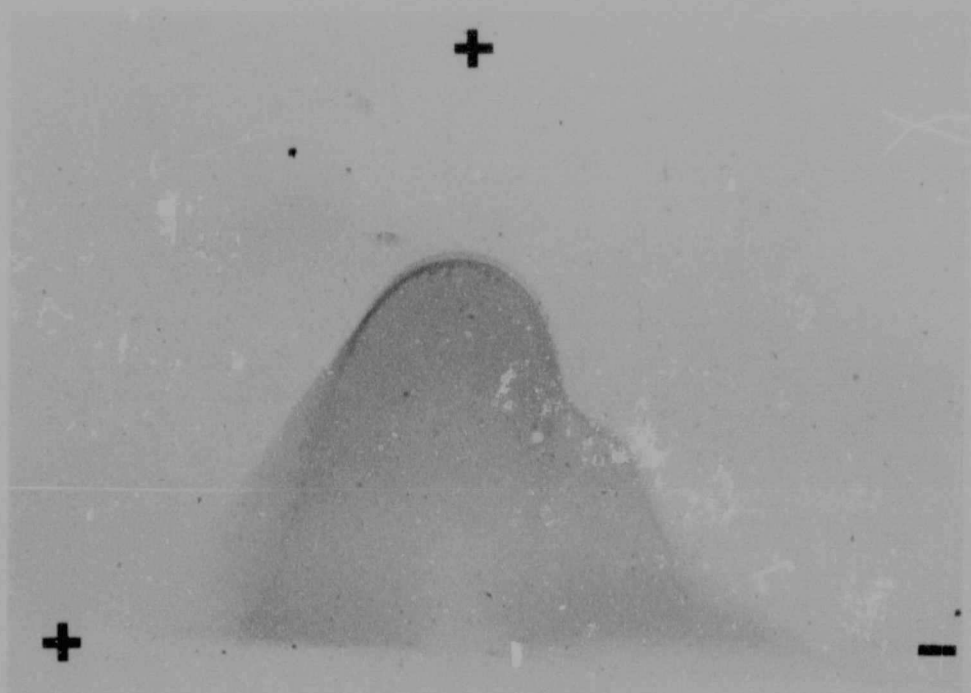



Figure 7

Crossed immunoelectrophoresis of human plasma separated in SAGE against monospecific anti-transferrin.

Haemopexin, transferrin and $\alpha_2$-macroglobulin were found to be close to each other after SAGE (see Fig. 2), and by mixing the three types of monospecific antibodies, the results shown in Fig. 8 were obtained. After labeling the plasma with radioactive iron and then doing CIE, it could be seen that a third shoulder of the transferrin rocket peak appears on the anodal side (Fig. 8). From Figs. 7 and 8 it is seen that transferrin normally has a rocket peak with a shoulder to the cathodal side after SAGE. The third (anodal) shoulder appearing after transferrin has been labelled with radioactive iron (Fig. 8), could indicate that not all the transferrins are transporting iron to the same extent. The charge of transferrin might change when transferrin binds to iron, and thus also the different relative mobilities of the transferrin during SAGE.

**Author** Coetzee N
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