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ISOLATION AND LIMITED CHARACTERISATION OF LION - *Panthera leo* - ALBUMIN

by

CAREL PETRUS JOOSTE

Submitted in fulfillment of the requirements

for the Degree of Master of Science

in the

Department of General Physiology

School of Dentistry

University of the Witwatersrand

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PREFACE

The experimental work described in this dissertaion was conducted in the Department of General Physiology, University of Witwatersrand under the supervision of Professor Johann Hattingh.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

C.P. Jooste

C.P. JOOSTE

13th day of November, 1987.

ABSTRACT

On routine plasma protein analysis of a variety of wild animal species, the electrophoretic separation pattern of lion albumin was found to be peculiar in the sense that it consisted of a double band of unequal concentration. This phenomenon was investigated in the following way:

1. Albumin was isolated from lion plasma by two different methods.

2. A variety of different analytical methods were employed to demonstrate the albumin pattern. In this regard cellulose acetate and disc gel electrophoresis with and without SDS as well as analytical and preparative isoelectric focusing and column exclusion chromatography were used.

3. Combinations of these analytical methods were used to ascertain whether the particular separation pattern was influenced by:
   a) geographic origin of donor lions,
   b) time period between lion immobilization and sample taking,
   c) various immobilizing drugs,
   d) specific anticoagulant,
   e) sample storing procedures,
   f) length of electrophoretic separation time,
   g) nutritional status of donor lions,
   h) degree of concentration of sample,
   i) wild or captive state of donor lions.

4. Specific antibodies to lion albumin were raised for immunological characterisation of this protein.

It was concluded that:
i) the separation pattern was indeed due to albumin,
ii) the electrophoretic picture was not influenced by analytical method or by sample handling,
iii) the two albumin bands exhibit differences in charge but not in molecular mass,
iv) the two bands are immunologically similar.

ACKNOWLEDGEMENTS

AT THE END OF THIS STUDY MY SINCERE THANKS AND DEEPEST APPRECIATION GO TO:

- Johann Hattingh for having the utmost patience for this part-practitioner part-teacher with academic ambition.
- Elaine Marcus for always, from capture to conclusion, being there to help.
- Tracy Marcus for spending holiday hours to type boring chapters.
- The research staff of: Kruger National Park
  Etosha Game Reserve
  Johannesburg Zoo
  for helping with sample collecting.
- The staff of the Department of General Physiology for creating a pleasant environment to work in.

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INTRODUCTION

During routine investigation of plasma samples of a variety of wild animal species a distinctly different albumin pattern was observed in lion plasma protein analysis. These differences were investigated in this study. Since these characteristics rely on albumin behaviour under different conditions it is necessary to discuss the influence that altered environments have on the properties of the albumin molecules.

A. SOLUBILITY IN SOLUTION

Albumin (named after coagulated egg albumin, albus being white in Latin) possesses certain characteristic properties which may be utilized in its isolation and purification. It is known to be an acidic and soluble but stable protein. The molecule, in the presence of stabilizers (such as caprylic acid), may withstand 60°C for 10 hours, a procedure routinely executed to rid clinical albumin preparations of hepatitis B virus. At pH 1 or 2 the molecule elongates reversibly to the so called expanded form (Foster, 1977). Albumin is relatively unharmed at pH 9 to pH 11 but may undergo denaturation or disulfide interchange under conditions of high pH (Wallevik, 1976a). The behaviour of albumin in an aqueous environment is dependent on the character of the residues attached to the alpha-carbon of each monomeric amino acid unit, since hydrophobic residues will be entropically driven to interact with one another in order to minimize water contact whereas ionizable and polar residues will interact with the water solvent. The three-dimensional structure of albumin is therefore a reflection of the interaction between amino acid monomeric units (of backbone peptides and side chain residues) with one another and with the solvent (Lumry and Biltonen,

1969). Solubility of protein in a given solvent is determined by the balance between protein-protein and protein-solvent interactions. Since all protein isolation methods involve some measure of altered solubility it is essential to summarize the parameters of protein solubility:

1 Molecular size of the protein

Both size and shape of a protein molecule, described by its Stokes radius, will determine its exclude (effective) volume (Tanford, 1961) and it is generally recognised that proteins with large exclude volumes are less soluble than smaller macromolecules.

2 Amino acid composition, sequence and conformation of the protein

The ratio of polar to non-polar residues, their distribution (in clusters) along the polypeptide chain and their accessibility to the solvent will influence the protein solubility. Apart from these inherent factors, combination with other non-protein substances, of which albumin is known to attract and bind many, will also affect solubility. These substances may be covalently linked, ionically complexed or hydrophobically bound by van der Waals forces to the protein. The solubility of these ligands may, for example, explain the different solubilities of glycoprotein and lipoproteins in aqueous solution.

3 Charge of the protein

Molecules of the same charge will repel one another in addition to interacting with dipolar water molecules and are thus more soluble in water solution than uncharged molecules. The charge of a protein is determined by a) total number of ionizable residues, b) their accessibility to the solvent, c) the pH of the solution and d) the dissociation constants of the ionizable groups which are, in turn, determined by the chemical composition of the groups

themselves, of their neighbouring groups and of the solvent as well as by the ionic strength of the solvent and the temperature of the solution (Edsall and Wyman, 1958). The net charge of protein in solution may therefore be manipulated by affecting changes in the solvent, like altering the pH. The pH of minimum solubility of a protein implies that the net charge of each molecule is zero at that point, which promotes the aggregation of the molecules. This is called the isoelectric pH. Binding of simple anions or simple cations will respectively decrease or increase the isoelectric pH but the binding of metals will have an effect that is dependent on the specific method of binding and its resultant alteration of the net charge (Edsall and Wyman, 1958). The addition of larger ionic organic substances will result in structural changes to the protein with resultant alteration of protein solubility which will overshadow the alteration caused by the change in net charge.

4 Solvent dielectric constant

This is a measure of dipole moment and molecular polarizability of the medium. In a solvent of high dielectric constant (eg.water) dissociation of electrolytes is promoted but in solvents with low dielectric constants (eg.ethanol) solubility is decreased (Frigerio and Herringer, 1962). Since albumin is known to be stable in the presence of such organic solvents, this becomes a viable option for precipitating albumin.

5 Ionic strength

Low concentrations of ions have a solubility effect on proteins ("salting in") and, conversely, high concentrations have a precipitating effect ("salting out"). In an aqueous solution (high dielectric constant) the salting out effect dominates while salting in would be greater in media of low dielectric constant (Kirkwood,1943). The salting out potential of various salts is strongly

dependent on the specific cationic and anionic composition of the salt (Hofmeister, 1888). These ion effects appear to be related to ion size, ion charge density and ion polarizability which all will influence ion-solvent as well as ion-protein interactions. In this way ions may act as conformational stabilizers which result in increased helix content (eg.sulphate) and thus will decrease protein solubility or they may act as denaturants which result in the unfolded coil state (eg.thiocyanate) and thus will increase protein solubility (Long and McDevit,1952). In comparing the efficiency of various salts in the salting out process it is essential to observe both the completion of precipitation of the first protein fraction as an indicator as well as the increment of salt required for each subsequent fraction. On this second basis of comparison it was found that the quantity of chloride required for each precipitation is of the order of five times the initial concentration for the corresponding sulphate (Howe, 1923). When comparing the various sulphate salts it was found that sodium and potassium are equally effective, followed by magnesium and then lithium. These differences seem to be a function of the degree of hydration of the ions of the salt solution with highly hydrated ions being more effective than less hydrated ions.

6 Temperature

In salt-free, low ionic strength and ethanol-water solutions the solubility of the protein is endothermic; it shows a positive temperature coefficient of solubility. The specific protein will determine whether the latter is positive or negative in concentrated ion solutions (Alderton, Ward and Fevold, 1945).

B. EXPERIMENTAL MANIPULATION OF PROTEINS

Induced molecular conformational changes may be affected by temperature,

pressure, pH, organic solvents or neutral salts. As a general rule fractionation is conducted under conditions of a) using the lowest possible temperature, especially when using organic solvents, b) restricting the pH as near to neutrality as possible and c) using salts and reagents conservatively (Tanford, 1970). Apart from these general principles some specific precautions have come to light in the recent past.

Proteins are surface active and are structurally affected by air-water or solid-water interfaces. Introduction of air into protein solutions and the resultant generation of foam should be avoided by careful handling of the samples (Bull, 1971).

Pumps and stirrers could lead to shear damage of the molecules and should be carefully employed in the handling process (Charm, 1971).
The techniques employed in the addition of reagents to protein solutions require careful consideration since local excesses may lead to denaturation of the protein. Liquid, rather than solid reagents, added beneath the surface of the solution in a multidirectional spray fashion seems to be an accepted method. The more gradual addition of reagents by dialysis has also been suggested (McMeekin, 1939).

When employing one of the separation techniques it is important to determine and adhere to the exact equilibration time of each precipitation step since the degree of aging of solutions after addition of reagents will alter the effiriency of the subsequent precipitation (Watt, 1970). Regarding the concentration of the protein solution due for fractionation it has been suggested that a reduced total protein concentration will increase the resolving power of any method. On the other hand, protein is much more stable

structurally in a concentrated solution or in the solid state because in dilute solutions the molecules are more exposed to an environment of high dielectric constant and may be denatured and form insoluble precipitates. There therefore exists an optimum dilution minimumizing the above effects of extremes of concentration (Cohn, 1925).

Oxidative processes could also alter the quality of the resultant precipitate and in some laboratories it has become custom to include metal chelators in the protein solutions to prevent this oxidation (Pennell, 1960; Ray, Davisson and Crepsi, 1954).

Plasma contains significant levels of proteolytic enzymes in active or inactive precursor forms. In order to avoid their action protease inhibitors may be added to the starting plasma or to the buffers employed. Since metal ions activate some of these enzymes, chelators will again be effective in preventing activation. Operation at the lowest possible temperature also reduces enzyme activity.

Protein solutions are generally excellent media for bacterial growth and precautions to prevent contamination like using a closed system, working at low temperatures and adding bacteriocidal or bacteriostatic agents to the solution will reduce this.

Keeping these operational constraints in mind, it is clear that no single method of separation will result in pure albumin. Usually a combination of different methods will result in the highest yield relatively pure albumin. Possible fractionation methods are thus based on one of the following principles

a) interaction between the protein and soluble reagents or solvents. This may

consist of either major changes affected to the solvent or of major changes to the protein itself caused by the binding of reagents,

b) interaction of protein with insoluble media,

c) interaction of protein with physical fields.

C. APPROACH OF THE PRESENT STUDY

In this study the albumin was first of all isolated from the lion plasma. This had to be undertaken in order to ascertain that the peculiar pattern obtained in the pilot experiments were indeed due to an unusual albumin configuration. To this end two different combinations of methods were utilised and their results compared.

Secondly the albumin was characterised by investigating its electrophoretic, chromatographic and antigenic properties. A variety of different methods were employed for demonstrating each property.

For comparative purposes the methods selected were chosen to be closest to the routine investigations regularly performed in clinical and experimental protein analysis. Since some results could be attributable to a specific methodological cause, different methods with similar aims were used to rule out the possibility of ascribing a certain characteristic to the investigated protein on the basis of one analytical method only.

MATERIALS AND METHODS

Blood was obtained by venopuncture from immobilised and anaesthetised lions of a range of origins all over Southern Africa. Since the method of sampling, origins of donors and handling techniques of the material formed a basis fo. comparison, these aspects are discussed separately in the section entitled "Results".

A. ISOLATION OF ALBUMIN FROM LION WHOLE PLASMA

1. Ethanol fractionation (Hao, 1979)

Plasma diluted threefold with isotonic saline, was placed in a water bath at -5° C and the pH was adjusted to 5,6, using a 0,8M acetate buffer, pH 4, prepared from 2,5 times diluted glacial acetic acid and 160g/l solution of NaOH in the ratio of 4:1 (Cohn, Heyroth and Menkin, 1928).

After stirring for one hour, precooled (-5°C) 95% ethanol was added in a thin stream fashion (to avoid heat production) to a final concentration of 42% (V/V). The temperature of the mixture was kept as close to -5°C as possible but, in any case, throughout the procedure never exceeded 0°C. After the ethanol addition the pH was 5.8 and the mixture was continuously stirred for at least one further hour. This constant low temperature was achieved by using a circulating bath filled with glycol into which the mixture was submerged. The bath was set up over a magnetic stirrer.

Centrifugation for one hour at -5°C was performed in a Sorvall refrigerated centrifuge operating at 12000 x g. Afterwards the precipitate was discarded and

the supernatant used for further fractionation. The pH of the latter was adjusted to 4.8 using acetate buffer and, after stirring for one hour the mixture was allowed to stand for at least three hours (Cohn et al., 1946). After this ageing the mixture was again centrifuged for one hour. The resultant precipitate was similar to Cohn fraction V in texture and was the albumin paste used further. The paste was reconstituted in distilled water and then lyophilized. The powder product was stored and subsequently was dissolved for purity checks and further analysis. By using electrophoretic techniques the separation efficiency of the procedure was assessed in each step and by using protein estimation techniques, the yield of the final product was calculated. This was always higher than 90% and with no electrophoretically detectable impurities.

2. Polyethelene glycol precipitation (Curling et al., 1977).

The pH of the starting plasma was adjusted to 8.0 by the addition of 1M NaOH. Polyethelene glycol (PEG) 4000 was made up to a 50% W/V concentration (50g made up to 100ml) and added to the plasma (which was continuously stirred) up to a final concentration of 12% W/V.

This mixture was stirred at 4°C for one hour and then centrifuged for 15 minutes. The supernatant collected was adjusted to pH 4.6 and solid PEG was added while stirring, to a final concentration of 25% W/V. After one hour of stirring, centrifugation for 15 minutes yielded a precipitate which was washed in 25ml of cold distilled water and again centrifuged. The final precipitate was dissolved in 20ml cold distilled water and the pH adjusted to 7.0.

DEAE Sephadex A-50 was swollen in excess volumes of 1M sodium acetate at 96°C for two hours and then transferred to a 0.05M sodium acetate acetic acid buffer

of pH 5.2 and packed in a column (15cm x 1cm). This packed column was equilibrated with two column volumes of the buffer.

The pH of the last solution was reduced to 6 and it was then applied to the column in the ratio of 5ml solution for each 0.5g dry ion exchanger. The column was then washed with one column volume of buffer to remove residual PEG and unbound protein. The buffer pH was lowered to 4.6 with an accompanying increase of ionic strength. These conditions then eluted the albumin.

Fractions of 1ml were collected at a flow rate of 0.5ml/min and their light absorbance was monitored at 254nm wavelength. In this way the fractions during a peak of increased absorbance could be collected and stored for further use.

Quality control was again executed by means of electrophoretic techniques to exhibit purity of separation and by protein concentration estimations for percentage albumin yield. The former was occasionally quite obscure because of the dilution of the protein but the latter measured a yield consistently above 80%.

B. CHARACTERISATION OF THE ALBUMIN.

Since the majority of investigations into the properties of albumin involve some type of electrophoretic technique in this study, it would be appropriate to discuss the principles of electrophoretic separation as they can influence the conclusions reached about the specific albumin condition studied here.

Electrophoretic techniques

Many biological molecules possess ionizable groups and may thus be made to exist in solution as electrically charged entities. Molecules with a similar charge but with different molecular masses still differ on the basis of the charge/mass ratio. The differential migration seen in such molecules when they are placed in an electric field forms the basis of electrophoresis.

The rate of movement of cations to the cathode and anions to the anode is determined by the balance between the attracting forces of the electrical field and the frictional and electrostatic effects of the movements of charged ions in a medium. For movement to take place the sample must be dissolved in a buffer solution and the medium must be saturated with buffer in order for the current to be conducted.

The current is maintained by electrolysis taking place at the electrodes so that, at the cathode, hydroxyl ions and hydrogen are produced and oxygen and hydrogen ions at the anode. The cathodal hydroxyl ions cause increased dissociation of the weak acid in the buffer with the resultant increase in anions which will conduct the current to the anode. There the anions combine with hydrogen ions to reform the acid while the electrons are refed to the electric circuit. The sample ions join these buffer ions in their migration and are separated on the basis of their electrophoretic mobility. When allowing these migrations to take place on an inert supporting medium one causes the ions to separate into distinct zones. The nature of the supporting medium will determine the interaction with the ions being separated and could exploit the differences in charge/mass ratios or retard the sample.

Migration rate is influenced by various factors. The rate increases with an increase in net charge of the sample ions which, in turn, was determined by the

pH of the sample. The rate decreases for larger molecules because of increased frictional and electrostatic forces. Molecules of the same size but with different shapes will migrate at different rates; fibrous and globular proteins migrate differently. Changes in the electric field will also exert an effect in the sense that an increase in the potential gradient (measured in volts) will increase the rate of migration. Similarly, an increase in current will increase migration rate. Conversely, any resistance exerted by the support medium will retard the rate of migration. An increase in temperature will cause increased mobility of ions as a result of evaporation of solvent from the support medium which will decrease resistance. This is, however, overbalanced by the increased buffer ion concentration with a net resultant decrease in migration rate. Direct current was supplied through power packs which deliver either constant voltage or constant current which makes subsequent comparisons possible. With a constant voltage applied one observes a rising current during electrophoresis due to a decrease in resistance of the medium because of a rise in temperature. While this may not be problematic in low voltage paper electrophoresis the heat generated is not easily dissipated with gels or cellulose acetate and in these conditions constant current should rather be used. A closed chamber will reduce evaporation and a cooling system will help reduce overheating. A group of media running in parallel will also cause too much heat when run with a constant voltage. Constant current is again preferred but should be increased in proportion to the number of media used. For absolute reproducable conditions the voltage drop per cm length of medium, the current density per cm width of medium and the temperature should be accurately measured.

The buffer used has to, first of all, dissolve the sample so that the operator should be wary of sample diffusion, especially if the sample ions are small. Low ionic strength buffers will result in a smaller portion of the current being

carried by the buffer ions and, consequently a larger portion carried by the sample ions. This will increase the rate of migration. It also causes less heat production but sample diffusion and loss of resolution is higher. On the other hand, high ionic strength buffers will decrease the portion of current carried by the sample ions and hence reduce the migration rate. In addition it will increase the overall current and heat production. The eventual choice of buffer ionic strength is a compromise between these two extremes.

The pH of the buffer has little effect on fully ionised compounds such as inorganic salts but for organic compounds pH will determine the degree of ionization. Increasing buffer pH levels will cause increased ionization of organic acids and decreased ionization of organic bases; the reverse applies to decreasing buffer pH levels. In most electrophoretic systems there is a continuous buffer system over the medium but in certain disc gel systems another buffer is used in the gel itself; this is thus a discontinuous buffer system.

Regarding the support medium, it is usually considered to be relatively inert but nevertheless can interact to some extent with the sample ions and therefore can be employed to manipulate mobility. Sample ions can be adsorbed to the medium, causing a "tailing" instead of movement in a band shape and thus reducing rate and resolution of separation. Surface adsorption of ions from the buffer or the presence of stationary carboxyl groups on paper or sulphonate groups on agar can result in a relative charge existing between the medium thus charged and the water molecules in the buffer. This generates a motive force for fixed anions to the anode and results in movement of hydronium ions to the cathode, carrying along neutral substances by solvent flow. In this way so called electro-endosmosis accelerates the movement of cations but retards that of anions. This interferes with determinations based on obtaining the

isoelectric points of compounds and for correction purposes the migration of neutral substances such as urea or glucose must be measured in the same system. Gel media, which all have some randomly intertwined molecular chain structure, can act like a sieve. In this way agar, starch and polyacrylamide gels retard the movement of larger molecules which utilize them with more ease.

In view of the numerous variations in pattern obtainable from the same sample due to experimental manipulation and the need to restrict characterization to often used standard techniques in order to compare results with published routine results a few applications of the above concepts should be considered for effective characterization.

ELECTROPHORETIC METHODS.

1. Cellulose acetate electrophoresis

Cellulose acetate strips have a very homogeneous pore structure and allow very little adsorption of macromolecules. It is less hydrophylic than paper, holds less buffer and therefore allows a better resolution in a shorter time. This, however, can lead to heat being produced but, by using a closed chamber, drying of the medium strips was avoided. An added advantage is that the background can be rendered transparent by treatment with a clearing agent followed by heat drying. It is thus generally considered superior to paper and has become very popular clinically. In this study tris-barbital-sodium-barbital buffer (pH 8.8) was made up and 100ml buffer with 0.5ml ethanol used as a wetting solution to soak the cellulose acetate strip for at least 10 minutes. A volume of 200ml buffer was used in the electrophoresis chamber with both sides equally filled. After the removal of the strip from the soaking solution it was placed on a sheet of absorbent paper. Meanwhile the application block was loaded with

samples and standards with the use of a Pasteur pipette. The block may be covered with a lid in between loading procedures to prevent evaporation. From there the test solutions were picked up by the applicator which absorbed the samples between the two parallel wires when the applicator key was depressed for 10-15 seconds. This was then used to transfer the samples and standards to the medium strip which has to be lightly blotted with another sheet of absorbent paper and then placed in the chamber before loading. The electrodes were placed over the chamber in their fixed position and plugged into a Vokham power pack on which the voltage was adjusted to 225V. Electrophoresis was allowed to proceed for 20 minutes. Immediately after switching off the power supply the strip was removed from the chamber and floated on top of the sta on (0,5% Ponceau S in 7,5% aqueous trichloroacetic acid). When npletely wet it was submerged and stained for 10 minutes. Using forceps, the strip was removed from the stain and placed in a 5% acetic acid rinsing solution which was then agitated to remove excess stain. This rinsing procedure was then repeated in a second and third acetic acid rinse for a total time of about 10 minutes until a clear background was obtained. The strip was then removed from the final rinse tray and allowed to drip off the excess rinse solution before being put in Sepra Clear® clearing solution for 5 minutes. After this the strip was placed lengthwise on a glass slide and a second glass slide edge was pulled over this at 45° angles to remove all the air bubbles trapped underneath the strip. The free edges were then folded over and the slide was placed in an oven preheated to 80°-90°C and left for 20 minutes to produce a completely transparent and dry background. After cooling down the slide was cleaned with water and could then be stored for further analysis later. Densitometric (Gelman scanning computerised densitometer) analysis could provide information regarding the relative contribution of the various protein fractions separated on the paper).

2. Polyacrylamide disc gel electrophoresis

The following four solutions were made up:

1. 20g acrylamide and
   0,8g N'N' methylene bisacrylamide
   in 100ml distilled water

2. 1,6g or 1,84ml dimethylaminopropionitrate and
   40ml stock buffer (below)
   in 100ml distilled water

3. Stock buffer: 3g Tris (methylamine) and
   14,4g glycine in
   100ml distilled water
   (pH should be 8,5)

   Working buffer: Stock buffer diluted 10-fold

4. 0,5% ammonium persulphate

These solutions can be stored in dark bottles in the fridge. One end of the special 2ml glass tubes was sealed with parafilm or a close fitting plug. Eight of these tubes were placed in a gel casting stand and kept vertical. 5ml of each of the four solutions was mixed and then sucked up in a large syringe. The latter was used to fill each tube for about 75% of its length and very carefully about two drops of distilled water was lowered onto the above mixture in each tube to seal it off and cause solidifying. After about 15 minutes the tubes were transferred to the discontinuous electrophoretic chamber with the bottom

chamber fil d with working buffer to cover the electrode sufficiently. Tubes were then unplugged at the bottom and the water layer at the top removed before being lowered into the buffer, ensuring that no air bubbles are trapped underneath each tube. The upper chamber was then also filled with working buffer and, using a syringe, air trapped on top of the gels was displaced by buffer. The top electrode was lowered into the buffer and should be completely submerged. The electrodes were then connected to the Vokham power pack so that the negative terminal was at the top and the positive at the bottom. A constant voltage of 20V was applied for 1-2 minutes. On a sheet of parafilm 5µl of sample was used to dissolve a few crystals of sucrose to weigh the sample down onto the gel surface when placed under the level of buffer. Blank tubes could be loaded with protein solution stained with bromophenol blue to secure a visibly migrating front in order to judge range of migration.

After applying all the samples on the top surface of the gels and replacing the top electrode, the power pack was switched on to supply 90V for 20 minutes and then 160V for again 20 minutes to the chamber. The stained protein could be visually checked to ensure adequate distance of movement. After removal of the electrodes the tubes were removed from the chamber and, using a long needle and syringe to introduce distilled water between the gel and inner glass surface to loosen the gels, they were removed from the glass tubes. Gels were then placed in individually numbered compartments of a staining rack and stained with 3g/l amido black in 5% acetic . for at least 30 minutes. Destaining could be performed electrophoretically in the Shandon transverse gel destainer by placing the stained gels in between two pads in a destain frame and firmly wedging it into the centre of the destaining chamber. Acetic acid (10%) was introduced on one side of the gel stack and allowed to flow through the pads. The power pack

was connected to the destaining chamber with the negative electrode to the clear side and the positive electrode to the side with stained acetic acid. Constant current of 0,2A was applied for about 2 hours and then the gels were removed from the holder and stored in 5% acetic acid. Destaining may also be accomplished by diffusion, by leaving the gels for a day or more in 10% acetic acid while being stirred continuously. This, however, was very time-consuming.

Modification of pore sizes in polyacrylamide gels

Porosity of these gels is determined by the relative proportion of acrylamide monomer to cross-linking agent. The total mass per volume acrylamide and cross-linking agent is usually expressed as a percentage. In this way it is possible to have gels ranging from 3% to 30% which yield corresponding pore sizes of 0,5nm to 0,2nm in diameter.

With this in mind the following percentage gels were prepared:

5% - 20g acrylamide
0,8g NN Methylene bisacrylamide

7,5% - 30g acrylamide
1,2g NN Methylene bisacrylamide

8,5% - 34g acrylamide
1,36g NN Methylene bisacrylamide

10% - 40g acrylamide
1,6g NN Methylene bisacrylamide

3. SDS - polyacrylamide disc gels (Fairbanks, 1971)

This variation on the disc gel electrophoresis enables one to separate samples on the basis of molecular mass alone. In this way calibration curves indicate a linear relationship between migration distance and the log of the molecular mass of the protein samples.

In this modification the samples are given the same charge/mass ratio by solubilizing it in the following solution:

10g/l sodium dodecyl sulphate (SDS)

100g/l sucrose

10mM Tris - HCl (pH 8)

1 mM EDTA (pH 8)

0,5g/l mercaptoethanol

10mg/l Pyronin - Y (tracking dye)

This process was carried out at 100°C for 5 minutes. The mercaptoethanol breaks the disulphide bonds and the SDS acts as a detergent to cause the solubilization and attaches an ionic group at regular intervals.

Stock solutions

1 Concentrated acrylamide bisacrylamide

acrylamide 40g

NN Methylene bisacrylamide 1,5g

in 100ml distilled water

2 Buffer (pH 7,4)

1,0 M Tris-HCl

2,0 M sodium acetate

in 100ml distilled water

(adjusted with acetic acid to pH 7,4)

Gels were then prepared as follows:

Conc.acrylamide bisacrylamide	7ml
Buffer	5ml

20% SDS	2,5ml
Water	28ml
1,5% ammonium persulphate	5ml
0,5% TEMED	2,5ml

Glass tubes were used to contain the individual gels and these were gently overlaid with 0,1% SDS

0,15% ammonium persulphate

0,05% TEMED

and left for thirty minutes at room temperature to polymerize.

Running buffer was made up as follows:

Gel buffer	100ml
20% SDS	50ml
Distilled water	850ml

The gels were left in the chambers with the running buffer overnight in order to promote equilibration.

Electrophoresis took place at a potential difference of 50V/cm and until the tracking dye has migrated about 8cm. After removal of the gels from the glass tubes they were placed in slotted test tubes suspended in a beaker containing 25% isopropranolol and 10% acetic acid which was being stirred continuously for twelve hours.

Staining took place overnight in 10% isopropanol and 10% acetic acid containing 0,025% (W/V) Coomassie Blue 250. Destaining was executed in the same way as normal disc gels by electrophoresis or diffusion.

4. SDS Polyacrylamide slab gels

Slab gels were prepared in a commercially available casting frame so that the gel was poured in the same vertical position that it was used in, making transfer of the gel unnecessary.

Samples were prepared according to a modified method (Laemmli, 1970) where the final sample solution contains

0,0625 M Tris-HCl (pH 6,8)

2% SDS

10% Glycerol

5% 2-mercaptoethanol and, for a marker sample, also

0,001% bromophenol blue.

These samples solutions were then immersed in boiling water for 1,5 minutes to ensure complete dissociation before quantities of 0,3 ml were loaded into the sample hollows made into the gel by applying a comb to the unpolymerized surface.

The gel consisted of a separating portion made up of 8% acrylamide and NN bismethylene acrylamide with a final concentration of 0,375 M Tris-HCl (pH 8.8) and 0,1% SDS as well as a stacking gel made up of 3% acrylamide and NN bismethylene acrylamide with a final concentration of 0,125 M Tris-HCl (pH 6.8) and 0,1% SDS. Both gels were polymerized in turn by the addition of 0,025% tetra-methyl-ethylene-diamine (TEMED) and ammonium persulphate.

The electrode buffer (pH 8.3) contained 0,25 M Tris and 0,192 M glycine and 0,1%

SDS. Cooling of the gel during separation was necessary and the electrophoresis was continued until the marker has reached the bottom of the gel.

After electrophoresis the proteins were fixed in 50% trichloroacetic acid (TCA) overnight, stained for one hour at 37°C with 0,1% Coomassie brilliant blue made up in freshly prepared 50% TCA. Subsequently it was diffusion - destained in 7% acetic acid.

5. ANALYTICAL ISOELECTRIC FOCUSING (I.E.F.)

Gel preparation 0,3g agarose - IEF
3,6g sorbitol
29ml distilled water
is mixed in a 50 ml flask and heated on an electrically heated magnetic stirrer until the agarose is dissolved.

Mould preparation

A levelling casting platform was adjusted to the horizontal and 2 ml of water is poured onto it. A sheet of GelBond, with its hydrophilic surface upwards was placed on the table, being handled by its edges only. (The hydrophilic side can be identified as the side on which a water drop will "spread" and not "bead". Excess air bubbles and water were removed from underneath the film by rolling it flat with a roller or glass rod and the expressed water is removed from around the edges with tissue paper.

The casting frame was then fastened over the film and held on by spring clips. With the use of a hairdryer the film was then thoroughly prewarmed.

Gel moulding

Once all the agarose was dissolved, the mixture was allowed to cool to about 75° C before 1,9 ml of the carrier ampholyte, Pharmalyte, was added. The mixture was then stirred and quickly poured into the mould, ensuring that the solution flowed into all corners and that no air bubbles existed in the gel (a hot needle will "pop" these before the gel sets).

After 10-15 minutes the gel had set and could be separated from the frame by pulling a scalpel around the edges. Having removed the casting frame, the film with gel could be lifted off the casting platform. Subsequently gel was allowed to harden at 4°C for one hour or overnight at room temperature. During this it should always be stored in an airtight container with moist tissue paper to avoid drying out.

Separation

A small quantity (2 ml) of water is placed on the cooling platform (Pharmacia FBE 3000) so that, when placing the gel-carrying film, there is a thin layer underneath over the whole surface.

The electrode strips were then each soaked in either 1M sodium hydroxide or 0,05 M sulphuric acid and subsequently blotted for one minute to eliminate excess solution before being placed at the cathode and anode respectively.

Plasma samples of 20 µl were applied to the gel by means of paper applicator strips. The electrodes in the lid should be adjusted to ensure contact with the

electrode strips when the gel is covered.

The constant power supply (Pharmacia ECPS 3000/150) was then set to deliver a maximum of 15 W and 1500 V with unlimited current for one and a half hours. The cooling platform was connected to a circulating water bath which keeps the temperature of the gel environment at 10°C. Sample applicator strips could be removed after 45 minutes.

Fixing and staining

Immediately after completion of the run the gel was immersed in fixing solution for 30 minutes. This solution consisted of:

5% Sulphosalicylic acid

10% Trichloroacetic acid

in distilled water

The gel was now rinsed in two washings of destaining solution, each for 15 minutes. This solution consisted of:

35% ethanol

10% acetic acid

in distilled water

The gel was then dried by putting three layers of filter paper over it, followed by glass plate and a weight of about 1 kg. After about 15 minutes all this was removed and the gel dried with a hairdryer.

The dried gel was then placed in staining solution for 5-10 mminutes. This solution consisted of 0,2% Coomassie Blue R250 in destaining solution.

Subsequent destaining should be of 15-30 minute duration, sufficient to obtain a clear background. The gel film was then dried with a hairdryer and could be stored as a permanent record.

6. PREPARATIVE ISOELECTRIC FOCUSING (IEF)

Apart from the analytical separation by isoelectric focusing, the method can also be performed on a preparative scale. The medium chosen in this study was Sephadex - IEF, a specially prepared Sephadex G - 75 for use in preparative IEF.

Sephadex (15g) was swollen in 225 ml distiled water and 12 ml of the carrier ampholyte, Pharmalyte, was then added before the gel is deaerated.

The special gel trough (Pharmacia) was placed on a horizontal casting platform, supported by the glass plate supports. The gel slurry was poured into the trough and with the help of the gel spreader, it was evenly distributed. The opening of the Sephadex - IEF bottle was covered with a fine gauze held in place with an elastic band and additional Sephadex was sprinkled over the entire surface until the gel remained immobile when tilted by 45 degrees. The gel consistency would then be considered correct.

With the use of a strip from the fractionator grid a slice of 1 cm wide was removed from each end of the gel trough and the electrode strips were soaked in 0,1 M sulphuric acid for the anodal electrode and 1 M ethylenediamine for the cathodal electrode and placed in these spaces. Distilled water (5 ml), containing 1% detergent, was placed on the cooling plate under the gel, avoiding

air bubbles being trapped there. The cooling circulating water bath was then switched on at a temperature of 10°C.

Prefocusing of 45 minutes at 8 W is recommended before samples can be loaded. The latter was achieved by inserting the applicator spacer into the gel and scooping out all the separated gel into a beaker where it was mixed with the sample before being returned to the applicator space with the aid of a syringe. The power pack was set to deliver 50 W for 6 hours and switched on about 10 minutes after sample application to allow for equilibration.

The separated molecules could be detected with a replica technique, involving rolling over the gel surface an exactly fitting sheet of Whatman 1 MM filter paper and leaving it in position for 2 minutes. After drying, the paper was fixed and stained in the above solutions. From this replica separated zones were identified with the help of the fractionation grid which could then be introduced into the gel and, by inserting cutting strips, were cut out of the gel plate and scooped out. This gel was then resuspended in buffer in a suitably sized column from which the substance isolated could be eluted with one column volume of buffer.

7. Column exclusion chromatography

In this experiment commercial columns (Pharmacia) were used. Sephadex G 200 superfine gel particles are swollen and equilibrated for three days in the buffer solution consisting of 0,1 M Tris and 0,5 M NaCl in distilled water with the pH adjusted to 8.5 with HCl.

A 2 ml sample of albumin reconstituted in buffer solution (40 g/l) was added to

the top gel surface by removing all the solvent above the gel column and then applying the sample directly onto the top surface without the gel running dry. Buffer was then carefuly added to this layer to a height of about 10 cm before the reservoir was again connected up. This could easily be done and thus obviated the need to weigh the sample solution down with 1% sucrose dissolved in it or the need for direct application by means of a syringe inserted through the solvent above the gel surface.

Movement of solvent through the column was aided by a peristaltic pump (Pharmacia P - 1) set to provide a solvent flow of 5 ml/hour. Only this one solvent was used in eluting the molecules, making this an isocratic method of separation in contrast to the gradient elution that relies on a continuously changing pH of the solvent.

A single path ultraviolet monitor (Pharmacia) was used to monitor the passage of the proteins since they absorb the light maximally at 254nm wavelength.

3. Crossed Immunoelectrophoresis (Weeke, 1973)

This technique combines the use of electrophoretic separation of proteins in agarose gels followed by electrophoresis perpendicular to this into an antibody-containing gel. Immunological behaviour of each separated protein is thus demonstrated.

The following stock buffer was made up

44,3g Tris

22,4g Barbitone

0,53g Ca - lactate

0,65g Na azide made up to 1l with distilled water (pH 8,6..

This was diluted 1 + 7 with distilled water for the experiments.

With the use of this buffer a 1% agarose solution was made and fully dissolved by carefully heating the mixture to just boiling point and then measuring out with a pipette 15 ml which was run over the preheated (hair dryer) surface of a 90 x 110 x 1,5 mm glass plate which was held horizontal on a gel casting platform. Avoiding the formation of air bubbles and spreading the gel over the entire surface should result in a uniform homogeneous gel of about 1,5 mm in thickness.

After congelation of the agarose a well (2,5 mm in diameter) was cut in the one corner of the gel and the gel was placed on a cooling platform with tap water running through. The buffer tanks were filled with the diluted buffer and connected with the gel by means of three filter paper wicks. The gel was turned so that the direction of migration during electrophoresis was away from the well along the long side of the gel. The anode was thus connected to the far side and the cathode to the well side of the gel. 5 µl of sample was measured into the well, the apparatus was covered and electrophoresis at 350 V (constant voltage) was performed for 45 minutes. A marker drop of bromophenol blue could be placed on the gel and run along the opposite side as the sample.

After completion of this the top two-thirds of the gel (60 x 110 mm gel) was cut off from the 30 x 110 mm strip in which the sample ran and the former was peeled off the glass plate which was then returned to the casting platform.

The 1% agarose was again used by heating the mixture to just boiling point and then allowing it to cool off to about 55°C. At this temperature 12 ml of gel

was carefully mixed with the commercially obtained antibody (anti human albumin, Dako) which was measured out into test tubes in a water bath (50°C) beforehand to preheat. The exact amount of antibody solution determines the antigen / antibody ratio which is crucial for the second dimension range of migration. In these experiments 20 µl and 60 µl were respectively used in two sets of separations.

The gel was carefuly mixed with the antibody solution in the water bath without generating air bubbles. This was then poured over the denuded area of glass next to, and overlapping slightly with, the remaining sample-containing gel.

After congelation (10 - 15 minutes) the gel slide was transferred back to the electrophoresis tank and set up to allow migration out of the first gel (cathodal side) into the antibody containing second gel (anodal side) and the wicks were placed.

Electrophoresis was carried out under the same conditions mentioned above but only for 35 - 40 minutes or until a dye marker sample had run the whole width of the plate.

After completion the gel plate was removed and covered with saline and three sheets of filter paper and a glass slab with about 1kg mass on top. This was continued for 12 hours but the paper was replaced three times during that period.

Then the plate was dried in a preheated oven at 90°C for 30 minutes and then allowed to cool down before it was immersed in the following staining solution for 20 minutes.

5g Coomassie Brilliant Blue R250

450ml 96% ethanol

100ml glacial acetic acid

450ml distilled water

The solution was left standing overnight after mixing and was then filtered before use.

After this the gel was destained three times, each time with fresh destaining solution for a total time of 15 minutes in a mixture of:

450ml 96% ethanol

100ml glacial acetic acid

450ml distilled water

The gel was then dried at 90°C in the oven for 5 - 10 minutes.

Further modifications of this technique would apply different methods to the first dimension of separation. In this experiment isoelectric focusing, polyacrylamide disc gels and SDS-polyacrylamide disc gels were used for the initial separation.

For identification in this step the polyacrylamide gels were cut lengthwise (best performed when frozen) and the gel bond of IEF could be split. In both cases the other half was then be stained normally. Before pressing after the second dimension electrophoresis the half gels of polyacrylamide had to be removed from the glass but the IEF strip could remain and be pressed and dried with the agarose.

9. Plasma total protein determination

These estimations were done by using the method of Lowry et al (1951) using bovine serum albumin as a standard and reading the absorbances on a spectrophotometer (Spectroplus 20) at 660 nm.

10. Production of specific antibodies

For this purpose the method suggested by Harboe and Ingild (1973) was used. The purified antigen protein was diluted in saline to a concentration of 4mg/ml and 3-4 kg rabbits were used as experimental animals in which the antibodies were raised. On days 0, 14, 28 and 42 each rabbit was given a standard mixture consisting of 50µl antigen solution + 50µl Freund's incomplete adjuvant. This mixture was injected very superficially subcutaneously in the thicker part of the skin over the scapula. Or[illegible] sample of up to 45ml of blood was withdrawn from a lateral ear vein. [illegible] an alternative up to 60ml of blood was obtained by cardiac puncture in [illegible]etised rabbits. Blood sampling was repeated every six weeks, pr[illegible] animals had received a booster injection of the standard mixture 8-10 days before.

After centrifugation the plasma samples were frozen and stored. Plasma obtained from each rabbit on separate bleeding occasions was pooled for isolation of the IgE and IgA.

To each 100ml antiserum 25g ammoniumsulphate was added and the mix[illegible]as allowed to stand for about 20 hours at room temperature. After centrifugation for 30 minutes at 4,000 xg 98% of the antibody was precipitated and the supernatant could be discarded.

This precipitate was then washed with about 25ml 1,75M ammoniumsulphate solution, centrifuged and the supernatant again discarded. This washing was repeated once more. After dissolving the precipitate in a small amount of water the mixture was placed in a 25ml dialysis bag and dialysed at 4°C for two 12-hour periods against distilled water, one 24-hour period against an acetate buffer (0,05M Na-acetate + 0,021M H-acetate, pH = 5), two 12-hour periods against distilled water, and again one 24-hour period against the acetate buffer. The sample was then again centrifuged at 4,000 xg for 30 minutes and the precipitate (lipoproteins) discarded.

The supernatant was then trasnsferred to a column containing 25ml DEAE - Sephadex A50 equilibrated with the acetate buffer (pH = 5). The column was eluted with about 25ml acetate buffer. The eluate was then concentrated by a salting out process using 25g ammoniumsulphate per 100ml eluate and then dialysed against 0,1M NaCl for twelve hours at 4°C. The final solution was frozen and stored at -20°C.

RESULTS

ISOLATION OF ALBUMIN

Cellulose acetate electrophoresis was used to follow the success of t two albumin isolation methods and Lowry et al's total protein estimation method was used to calculate the percentage protein yield. The polyethylene gl precipitation method yielded above 80% of the available albumin but substantial amounts of impurities were present.

The ethanol fractionation method, on the other hand, yielded consistently more than 90% of the available albumin and contained no electrophoretically detectable impurities. For these reasons, and also because of the ease of methodology, this method was preferred and used for the isolation of the experimental samples. An example of the cellulose electrophoretic separation of lion plasma in the different stafes of isolation compared to that of a human sample can be seen in Fig 1. In both instances "pure" albumin in the characteristic configuration was obtained with only trace amounts of unwanted separation products.

EXPERIMENTAL MANIPULATION

In all the following experiments lion whole plasma as well as the isolated lion plasma albumin were used in all the different techniques.

A. Cellulose acetate electrophoresis

This separation method was used in the investigation of the following:

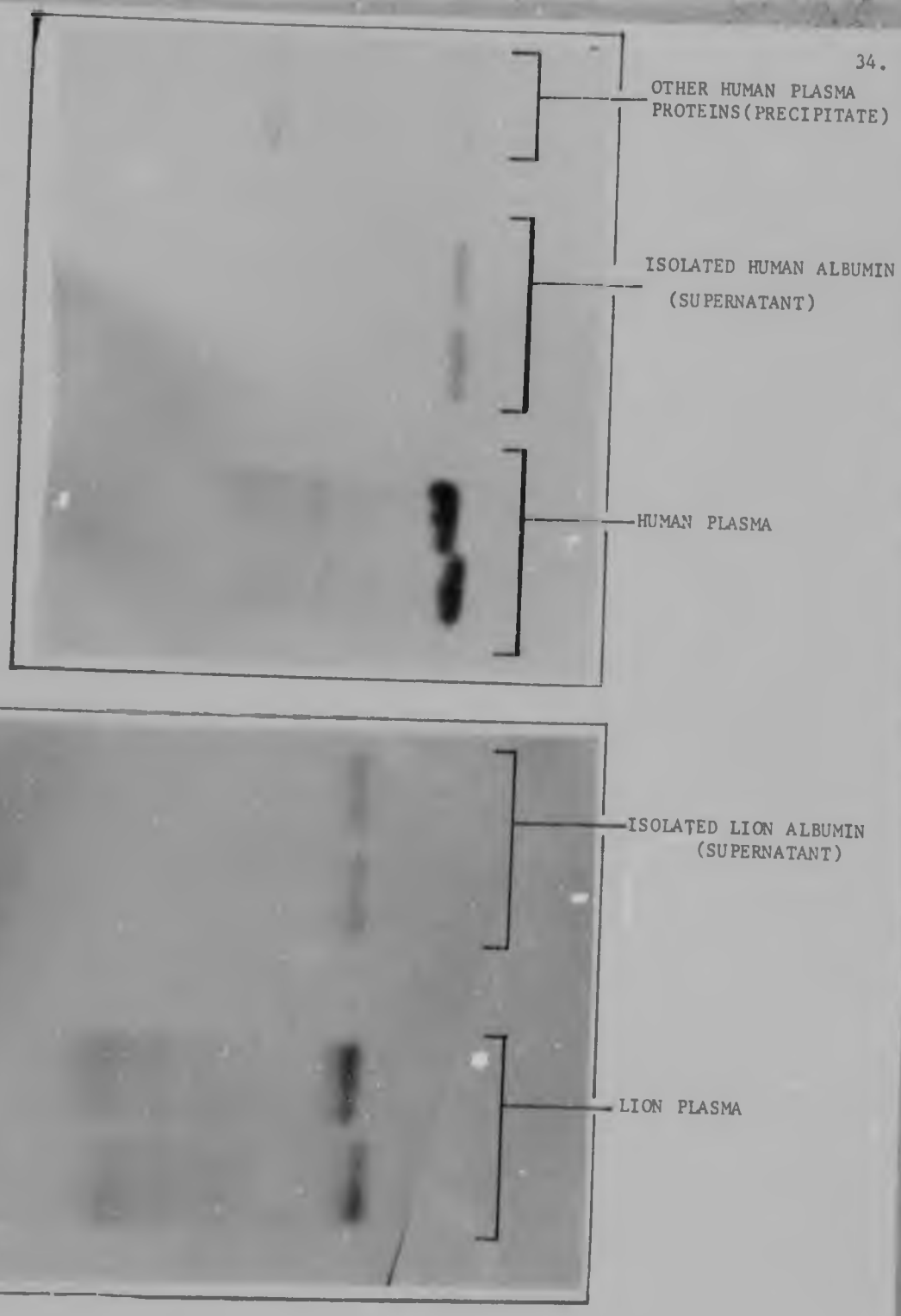


FIGURE 1. QUALITY CONTROL OF THE ISOLATION PROCEDURE.

1 Establishing the characteristic pattern

Plasma samples of eight lions were initially repeatedly separated and all separations showed the same pattern of a high concentration fast running albumin band followed by a low concentration slower running band (Fig.2).

2 Comparison with human plasma albumin

All eight lion plasma samples were compared to human plasma separated on the same strips and under identical conditions. This comparison highlighted the different patterns of albumin with the single human albumin band and the double lion albumin bands. It also revealed the consistent pattern of faster running lion albumin bands (Fig.3).

3 Comparisons with other species

Separations of plasma from cheetah and domestic cat were performed on the same strips as lion and human plasma. All the cat species had faster running albumins compared to the human (Fig.4).

4 Comparisons between lions of different areas

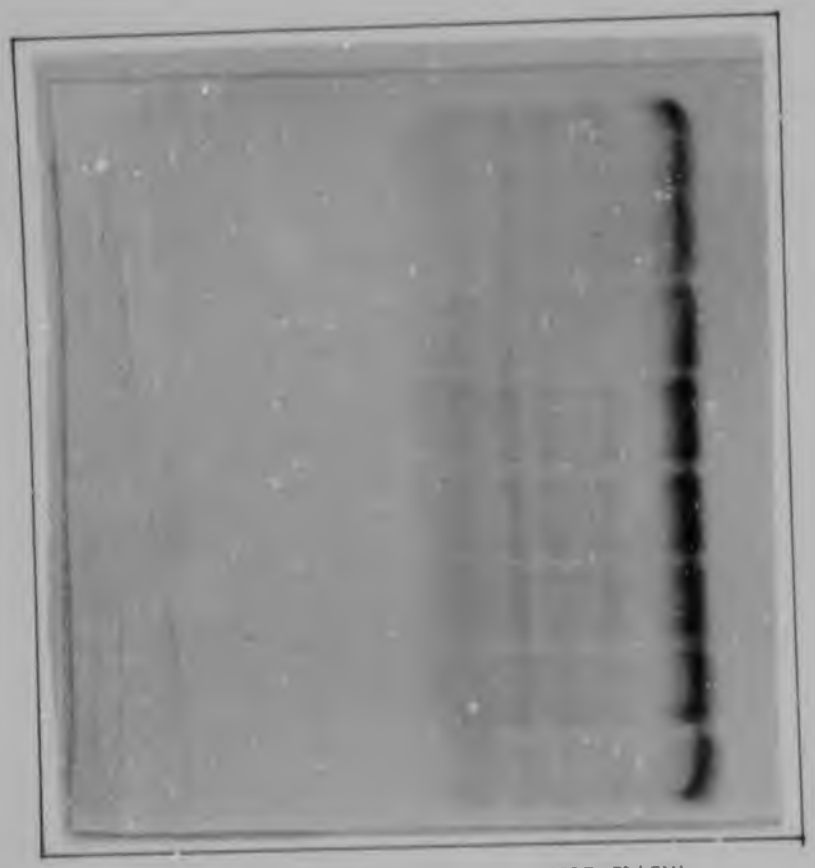
The sample population was extended to include lions from all over Southern Africa including the Eastern Transvaal (Kruger National Park), Northern Transvaal, Kalahari Desert and Northern Namibia (Etosha Game Reserve). This made up a sample size of 65 lions and their electrophoretic patterns were all identical.

5 Influence of time from immobilization to sampling

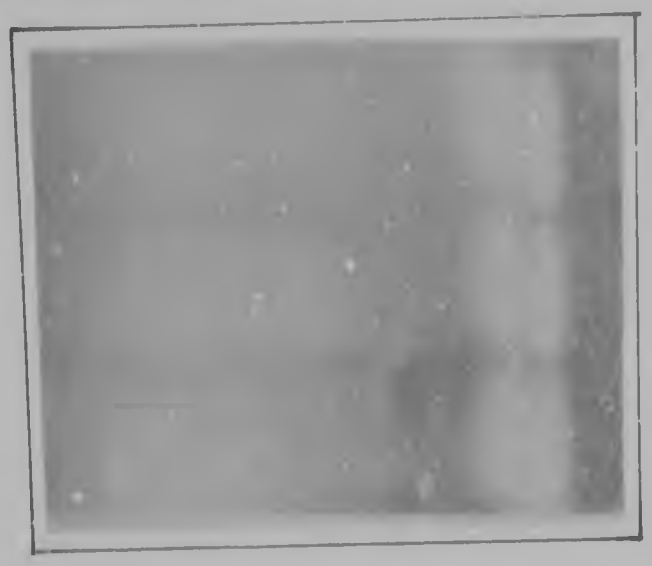
Previous workers (Melton et al, 1987) reported an effect of this time interval on the total plasma protein composition. The present investigation found no differences in the separation patterns regardless of the elapsed time (up to 1,5 hours).

6 Influence of immobilization drugs

The two drugs used in immobilizing these animals were xylazine hydrochloride



SEPARATION PATTERN OF LION WHOLE PLASMA.



SEPARATION PATTERN OF HUMAN BISALBUMINEMIC PLASMA

FIGURE 2. CELLULOSE ACETATE ELECTOPHORETIC SEPARATION.

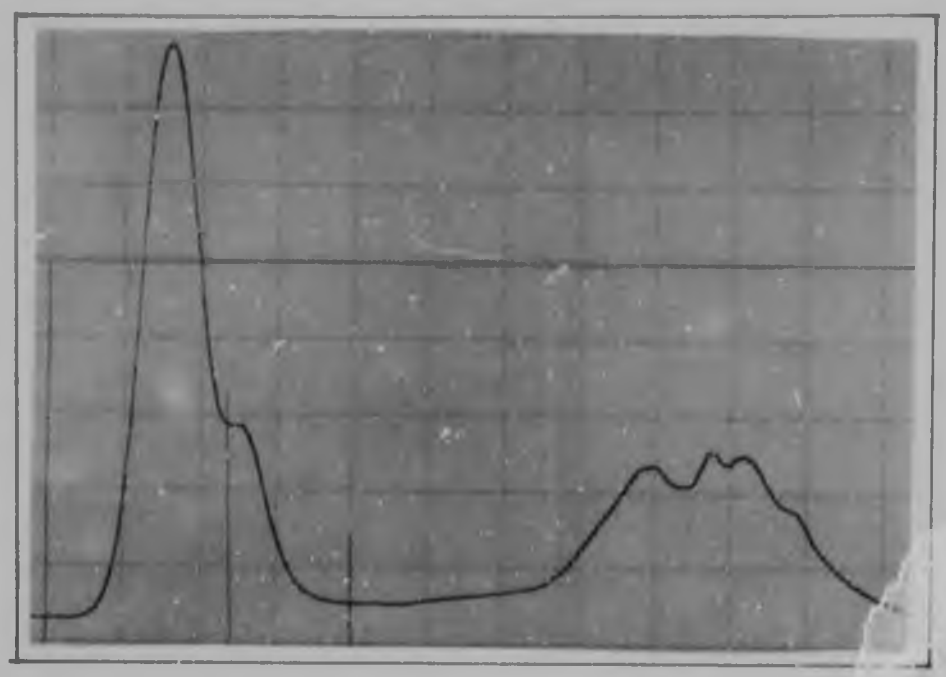


FIGURE 2b DENSITOMETRIC ANALYSIS OF ELECTOPHORETOGRAM.



LION ALBUMIN

HUMAN ALBUMIN



LION ALBUMIN

HUMAN ALBUMIN

DIRECTION OF SEPARATION

FIGURE 3. COMPARISON BETWEEN HUMAN AND LION ALBUMIN SEPARATED ON CELLULOSE ACETATE.



FIGURE 4. COMPARISON AMONG VARIOUS DIFFERENT SPECIES
AFTER CELLULOSE ACETATE ELECTROPHORESIS.

(Rompun; Bayer, West Germany) in combination with ketamine hydrochloride (Vetalar; Park-Davis, USA) and phencyclidine hydrochloride (Sernylan; Bio-Ceutic Laboratories Inc, USA). The effects of anaesthetic drugs on other blood chemistry and haematological parameters have been reported (Steyn, 1975). Although the protein content of plasma could be affected, this study showed no difference in protein separations.

7 Influence of anti-coagulant

In this investigation blood was collected in either heparin, oxalate or citrate before centrifugation or alternatively allowed to clot before centrifugation. The separation of the different plasmas as well as serum were as far as the albumin pattern was concerned, all similar.

8 The effect of storing

It is well known that storing plasma at -4°C in a freezer does not completely prevent samples undergoing changes associated with protein denaturation, possibly due to continued enzymatic activity. In addition to this, frequent thawing and refreezing may be part of the usual laboratory procedure where portions of samples are used and the remainder stored again. The effects of such intermittent freezing and thawing have been described for other wild animal species (Cheney, 1982) and in this investigation the same general changes as far as total protein and albumin concentrations could be observed in lion plasma. The electrophoretic pattern, however, was not altered. Frequent thawing and refreezing was avoided by dividing all samples into small aliquots which were stored, used once and discarded. Total protein and albumin concentrations could therefore only be affected by minimal changes occuring during long term storage at -4°C.

9 Effect of length of separation time

A possible methodological explanation for a certain electrophoretic separation configuration could be the time allowed for separation which would then

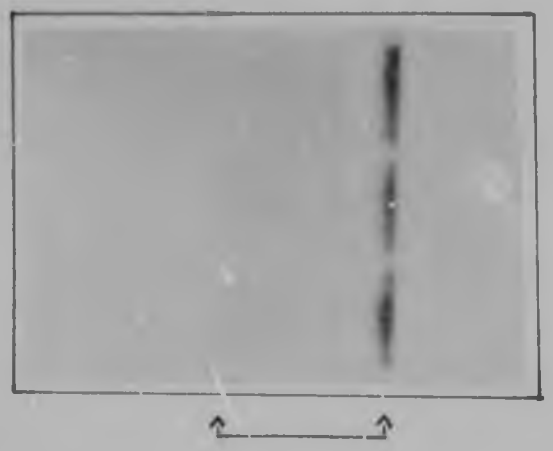
influence the distance of movement of various fractions. Short distances could thus result in inadequate separation with one fraction obscuring others and longer dis' nces may allow for splitting single-type proteins into subunits, making comparisons to standard patterns very difficult. Electrophoretic time was varied from 10 minutes to 45 minutes. In all separations lasting a shorter time than the standard 20 minutes an inadequate separation could be observed that not only obscured the second slower albumin band but also other plasma protein fractions (Fig.5). All the separations over a longer time revealed the two albumin bands but spread the bands of other fractions so wide that they became very indistinct and difficult to identify.

10 Effect of different concentrations of sample

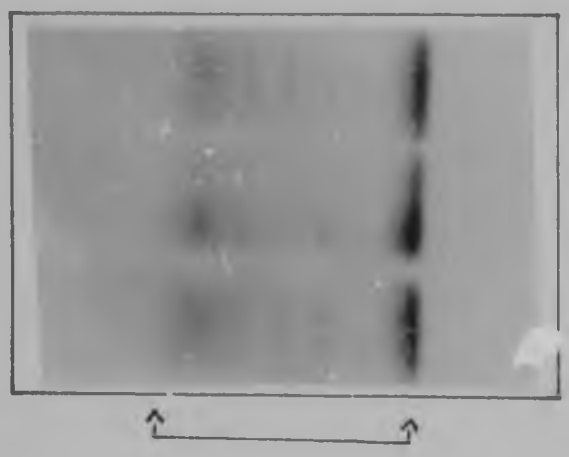
Since loading of different amounts and differences in concentration of samples can influence the clarity of electrophoretograms and change the behaviour of proteins (especially in very dilute concentrations) a comparison was made between the separation of dilute samples. Plasma was diluted with isosmotic saline and Fig.6 shows the separation of a 1 + 2 and a 2 + 2 dilution compared to undiluted plasma. In these two dilutions the proteins of smaller concentration become progressively fainter but the double-banded albumin is maintained. Further dilutions (1 + 3 or 1 + 4) yielded a very faint albumin picture that, however, still retained the same configuration. More concentrated samples (concentrated once and five-fold, respectively, in a Minicon microconcentrator) as well as increased amounts of sample loaded gave a very obscure electrophoretic pattern, totally impossible to identify.

11 Comparison between samples from wild and captive animals

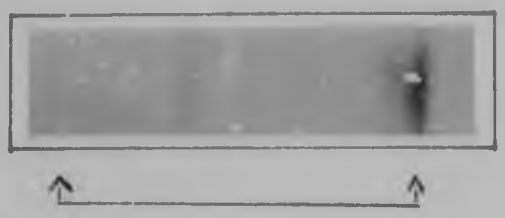
Reported values of the plasma total protein and albumin concentrations for wild lions (Melton et al, 1987) differ slightly from those of captive lions (Morse and Follis, 1974 and Fowler, 1978). In this study a small sample of eight lions were in fact from a captive population which may, mainly from the



10 MINUTES



20 MINUTES



45 MINUTES

FIGURE 5. THE EFFECT OF SEPARATION TIME ON CELLULOSE ACETATE ELECTROPHORESIS.

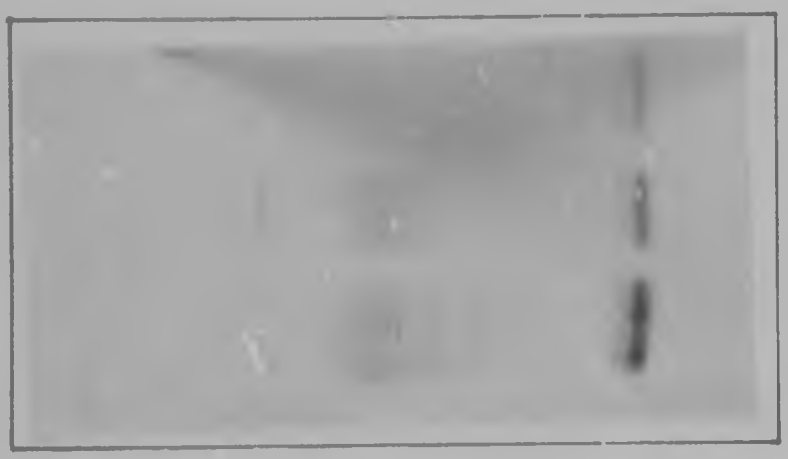


FIGURE 6. THE EFFECT OF SAMPLE DILUTION ON SUBSEQUENT CELLULOSE ACETATE ELECTROPHORESIS.

point of view of a different diet, have had a slightly modified plasma composition. Comparison of the electrophoretic patterns, however, did not yield any noticable differences.

12 Effect of feeding

Extending the investigation of the influence of diet, a small group of eight captive lions were used to observe possible changes caused by the quantity of food given at feeding sessions. These lions were starved for two days, given normal rations for two days and then double rations for two days with a suitable equlilbration time in between each session. Figure 7 shows the comparison between the electrophoretically separated samples of the plasma obtained after each of these three sessions. It is obvious that quantity of food eaten does not alter the protein pattern.

13 Percentage composition of the albumins

From the densitrometric scanning of the cellulose acetate strips (fig 2b) and the total protein estimations the following overall results for the whole population were obtained (Mean±S.D., n = 65)

Mean total protein concentration in plasma = 83,8 ± 3,3g/l

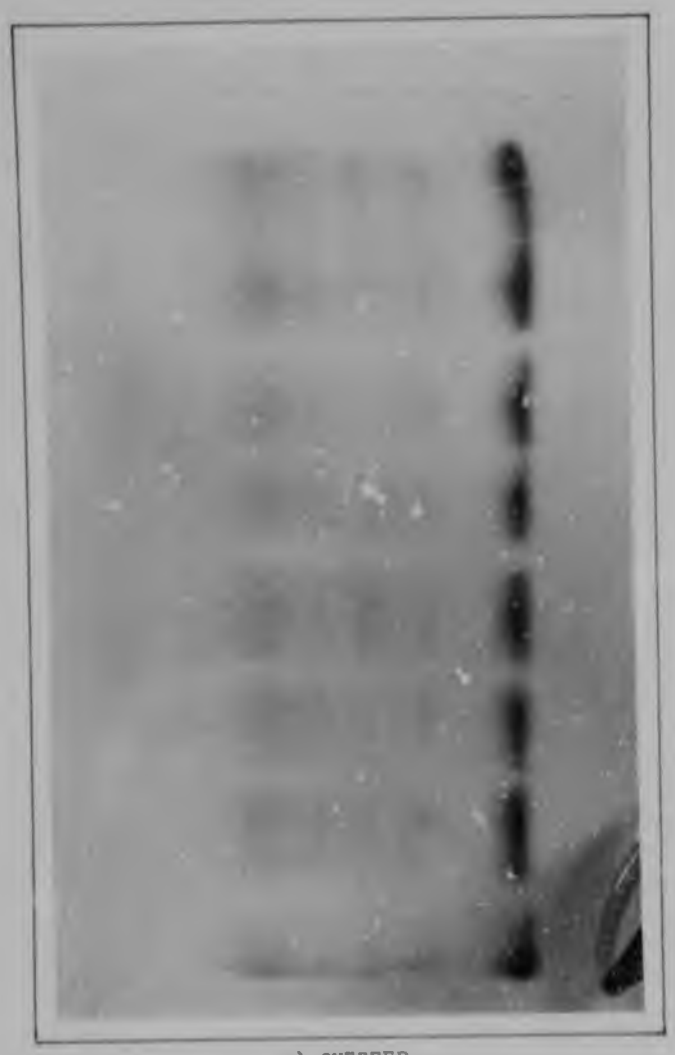
Mean albumin concentration in serum and plasma = 37,3 ± 7,2g/l

Mean concentration of anodal albumin band = 30,7 ± 5,98 g/l

Mean concentration of cathodal albumin band = 6,54 ± 3,22 g/l

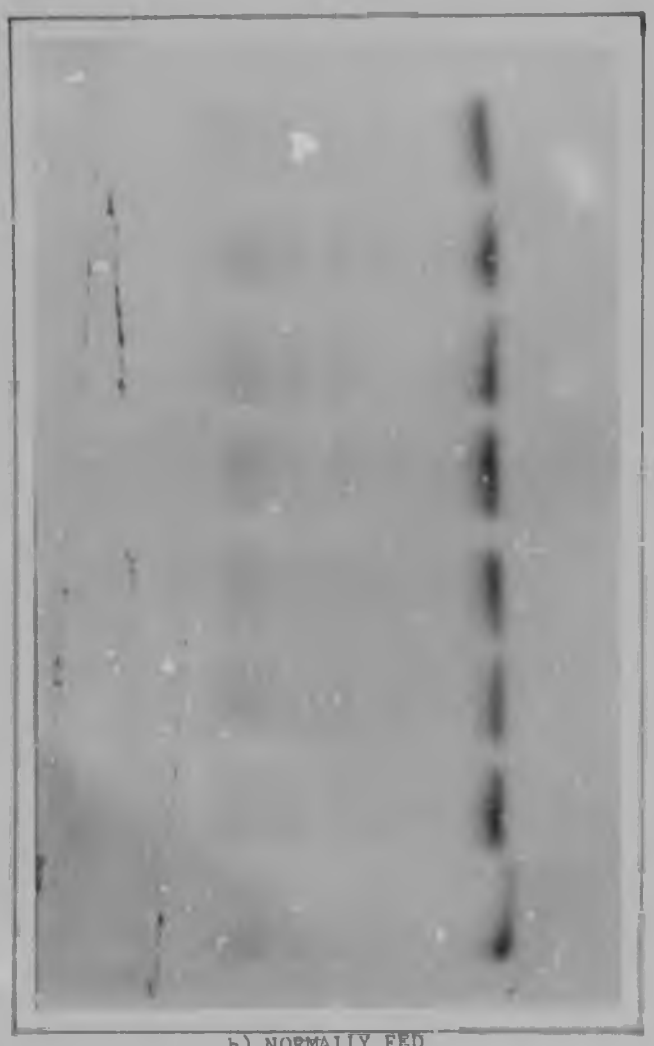
B. Polyacrylamide disc gel electrophoresis

In this separation technique it is possible to alter the gel concentration and hence the pore size of the gel. Initial separation of lion plasma was done repeatedly on a range of gel concentration media: 3%, 5%, 7,5%, 8,5% and 10%. The same double banded pattern was observed and it proved not to be a function of medium density (Fig.8a). The 7,5% gel yielded the clearest pattern and was

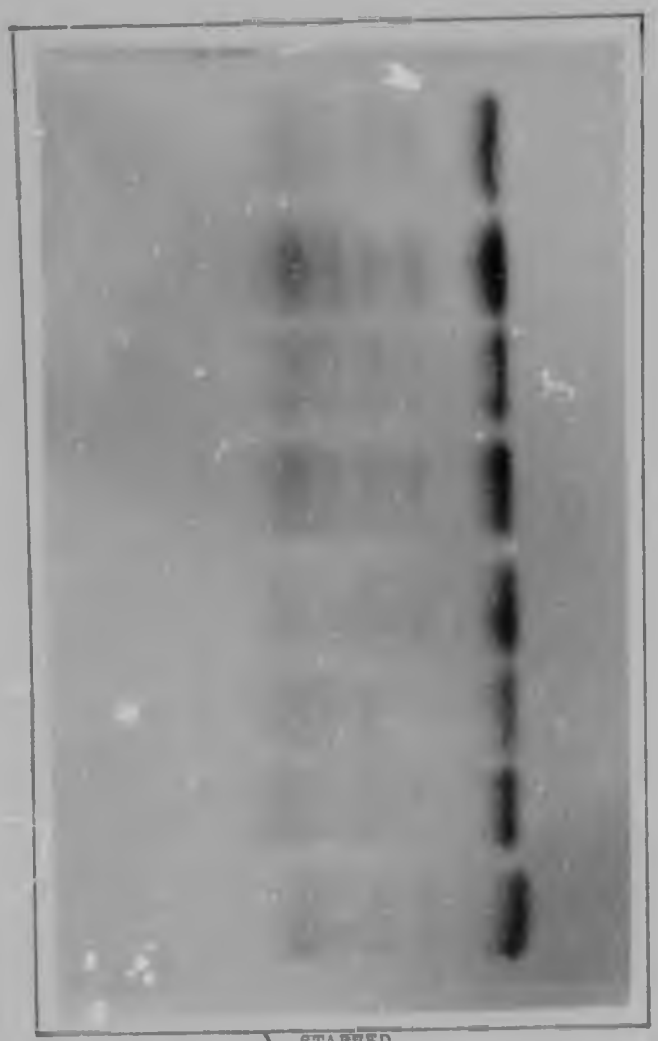


a) OVERFED

FIGURE 7. THE EFFECT OF FEEDING STATUS ON ELECTROPHORETIC SEPARATION PATTERN.



b) NORMALLY FED



c) STARVED



FIGURE 8a THE EFFECT OF DIFFERENT GEL CONCENTRATIONS ON DISC GEL ELECTROPHORESIS.

therefore chosen for subsequent separations, also including the separation of the purified albumin (Fig.8b)

This method was used to investigate some of the same influences tested on microzone cellulose strips:

1 Establishing the initial pattern in eight lion plasma samples

2 Comparison with human plasma

3 Comparisons between 65 lions from different geographical areas

4 Influence of length of period: immobilization to sampling

5 Influence of immobilization drugs

6 Influence of different anti-coagulants

7 Effect of storing of samples

8 Effect of length of separation time

9 Possible differences between wild and captive sample animals

10 Influence of amount of sample. Quantities of 1, 2 or 3 µl were compared.

In all these investigations the separation patterns were similar.

C. Analytical isoelectric focusing

This method separates proteins into very fine fractions which makes identification difficult since standard patterns are fairly irregular in configuration. In this study, on separating lion plasma, it was possible to identify the "albumin zone" by comparing it to the isoelectric points of human albumin and other marker proteins, eg haemoglobin. On this basis it is possible to state that the albumin zone for lion albumin is quite distinctly different from that of human albumin and further that this zone is similar in all lion samples (Fig.9). The possible differences in focusing pattern was investigated for the following influences:

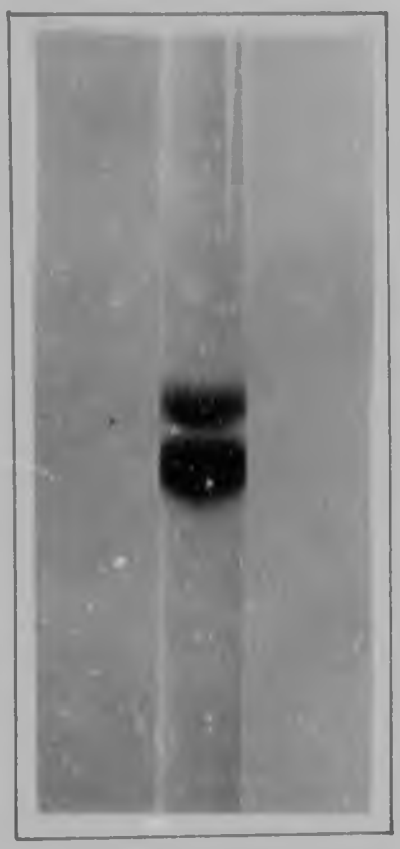


FIGURE 8b. SEPARATION OF LION ALBUMIN BY POLYACRYLAMIDE DISC GEL ELECTROPHORESIS.

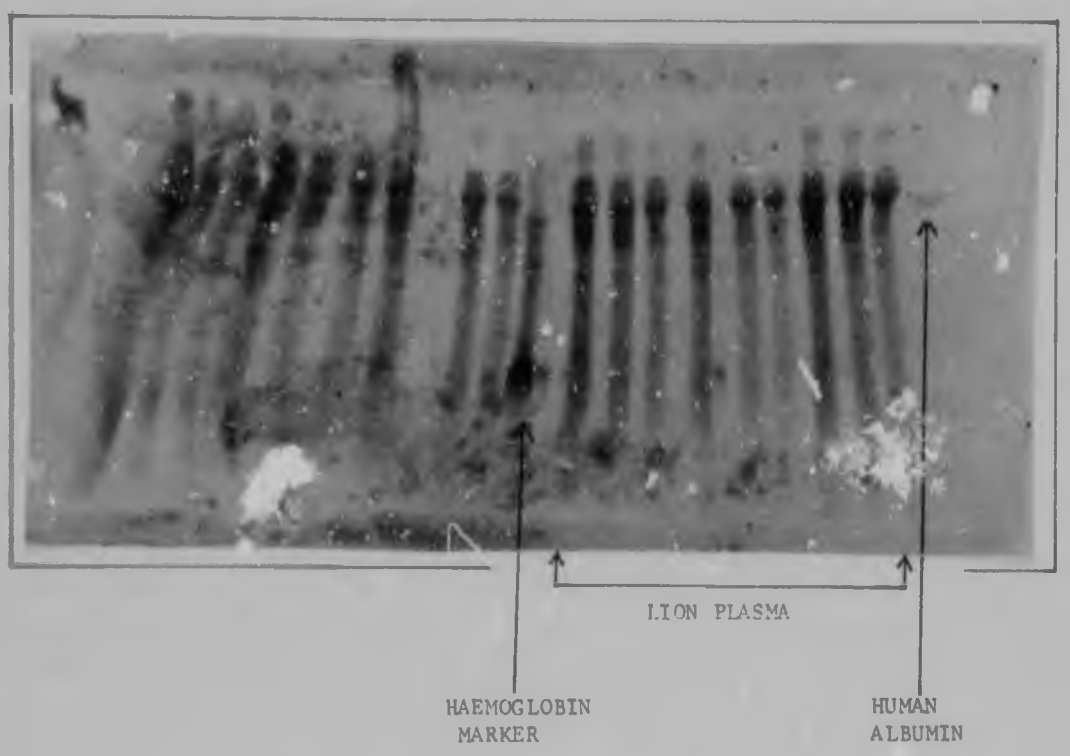


FIGURE 9. ALBUMIN ZONES OBTAINED BY ISOELECTRIC FOCUSING

1 Comparisons between 65 lions from different geographical areas

2 Comparis[illegible]uman albumin

3 Influenc[illegible] of period: immobilization to sampling

4 Influence of immobilization drugs

5 Influence of different anti-coagulants

6 Effect of storing of samples

7 Differences between wild and captive animals

8 Influence of length of focusing period

It is known that an increase in the duration of focusing will result in fatty acids being removed from albumin, so that the albumin will in fact undergo defatting (Basic et al, 1978). Should the focusing pattern be influenced by this combination of fatty acids and albumin, this separation of the two substances would alter the focusing pattern. Lengthening the focusing time to three hours had no influence and Fig.10 shows a typical isoelectric focusing plate.

D. Column exclusion chromatography

Recordings made of the lion plasma sample-containing buffer showed a very clear isolation of the albumin fraction as a whole but separation of the two components could not be achieved. Reducing the flow to 2ml/hour still did not result in producing any split in the albumin peak. Isolation of the two albumin fractions could thus not be achieved by column chromatographic methods, in spite of using superfine Sephadex 200 in a very large volume column at an extremely slow flow velocity (0.2 ml per hour).

E. Preparative IEF

By employing a blotting technique using a sheet of filter paper, the isoelectric

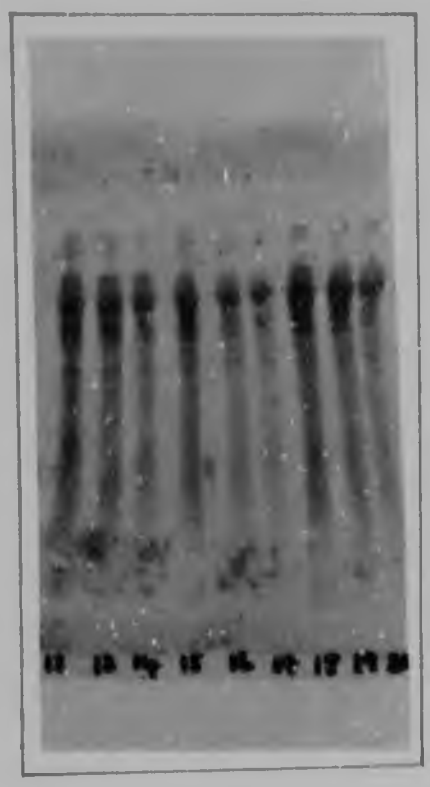


FIGURE 10. ISOELECTRIC FOCUSING OF LION PLASMA.

focusing pattern could be determined. From this it became clear that separation of the two albumin types would be difficult, they focused very close together and in a wavy front. Cellulose acetate electrophoresis of the resuspended "isolated" samples eluted from the medium showed the two-band pattern appearing in both fractions cut out of the preparative gel.

F. SDS disc gel electrophoresis

In this method the detergent covers the charged sites on the partially denatured proteins so that separation occurs more on the basis of molecular mass than charge. In this instance all the modifications of this technique (varying gel concentration by changing the amount of concentrated acrylamide bisacrylamide added and also varying the amount of sample added) still prooduced only a single albumin band (Fig. 11).

G. SDS slab gel electrophoresis

In this method various attempts were made to modify the procedure to elliminate any influence possibly caused by the procedure. For example, samples were either stained or not stained with the tracking dye (bromophenol blue) and were either subjected or not to heat denaturation. Only those subjected to both tracking dye prestaining and heat denaturation yielded a satisfactory separation pattern. These samples also divided into only one albumin band.

H. Crossed immuno-electrophoresis

This was done by using agarose slab gels, polyacrylamide disc gels, isoelectric focusing and SDS polyacrylamide disc gels separating both lion plasma and lion albumin into their respective subfractions in the first dimension. The second dimension consisted of agarose containing either anti-human albumin (Dako) or anti-lion albumin (obtained from rabbits in our laboratory) antibodies. The



FIGURE 11. SDS - POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

separation on the agarose was not distinct and revealed the true two-banded albumin pattern only when the application of the sample was done in thin strip form instead of the usual well. SDS seemed to interfere with the antibody binding reaction in the second dimension and thus the method of using SDS polyacrylamide disc gels was discarded.

Figures 12 and 13 show the pattern obtained by polyacrylamide disc gels in the first dimension separating human albumin and lion albumin respectively and using anti-human albumin antibody in the second dimension.

Figures 14 and 15 show the pattern obtained from first dimension separation in agarose gels of human and lion albumin respectively and then reacting that against anti-human albumin antibody.

Figures 16 and 17 show the pattern obtained from first dimension splitting of human and lion albumin respectively on IEF and then obtaining the reaction with anti-human albumin antibody.

Figures 18 and 19 show the pattern obtained from initial separation of human and lion albumin respectively on IEF and subsequent reactions with anti-lion albumin antibody.

In all of these, despite smaller differences due to varying amounts of background proteins not taking place in the specific reaction and also because of the relatively impure nature of the non-commercially manufactured anti-lion albumin antibodies, the configuration of the crossed reaction was similar. Human albumin separated into one distinct band that reacted in one smooth and regular peak with both anti-human and anti-lion antibodies. Lion albumin

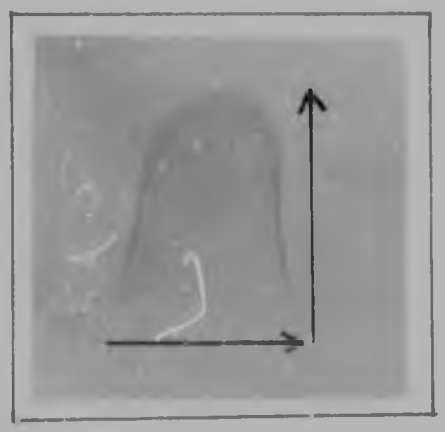


FIGURE 12. CROSSED IMMUNOELECTROPHORESIS OF HUMAN ALBUMIN AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.

FIRST DIMENSION: AGAROSE ELECTROPHORESIS.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

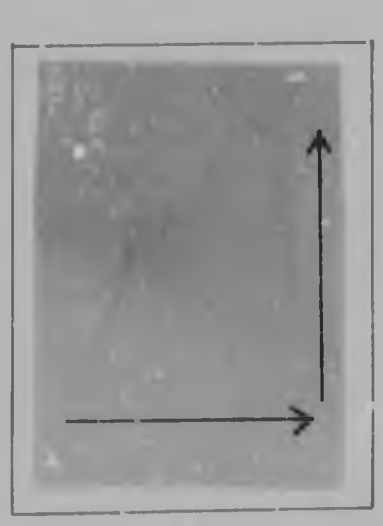


FIGURE 13. CROSSED IMMUNOELECTROPHORESIS OF LION ALBUMIN AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.

FIRST DIMENSION: AGAROSE ELECTROPHORESIS.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

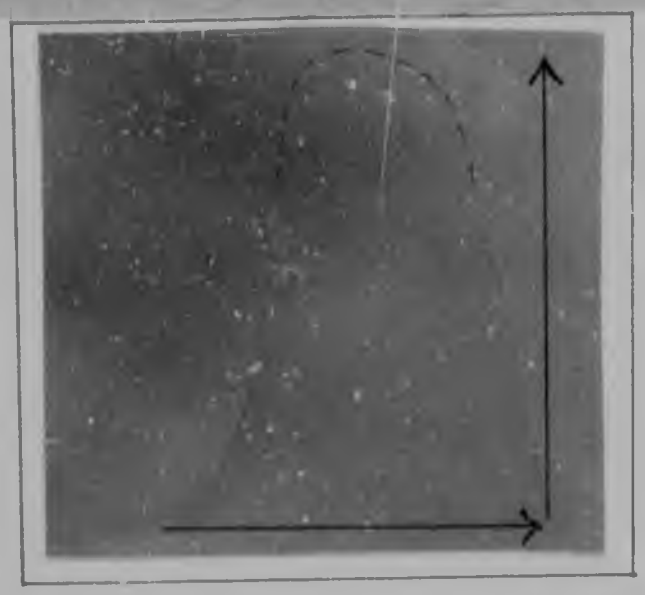


FIGURE 14. CROSSED IMMUNOELECTROPHORESIS OF HUMAN ALBUMIN AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.
FIRST DIMENSION: DISC GEL ELECTROPHORESIS
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

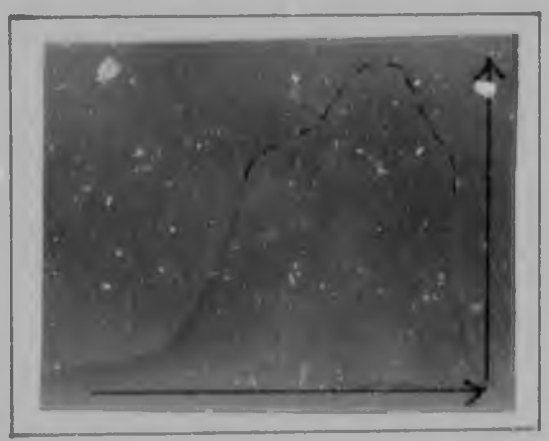


FIGURE 15. CROSSED IMMUNOELECTROPHORESIS OF LION ALBUMIN AGAINST AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.
FIRST DIMENSION: DISC GEL ELECTROPHORESIS.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.



FIGURE 16. CROSSED IMMUNOELECTROPHORESIS OF HUMAN ALBUMIN AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.
FIRST DIMENSION: ISOELECTRIC FOCUSING.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

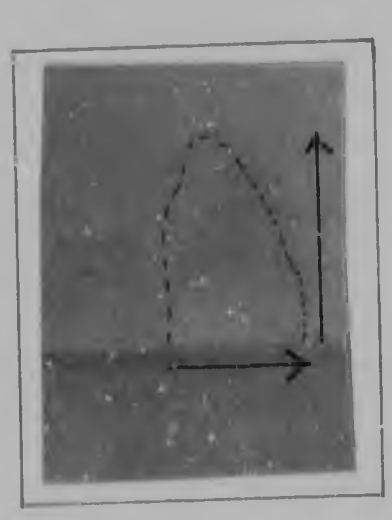


FIGURE 17. CROSSED IMMUNOELECTROPHORESIS OF LION ALBUMIN AGAINST ANTI-HUMAN ALBUMIN ANTIBODY.
FIRST DIMENSION: ISOELECTRIC FOCUSING.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

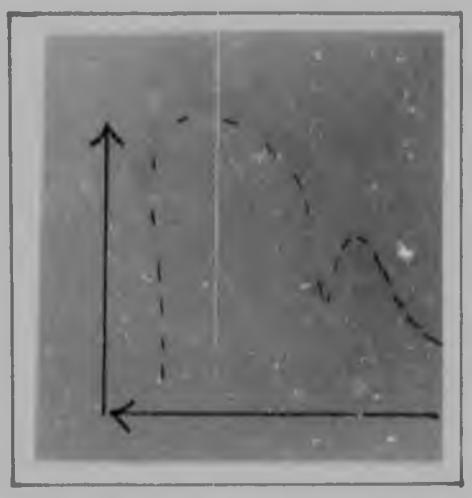


FIGURE 18. CROSSED IMMUNOELECTROPHORESIS OF HUMAN ALBUMIN AGAINST ANTI-LION ALBUMIN ANTIBODY.

FIRST DIMENSION: AGAROSE ELECTROPHORESIS.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

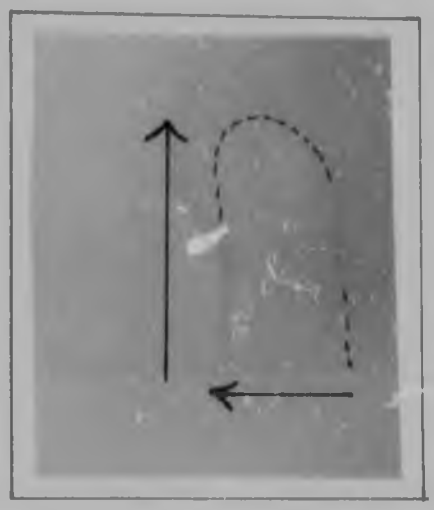


FIGURE 19. CROSSED IMMUNOELECTROPHORESIS OF LION ALBUMIN AGAINST ANTI-LION ALBUMIN ANTIBODY.

FIRST DIMENSION: AGAROSE ELECTROPHORESIS.
SECOND DIMENSION: AGAROSE ELECTROPHORESIS.

separated into the characteristic double-banded pattern which then reacted in one smooth and continuous peak with a "toe" indicating the reaction of the smaller more cathodal albumin band. This indicates the expected cross-reactivity between albumin and specific antibodies of different species (human and lion). More importantly, it reveals that the two lion albumin bands are immunologically similar.

DISCUSSION AND CONCLUSIONS

From the results of these investigations it is clear that the isolation procedures resulted in a reasonably purified albumin. Pure albumin in this context would mean a substance not accompanied by other (peptide or non-peptide) substances. It must be realised, however, that this scavenger-like protein would invariably be carrying ligands bound to it. Purity, thus, would be based on coherent general behaviour like precipitation characteristics, migration rate through gel columns and size-and charge-based electrophoretic separation patterns but would acknowledge the existence of bound particles not originally synthesized with the albumin. This was already noticed decades ago (Kendall, 1941) when even repeatedly crystallized human albumin did not give solubility curves characteristic of a pure substance. This is also the underlying cause for isoelectric focusing showing an albumin region of dissimilar bands rather than a single albumin fraction. The existence of these multiple albumin forms that differ slightly in charge or solubility is referred to as the microheterogeneity of albumin. Although the factors involved in causing these changes are numerous and varied, they have in common the fact that they all occur after synthesis of the molecule and thus cause post-translational modifications to albumin as it circulates through the body.

The following discussion of albumin genetics, synthesis, amino acid sequence, isomeric forms and the concept of microheterogeneity is based on the review articles available that deal with these overall principles on a general basis (Foster,1977 and Peters,1985).

MICROHETEROGENEITY OF ALBUMIN

Fatty acids are bound tightly enough to remain with the albumin during electrophoresis or ion-exchange chromatography. At the pH conditions of isoelectric focusing they are slowly removed from the albumin. Human defatted albumin will focus at pI 5.6 and fatty-acid containing albumin at pI 4.8. By lengthening the focusing period the former band would increase in intensity while the latter would decrease. This migration of fatty acids towards the anode most probably did occur with the lion samples but did not seem to alter the overall pattern of separation. These fatty acid molecules (mostly in their soap or salt form) bind with decreasing affinity to the albumin, usually one or two per albumin molecule, rising to not more than four in high titre fatty acid situations (eg.after exercise). The binding sites for these ligands on human albumin seem to be the middle of domain III, the border between domains II and III, middle of domain I and the middle of domain II for the first four fatty acid molecules respectively. The binding seems to occur in two distinct steps: firstly a rapid but loose attachment to the albumin molecular surface followed secondly by an opening of a hydrophobic pocket to allow the entry of the hydrophobic ligand portion into the interior of the molecule. This results in the formation of an ionic bond between the carboxyl of the fatty acid and a cationic amino acid side chain. This binding, of especially the first two fatty acid molecules, changes the shape of the fatty acid molecule from an elongated oval to a stubby and rounder shape which one would expect to be the prevalent molecular form in plasma.

Bilirubin can also result in an anodic extension of the albumin band on agarose electrophoretograms. There appears to be only one strong binding site for

bilirubin which is in domain II. Binding here results in unfolding of the bilirubin molecule by breaking the hydrogen bonds and then holding it in a tight, highly twisted orientation in a locus which is protected from the surrounding medium. This binding occurs as an almost instantaneous association of bilirubin with albumin, followed by a series of relaxation processes to reach the final configuration. The binding of the bilirubin is affected by the number of fatty acids bound to the albumin where large numbers of fatty acid molecules will compete with and depress the bilirubin binding whereas smaller numbers of fatty acids will actually enhance bilirubin binding due to a decreased alteration in shape of the albumin molecule. Albumin from the umbilical cord and in prolonged uremia also shows a decreased capacity (with normal affinity) for binding bilirubin due to an unknown ligand in each instance partially blocking the bilirubin site. Charcoal treatment at pH 3 clears the albumin of these ligands and restores the bilirubin binding capacity.

The single thiol group (on Cys.34) in domain I is a restricted hydrophobic site that can bind cystine and glutathione for transport from the gut to the peripheral tissues. This binding can cause heterogenicity at that site that can influence the patterns obtained after ion exchange chromatography.

Albumin is a non-glycoprotein, on of the few secreted proteins to lack carbohydrate, but nonenzymatic glucosylation inserts a monosacharide onto about 8% of albumin in the normal individual (and considerably more in the uncontrolled diabetic with prolonged hyperglycaemia). The binding site for this monosacharide is in domain III.

Treatment of rheumatic diseases very often involves prolonged exposure to acetyl salicylic acid which can cause acetylation of albumin in domain II. Similarly,

mostly transient, heterogeneity can be observed by electrophoresis because of treatment with certain penicillins, ethacrynic acid or mercaptopurine.

Circulating albumin will also exhibit an ever increasing degree of molecular structural changes. Isomeration by disulfide interchanges occurs with increasing age and this can affect up to 10% of circulating albumin.

Transient abnormalities can be observed in patients with pancreatic cysts where proteolytic enzymes alter the C-terminal of the normal albumin to create a faster-moving band on electrophoresis.

Gradual modifications also occur to side chains of amino acids, like in all circulating proteins, and deamination of asparagine and glutamine has been implicated in the cause of microheterogeneity. Nonenzymatic conversion of methionine residues to methionine sulfoxide also occurs in the circulating proteins of certain individuals.

These ligands, either bound tightly but reversibly or bound covalently to albumin or these structural changes of the albumin molecule can thus render the albumin very subtly heterogeneous and can influence certain analytical procedures. Only those not involving administration of specific drugs and those not resulting from a specific disease (like diabetes of pancreatic cysts) could possibly affect the entire lion population as described. For an explanation of the lion pattern attention should then shift to changes imparted to the albumin separation by a consistant interference from the binding of naturally occuring ligands in the circulation. This should then happen to such an extent that the albumin pattern will be affected not only in highly refined analytical systems like isoelectric focusing but also consistently in comparatively crude

separation analysis like cellulose acetate electrophoresis or polyacrylamide gel electrophoresis. Ligand binding or molecule modification having such a pronounced effect is virtually impossible and has not been reported in any previous work. Furthermore, the numerous different analytical methods and their many modifications should have illuminated the influence of any of these abovementioned factors in the sense of showing some changes in the separation configuration due to the different methods imposing differences in ligand binding characteristics on the albumin molecules. The few differences in the sample population (eg feeding pattern, age, sex) should also change some of these ligand attachments which should influence the eventual separation. The fact that the same pattern of separation was observed in spite of the different donor sources, degree of refinement of separation technique and sample manipulations points to more significant alterations to the albumin of lions than those imposed by slight molecular modifications or ligand binding influences.

SYNTHESIS OF ALBUMIN AND ITS AMINO ACID SEQUENCE

A more likely source of variation lies in the synthesis processes of albumin. These obviously have a genetic base or result from alterations of the normal process of albumin synthesis. Albumin RNA has been isolated from liver cell cytoplasm and from these, by cloning the complimentary DNA in bacteria, the nucleotide base sequence of the albumin gene has been established for a number of species. The position of the gene on the chromosome has been found to be in the same region as that of alpha fetoprotein, the embryonic counterpart of the adult albumin which is transcribed from the same strand of DNA double helix. These genes are about ten times as long as the coding segment and in both albumin and alpha fetoprotein coding segments there are fourteen introns,

creating fifteen exons. The lengths of these exons form a pattern of triplet homology that corresponds to the three albumin domains while the sites of insertion of the introns correspond to regular loci in the albumin chain. One finds that the internal threefold similarity pattern is much more homologous in the DNA sequence than in the protein amino acid chain, strengthening the concept that the present day albumin arose from a single domain or even just a single loop, the genes for which underwent replication.

Albumin synthesis in the hepatocyte follows the pattern of all secreted proteins when 19 ribosomes attach to one albumin mRNA to form a large polysome. This complex is guided to the membrane of the endoplasmic reticulum by a so called signal peptide, which is the first translated sequence and which shows a remarkable inter-species homology. This signal peptide directs the growing peptide chain through the membrane of the rough endoplasmic reticulum and is then cleaved off, even before the translation of the mRNA is completed.

Such newly formed albumin, still in the secretory channels of the liver cells, is initially in the form of proalbumin which is normal albumin with a hexapeptide attached at its amino terminus. In this form the protein migrates from rough to smooth endoplasmic reticulum and to the Golgi complex where the leader peptide is cleaved off just before release from the cell. Proalbumin is not normally found in the circulation and only mutations of the hexapeptide sequence that cause a blocking of the cleavage will result in this leader peptide being retained on the secreted molecule. Cleavage then takes place in the circulation, seemingly with the liver as the responsible organ.

This then results in the single chain molecule of which the peptide sequential composition is known in a few species. The existence and alignment of 17

disulphide bridges create the basis for the unique albumin molecular configuration of a series of 9 loops repeated in a triplet fashion of large-small-large loops making 3 homologous domains of 3 loops each. The amino acids are not distributed evenly; some favour the amino half of the chain and others the carboxyl half. The molecule is also not uniformly charged along its length with the amino head highly negative and the carboxyl tail less negative or neutral. Loop homology indicates that the large molecule could have evolved from a precursor of one-third or even one-ninth the present size, as was mentioned above.

Interspecies similarity is quite high between bovine and human and between rat and human (about 80% each) but slightly lower (about 63%) among all 3 species. Similarity between alpha-fetoprotein and albumin is a lot lower (less than 40%) in both humans and rats, with the homology more in the carboxyl end part of the molecules. Alpha-fetoprotein has no propeptide stage during its biosynthesis and has, apart from about 4% carbohydrate, a much more selective affinity for ligands than albumin.

The study of the effects of breaking peptide bonds without breaking disulphide bonds revealed that a variety of fragments can be obtained in this way. Isolation of these peptides has confirmed the disulphide-bonded loop structure, has helped to localise binding functions, has demonstrated the autonomy of tertiary structure of certain parts of the molecule and has helped to localise antigenic determinants. Loop 9 contains no tyrosine or tryptophan but contains antigenic sites to the extent that isolated bovine loop 9 fragments can prime animals for a secondary immune response to whole albumin or, when injected in the developing immune system, can give tolerance to the whole albumin molecule. In human loop 9 the active region has been determined as amino acids 345-373 and

monoclonal antibodies to the region have been manufactured.

Precise knowledge of the exact amino acid sequence has enabled researchers to calculate the accurate molecular mass and, in species where this has been possible, these calculated masses are remarkably consistent with estimations made previously on a hydrodynamic basis. Likewise, the nitrogen content of albumin can then be calculated more accurately and slight modifications can be made to the assumed 16% conventionally adhered to. For lion albumin these values, historic or altered by specific molecular mass calculations, are not known.

Evidence obtained from sedimentation studies, dielectric dispersion, electric birefringence, low angle X-ray scattering and electron microscopy confirmed the outline shape of the albumin molecule as being ellipsoid, not totally filled by polypeptide but having a hydration shell of water molecules, some at specific fixed loci and others more widely distributed but still tightly enough bonded to the protein by hydrogen bonds to render this water non-freezable. In natural albumin there is most probably 1 - 2 water molecules per amino acid residue.

The refractive index increment of albumin is very typical of a simple protein, consisting almost only of polypeptide chains. Ultraviolet light can be used to test the absorbance of amino acid aromatic side chains or peptide bonds, depending on how the protein is prepared before spectrophotometry. Fluorescence of albumin is determined by its tryptophan residues and the rotation of polarized light by an albumin solution is typical of a globular protein.

From this brief description of albumin synthesis it should be evident how genetic alterations causing changes in normal polypeptide structure could

influence the chemical, physical and immunological properties of the protein. Depending on the methods employed certain analytical results will indicate changes in some properties which, when viewed collectively, should point to the fundamental abnormality in basic structure.

ALBUMIN GENETICS

Expression of the albumin gene is codominant with the two alleles both showing complete penetrance. Genetic abnormality of one allele would then be controlling the synthesis of an abnormal albumin fraction which will co-exist with the normal fraction in the circulation. In humans this was the first category of this kind of albumin abnormality discovered in the mid 1950's.

About a decade later the homozygote condition where both alleles are abnormal and code for the same different albumin was described in North American Indian populations. Where bisalbuminaemia was the term used to describe the heterozygote situation of two different albumin molecules, alloalbuminaemia is used to describe both homozygote (one abnormal) and heterozygote (one normal, one abnormal) albumin conditions. In humans the total albumin concentration in plasma would be within normal limits and the two types of albumin (in the heterozygote situation) would each comprise about half the total albumin. The antigenic specificity of the human variant fractions has been tested by immunoelectrophoretic methods using anti-whole human serum and anti-human albumin antibodies raised in a variety of animals (horse, goat, sheep and rabbit). All these indicate that the fractions are immunologically identical to normal albumin.

Variant albumin types have only been described on the strength of their

electrophoretic behaviour with the basis of the difference thus being an alteration in amino acid make-up such that the overall electric charge changed. Any amino acid alteration that does not involve electric changes will not be detectable electrophoretically. The almost 100 human variants already described are based on a bewilderingly divergent group of electrophoretic methods so that some seemingly different variants may in fact be the same abnormality demonstrated in different ways. The choice of support medium and pH of separation influences the kind of electrophoretogram that can be obtained and, for human albumin at least, attempts have been made to standardise these two factors for the purpose of detecting and classifying variant fractions.

Only a few of the human albumin variants have had their specific abnormality identified. Of these some had one amino acid substituted in loop 9 and others had a substitution in the N-terminal hexapeptide which then did not prevent secretion but prevented the normal presecretion proteolytic splitting off of this segment. They are thus abnormal proalbumins in circulation.

Studies of the human albumin gene have revealed many more prevalent variations than eventually get expressed in the phenotype albumin molecule, indicating that these alterations must occur to a large extent in the untranslated intron regions. This would explain why polymorphism of the albumin gene is estimated at about a 1% occurance but albumin polymorphism is quoted at only about 0,1%.

ISOMERIC FORMS OF THE ALBUMIN MOLECULE

Another source of albumin variation that warrants mention is the existence of isomeric forms of te protein. Human albumin, when titrated with HCl, shows the appearance of a so called F (fast) form between pH 4 - 4,5 which has altered

electrophoretic mobility, precipitation ability, optical characteristics, helical content revealed and UV absorption. All these changes seem to be because of a partial opening of the albumin molecule exposing groups previously hidden from external solvents. Below pH 4 the molecule becomes fully uncoiled within the limits of its disulphide bonds which causes the threadlike molecules to increase the viscosity of their solutions tremendously. This is commonly called the expanded, E form. The uncoiling seems to be based on the mutual repulsion of the newly acquired positive charges on the molecule.

On the alkaline side of neutrality two more isomeric forms exist, namely the B (basic) and A (aged) form. The former is obtained at pH 9 and is characterized by a drop in helical content, slower electrophoretic migration and an increase in the binding capacity for calcium due to the exposure to the outside of the molecule of numerous hydrogen atoms that now become exchangeable. The latter aged form is obtained by keeping albumin solutions at low ionic strength at pH 9 and at 3°C for 3-4 days. This form then has an even slower electrophoretic migration rate and a decreased solubility. These isomers do not represent the limits of the conditions to which albumin can be exposed; titration can be conducted reversibly between pH 2 and 12 if the time spent at the extremes is minimised. Even exposure to 8M urea or 6M guanidinium chloride will not lead to permanent structural damage. Damage done by a high pH environment seems to be mostly directed towards the disulphide bonds whereas low pH damage is based on peptide bond cleavage. Heat seems to affect the fatty acid free albumin more than those molecules with these lipid ligands attached. Even after reduction of all 17 disulphide bridges reoxidising them will lead to refolding of the molecule, starting in each domain simultaneously and independently, with restoration of the major ligand binding capacities to some degree again.

With this as the general background within which any detected albumin variation can exert its effect and thus make itself evident, one can turn to some reports of albumin variants to observe where the specific alteration fits into the larger picture.

ANIMAL STUDIES

The analysis of genetic material only fairly recently gained momentum with the advent of many new techniques. The effects of specific restriction enzymes to digest the prepared DNA are discussed by de Sousa et al (1984) who described the existence of two intronic variant gene fractions with the use of Hae III enzyme which therefore do not become represented in the synthesised albumin. Lucotte et al (1984) described the use of Eco R 1 and Hind III restriction endonuclease enzymes to illustrate three types of variant patterns of both the albumin and the alpha fetoprotein genes in two inbred strains of rats. Gal et al (1982) also described similar patterns obtained in Sprague-Dawley and Buffalo strains of inbred rats and followed the inheritance of crosses between these strains. Stormont and Suzuki (1963) explained the existence of a pair of codominant autosomal alleles for albumin in horses on the basis of inheritance of phenotypes in two breeds. Juneja et al (1984) used two dimensional agarose-polyacrylamide gel electrophoresis to obtain a picture of polymorphism in the post-albumin region of laboratory rabbits. They could link that to polymorphism previously reported in the pretransferrin region, but not to albumin as such.

Osterhoff and co-workers have described polymorphism in various wild and domestic African animals. They used albumin gene frequencies to indicate the clear difference between indigenous and exotic breeds of goats (Osterhoff and

Ward-Cox, 1972) and confirmed the functioning of two codominant autosomal alleles in the albumin translation in Equidae (Osterhoff, 1969). The different genetic backgrounds of white and black rhinocerosses was also highlighted by each exhibiting one variant fraction of albumin (Osterhoff and Keep, 1970) but electrophoresis of serum of the South African b'esbok revealed only one band (Osterhoff et al, 1972). No variation could be found in African elephant (Osterhoff et al, 1972) and the same was found for African buffalo (Osterhoff et al, 1970). In the gerbil from Lesotho a high degree of genetic variation was found (Maurer et al, 1976) but in the South African impala the operation of two alleles giving rise to three phenotypes could be demonstrated (Osterhoff et al, 1972). Studies on South African cattle breeds also showed this operation of two different alleles (Osterhoff and Pretorius, 1967).

Worldwide evidence of animal polymorphism is quite abundant. Morera et al (1983) described two albumin phenotypes in Spanish merino sheep but Kaminski et al (1984) found only one type in the rodent Arvicantus niloticus whereas Kimura and Yamamoto (1982) reported the same for the domestic pigeon. Three phenotypes, caused by two different alleles were demonstrated in East African cattle (Ashton, 1964) and Guszkiewicz et al (1984) used the genetic polymorphism of thoroughbred horses to describe blood line differences in France and Poland. Maclaren and Petras (1976) isolated house mouse albumin and found that only one albumin type could be isolated even though unwanted polymers had to be removed with the aid of exclusion chromatography. Comparison of this albumin to a rare variant mouse albumin showed a difference in electrophoretic mobility only. Haley (1965) designated the major protein fraction in quail blood albumin and described its variations in chicken-quail hybrids.

In the border area between animal and man Lorey (1984) compared the bilirubin binding capacity of the polymorphic albumin fractions of Rhesus monkeys and that of American Indians. Since this binding did differ in the monkeys he postulated a selection in that species that would maintain the polymorphic form. In Indians no such binding capacity gain could be demonstrated.

In humans transient bisalbuminaemia has been demonstrated as a proteolytic product in numerous case reports (Renversez et al, 1984 and Dauth et al, 1984) of pancreatitis or pancreatic cysts and also in the nephrotic syndrome (Anmad et al, 1984). The hete[illegible]f human serum albumin due to various factors was discussed[illegible]1969) and Rousseaux et al (1982) described a variant albumin that was unstable upon storage at 4 C and attained the mobility of normal serum albumin. Salaman and Williamson discussed the anomalous behaviour of serum albumin when undergoing isoelectric focusing in the native and oenatured states while Spencer and King (1971) evaluated the isoelectric heterogeneity of bovine plasma albumin. Both these reports centre around the so called microheterogeneity of albumin and note should be taken not to confuse changes thus seen with inherited variant forms. Tillyer et al (1984) reported on the use of immunofixation methods to detect genetic albumin variants that have very similar electrophoretic mobilities. Au et al (1986) more recently isolated some variant human albumin and investigated its properties. They found similar antigenic reactions, similar binding properties and similar uptake capacities for dyes when comparing variant to native albumin.

THE PRESENT STUDY

Regarding the degree to which the results of the lion albumin investigations agree with the wealth of information dealt with above care has to be exercised. From the electrophoresis results of the various techniques one can state that the mobility difference between the two fractions is maintained in spite of changes in sample collection, sample handling and donor differences. The SDS electrophoresis techniques would separate the proteins on the basis of size much more than charge and those results indicate the difference between the albumin fractions to be one of charge. The inability of the exclusion columns to separate the two fractions would also point to a very similar molecular mass strengthening the concept that the charge is the most obvious distinguishing feature. Thus charge could be created by a specific ligand binding or by some genetic coding for two different types of sequences. Regarding the first possibility one can state that such binding has to be extremely tight so that no alteration in separating condition or in donor selection would affect it in any way. Furthermore, the probability that ligand binding can cause such a dramatic difference has never been recorded. Microheterogeneity could, however, result from such ligand attachments and these were indeed demonstrated by the isoelectric focusing results, yielding an albumin zone rather than single bands. The second possibility seems therefore a more feasable explanation. Only amino acid sequencing can reveal the exact nature of the alteration but similar human analysis has shown the substitution to involve only one amino acid, thereby changing the overall charge slightly but not affecting molecular mass. This alteration has to be genetically determined but can affect the polypeptide proper or the leader hexapeptide of proalbumin. The fact that this substitution only involves one amino acid also explains why the immunological character is not affected; the chances of altering the specific antigenic zone are very

r mote and it has not been reported for any species. Any analytical procedure bas d on anything else but molecular charge will fail to reveal the variant fraction.

The genetic background to this is difficult to explain since the normal two codominant gene situation would result in a 50% occurence of each fraction. In this case the ratio is very different, something also reported in one human variant fraction as well as in the reports on rhinocerosses by Osterhoff. It is possible that the genetically determined alteration is in the proalbumin region so that the splitting off continues in the circulation, keeping the variant fraction at a lower concentration than the normal fraction. Another possibility, already mentioned by Osterhoff in the other animal results, is that the two-allele system is most probably too simplistic and can be extended to include other gene pairs. This was already offered as an explanation for the existence of five phenotypes of plasma protein and one abnormal allele here could then code for a variant fraction which will exist at a lower concentration than 50% of the total albumin.

In humans the alloalbuminaemia occurs only at a very low frequency, making an argument for naming the condition a spontaneous mutation or calling it a polymorphic variation. The latter would involve some selection providing a benefit to the animal. In lions the frequency is 100% so that this is definitely based on natural selection. The possible benefit could lie in the different binding properties of the variant forms, something hinted at in previous animal studies.

REFERENCES

AHMAD J KHAN AS SIDDIQUI MA TEWARI SG AND KHAN RU (1984) BISALBUMINAEMIA IN NEPHROTIC SYNDROME JAP J MED 23 1 45-47.

ALDERTON G WARD WH AND FEVOLD HL (1945) ISOLATION OF LYSOZYME FROM EGG WHITE J BIOL CHEM 157 43-58.

ASHTON CC (1964) SERUM ALBUMIN POLYMORPHISM IN CATTLE GENETICS 50 1421-1426.

AU HAN BRAND S HUTCHINSON DW AND MATEJTSCHUK P (1984) STRUCTURES AND LIGAND-BINDING PROPERTIES OF HUMAN SERUM ALBUMINS BIOCHEM SOC TRANS 12 659.

BULL HB (1971) AN INTRODUCTION TO PHYSICAL BIOCHEMISTRY 2ND EDITION 229-260 DAVIS CO PHILADELPHIA.

CHARM SE (1971) FOAM SEPARATION OF ENZYMES AND OTHER PROTEINS IN:"ADSORPTIVE BUBBLE SEPARATION TECHNIQUES R LEMLICH ED 157-174 ACADEMIC PRESS NEW YORK.

CHENEY CS (1982) EFFECTS OF INTERMITTENT FREEZING AND THAWING ON STORED SERUM PROTEINS OF .IX WILD ANIMAL SPECIES BSC (HONS) PROJECT DEPARTMENT OF GENERAL PHYSIOLOGY DENTAL SCHOOL UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG.

COHN EJ (1925) THE PHYSICAL CHEMISTRY OF THE PROTEINS PHYSIOL REV 5 349-437.

COHN EJ HEYROTH FF AND MF MENKIN (1928) J AM CHEM SOC 50 696.

CURLING JM BERGLOF J LINDQUIST LO AND ERIKSSON P (1977) A CHROMATOGRAPHIC PROCEDURE FOR THE PURIFICATION OF HUMAN PLASMA ALBUMIN VOX SANG 33 97-107.

DAUTH J DE CONING JP AND DREYER MJ (1984) ALLOALBUMINAEMIA IN FIVE HOSPITALIZED PATIENTS S AFR J MED LAB TECH 30 4 69-70.

EDSALL JT AND WYMAN J (1958) BIOPHYSICAL CHEMISTRY VOL I ACADEMIC PRESS NEW YORK.

FAIRBANKS G STECK TL AND WALLACH DFH (1971) ELECTROPHORETIC ANALYSIS OF THE MAJOR POLYPEPTIDES OF THE HUMAN ERYTHROCYTE MEMBRANE BIOCHEMISTRY 10 13 2606-2617.

FOSTER JF (1977) IN: "ALBUMIN STRUCTURE FUNCTION AND USES" ROSENOER ORATZ AND ROTHSCHILD EDS 53-84 PERGAMON OXFORD.

FOWLER ME (1978) ZOO AND WILD ANIMAL MEDICINE WB SAUNDERS PHILADELPHIA.

FRIGERIO NA AND HETTINGER TP (1962) PROTEIN SOLUBILITY IN SOLVENT MIXTURES OF LOW DIELECTRIC CONSTANT BIOCHIM. BIOPHYS. ACTA 59 228-230.

GAL A LUCOTTE G NAHON JL AND SALA-TREPAT JM (1982) POLYMORPHISM OF THE RESTRICTION FRAGMENTS OF THE ALBUMIN AND ALPHA-FETOPROTEIN GENES IN DIFFERENT INBRED STRAINS OF RATS ACTA BIOCHIM BIOPHYS (HUNGARY) 17 1 24.

HALEY LE (1965) SERUM ALBUMIN POLYMORPHISM IN QUAIL AND CHICKEN-QUAIL HYBRIDS GENETICS 51 983-986.

HAO Y (1979) A SIMPLE METHOD FOR THE PREPARATION OF HUMAN SERUM ALBUMIN VOX SANG 36 313-320.

HARBOE N AND INGLID A (1973) IMMUNIZATION ISOLATION OF IMMUNOGLOBULINS ESTIMATION OF ANTIBODY TITRE IN:"A MANUAL OF QUANTITATIVE IMMUNOELECTROPHORESIS" AXELSEN NH KROLL J WEEKE B EDS UNIVERSITETSFORLAGET OSLO.

HOFMEISTER F (1888) ZUR LEHRE VON DER WIRKUNG DER SALZE ARCH EXPTL PATHOL PHARMACOL 24 247-260.

HOWE PE (1923) PROC SOC EXP BIOL AND MED XX 91.

JUNEJA RK VAN DE WEGHE A AND GAHNE BO (1984) GENETIC POLYMORPHISM OF SERUM POSTALBUMIN AND PRETRANSFERRIN IN LABORATORY RABBIT HEREDITAS 100 11-15.

KAMINSKI M ROUSSEAU M AND PETTER F (1984) ELECTROPHORETIC STUDIES ON BLOOD PROTEINS OF ARVICANTHIS NILOTICUS BIOCHEM SYSTEMAT ECOL 12 2 215-224.

KENDALL FE (1941) J BIOL CHEM 138 97-109.

KIMURA M AND YAMAMOTO S (1982) PROTEIN POLYMORPHISM IN A FERAL POPULATION OF THE PIGEON COLUMBA LIVIA DOMESTICA ANIM BLOOD GRPS BIOCHEM GENET 13 299-303.

KIRKWOOD JG (1943) THE THEORETICAL INTERPRETATION OF THE PROPERTIES OF SOLUTIONS OF DIPOLAR IONS IN:"PROTEINC AMINO ACIDS AND PEPTIDES" EJ COHN AND JT EDSALL EDS 276-303 REINHOLD PUBLISHING CORP. NEW YORK.

LAEMMLI UK (1970) PROTEIN FRACTIONS IN BACTERIOPHAGES NATURE 227 681-685.

LAVEREDA DE SOUZA S FRAIN M MORNET E SALA-TREPAT JM AND LUCOTTE G (1984) POLYMORPHISMS OF HUMAN ALBUMIN GENE AFTER DNA RESTRICTION BY HAEIII ENDONUCLEASE HUM GENET 67 48-51.

LOREY FW (1984) THE ALBUMIN POLYMORPHISM AND BILIRUBIN BINDING IN RHESUS MONKEYS AND AMERICAN INDIANS AM J PHYS ANTHROPOL 63 2 187.

LOWRY OH ROSEBROUGH NJ FARR AL AND RANDALL RJ (1951) PROTEIN MEASUREMENT WITH THE FOLIN PHENOL REAGENT . OL CHEM 193 265.

LUCOTTE G GAL A AND SALA-TREPAT JM (1984) RESTRICTION-SITE POLYMORPHISM OF THE ALBUMIN AND THE ALPHA-FETOPROTEIN GENES IN TWO INBRED STRAINS OF RATS BIOCHEMICAL SYSTEMATICS AND ECOLOGY VOL 12 NO 2 225-230.

LUMRY R AND BILTONEN R (1968) THERMODINAMIC AND KINETIC ASPECTS OF PROTEIN CONFORMATIONS IN RELATION TO PHYSIOLOGICAL FUNCTION.IN:"STRUCTURE AND STABILITY OF BIOLOGICAL MACROMOLECULES"S N TIMASHEFF AND GD FASMAN EDS 65-212 MARCEL DEKKER,INC. NEW YORK.

MACLAREN IA AND PETRAS ML (1976) ISOLATION AND PROPERTIES OF SERUM ALBUMIN FROM THE HOUSE MOUSE BIOCHIM BIOPHYS ACTA 427 238-250.

MAURER FW OP'T HOF J AND OSTERHOFF DR (1976) BIOCHEMICAL GENE MARKERS IN THE GERBIL TATERA BRANTSII FROM LESOTHO ANIM BLOOD GRPS BIOCHEM GENET 7 231-239.

MCMEEKIN TL (1939) SERUM ALBUMIN 1 THE PREPARATION AND PROPERTIES OF CRYSTALLINE HORSE SERUM ALBUMIN OF CONSTANT SOLUBILITY J AM CHEM SOC 61 2884-2887.

MORERA L LLANES D BARBANCHO M AND RODERO A (1983) GENETIC POLYMORPHISM IN SPANISH MERINO SHEEP ANIM BLOOD GRPS BIOCHEM GENET 14 77-82.

MORSE RW AND FOLLIS TB (1974) PHYSIOLOGIC NORMALS RESEARCH IN LIONS IN:"THE WORLDS CATS" VOL 2 EATON R! ED FELINE RESEARCH GROUP SEATTLE.

MELTON DA BERRY HH BERRY CU AND JOUBERT SM (1987) ASPECTS OF THE BLOOD CHEMISTRY OF WILD LIONS, PANTERA LEO S AFR J ZOOL 22(1) 40-44.

OSTERHOFF DR (1969) HAEMOGLOBIN TRANSFERRIN AND ALBUMIN TYPES IN EQUIDAE (HORSES MULES DONKEYS AND ZEBRAS) MADOQUA 1 53-56.

OSTERHOFF DR AND KEEP ME (1970) NATURAL VARIATION IN THE BLOOD PROTEINS OF WHITE AND BLACK RHINOS LAMMERGEYER 11 50-53.

OSTERHOFF DR AND PRETORIUS AMG (1967) SERUM ALBUMIN VARIANTS IN CATTLE PROC 3 AFR SOC ANIM PROD 6 224-229.

OSTERHOFF DR AND WARD-COX IS (1972) SERUM POLYMORPHISM IN THREE SOUTH AFRICAN GOAT BREEDS XII EUROP CONF ANIM BLOOD GROUPS BIOCHEM POLYMORPH BP 579-582.

OSTERHOFF DR WARD-COX IS AND EMSLIE V (1972) A STUDY OF GENETIC MARKERS IN THE SOUTH AFRICAN BLESBOK KOEDOE 15 49-53.

OSTERHOFF DR WARD-COX IS AND YOUNG E (1972) BIOCHEMICAL POLYMORPHISM IN THE SOUTH AFRICAN IMPALA KOEDOE 15 55-59.

OSTERHOFF DR YOUNG E AND WARD-COX IS (1970) A STUDY OF GENETIC BLOOD VARIANTS IN AFRICAN BUFFALO J S AFR VET MED ASS 41 1 33-37.

OSTERHOFF DR YOUNG E AND WARD-COX IS (1972) NATURAL VARIATION OF THE BLOOD PROTEIN TYPES OF THE AFRICAN ELEPHANT XII EUROP CONF ANIM BLOOD GROUPS BIOCHEM POLYMORPH BP 683-686.

PENNELL RB (1960) FRACTIONATION AND ISOLATION OF PURIFIED COMPONENTS BY PRECIPITATION METHODS IN:"THE PLASMA PROTEINS" VOL 1 FW PUTNAM ED 9-50 ACADEMIC PRESS NEW YORK.

PETERS T (1985) SERUM ALBUMIN ADVANCES IN PROTEIN CHEMISTRY VOL 37 161-245 ACADEMIC PRESS INC NEW YORK.

RAY BR DAVISSON ED AND CREPSI HL (1954) EXPERIMENTS ON THE DEGRADATION OF LIPOPROTEINS FROM SERUM J PHYS CHEM 58 841-846.

RENVERSEZ JC ROUSSEL S AND ROLLUX A (1984) ACQUIRED BISALBUMINAEMIA IN PANCREATITIS DEMONSTRATION OF A PROTEOLYTIC PROCESS IN TEN PATIENTS LA RICERCA CLIN LAB 14 417-423.

ROUSSEAUX J ABDO Y COANON G VITTU C AND DAUTREVAUX M (1982) STUDIES OF AN ABNORMAL SERUM ALBUMIN UNSTABLE UPON STORAGE CLIN CHIM ACTA 124 293-302.

SALAMAN MR AND WILLIAMSON AR (1971) ISOELECTRIC FOCUSING OF PROTEINS IN THE NATIVE AND DENATURED STATES ANOMALOUS BEHAVIOUR OF PLASMA ALBUMIN BIOCHEM J 122 93-99.

SPENCER EM AND KING TP (1971) ISOELECTRIC HETEROGENEITY OF BOVINE PLASMA ALBUMIN J BIOL CHEM 246 1 201-208.

STEYN DG (1975) THE EFFECT OF PHENCYCLIDINE ANAESTHESIA ON THE BLOOD CHEMISTRY AND HAEMATOLOGY OF THE CHACMA BABOON (PAPIO URSINUS) J S AFR VET ASS 46 235-239.

STORMONT C AND SUZUKI Y (1963) GENETIC CONTROL OF ALBUMIN PHENOTYPES IN HORSES PROC SOC EXP BIOL MED 114 673-675.

TANFORD C (1961) PHYSICAL CHEMISTRY OF MACROMOLECULES 196-197 WILEY NEW YORK.

TANFORD C (1970) PROTEIN DENATURATION THEORETICAL MODELS FOR THE MECHANISM OF DENATURATION ADV PROTEIN CHEM 24 2-95.

TILLYER CR BAYLISS VM AND TARNOKY AL (1984) DETECTION OF GENETIC VARIANTS OF ALBUMIN BY IMMUNOFIXATION ELECTROPHORESIS IRCS MED SCI 12 262.

TOMASZEWSKA-GUSZKIEWICZ K URBANSKA-NICOLAS H AND KAMINSKI M (1984) BLOOD GENETIC POLYMORPHISM OF THOROUGHBREDS BRED IN POLAND AND FRANCE GENETICA POLONICA 25 1 95-102.

VALMET E (1969) THE HETEROGENEITY OF HUMAN SERUM ALBUMIN PROTIDES BIOL FLUIDS 17 443-448.

WALLEVIK K (1976a) BIOCHIM BIOPHYS ACTA 420 42-56.

WATT JG (1970) AUTOMATICALLY CONTROLLED CONTINUOUS RECOVERY OF PLASMA PROTEIN FRACTIONS FOR CLINICAL USE VOX SANG 18 42-61.

WEEKE B (1973) CROSSED IMMUNOELECTROPHORESIS IN:' A MANUAL OF QUANTITATIVE IMMUNOELECTROPHORESIS AXELSEN NH KROLL J WEEKE B EDS UNIVERSITETSFORLAGET OSLO.

TILLYER CR BAYLISS VM AND TARNOKY AL (1984) DETECTION OF GENETIC VARIANTS OF ALBUMIN BY IMMUNOFIXATION ELECTROPHORESIS IRCS MED SCI 12 262.

TOMASZEWSKA-GUSZKIEWICZ K URBANSKA-NICOLAS H AND KAMINSKI M (1984) BLOOD GENETIC POLYMORPHISM OF THOROUGHBREDS BRED IN POLAND AND FRANCE GENETICA POLONICA 25 1 95-102.

VALMET E (1969) THE HETEROGENEITY OF HUMAN SERUM ALBUMIN PROTIDES BIOL FLUIDS 17 443-448.

WALLEVIK K (1976a) BIOCHIM BIOPHYS ACTA 420 42-56.

WATT JG (1970) AUTOMATICALLY CONTROLLED CONTINUOUS RECOVERY OF PLASMA PROTEIN FRACTIONS FOR CLINICAL USE VOX SANG 18 42-61.

WEEKE B (1973) CROSSED IMMUNOELECTROPHORESIS IN: A MANUAL OF QUANTITATIVE IMMUNOELECTROPHORESIS AXELSEN NH KROLL J WEEKE B EDS UNIVERSITETSFORLAGET OSLO.

Author Jooste C P
Name of thesis Isolation and Limited Characterisation of Lion - Panthera Leo Albumin 1987

PUBLISHER:
University of the Witwatersrand, Johannesburg
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