

A COMPARATIVE STUDY OF THE EFFECTS OF
IN THE BODY FLUIDS OF ANIMALS.

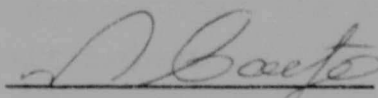
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A Thesis Submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
for the Degree of Doctor of Philosophy.

Johannesburg, 1981.

DECLARATION

I hereby declare that the work reported in this thesis was carried out by me in the Department of Physiology, University of the Witwatersrand, Johannesburg. It has not been submitted as a requirement for any other degree.


Naudine Coetzee

ABSTRACT

A new gel medium for the electrophoretic separation of proteins was developed and the separation properties were compared with that of polyacrylamide gel.

The new medium is based on the combination of Sephadex G-25 Superfine and acrylamide (1,4:1) into one separating gel. It separates certain body fluid proteins into more components than can be attained with conventional polyacrylamide gel electrophoresis. This new technique was used to study the proteins of various animals and the possible influence of physical exercise, heat exposure and fever on the electrophoretic properties and concentrations of proteins in the body fluids of these animals.

Emphasis was laid on albumin since this protein separated into two main fractions, an upper and a lower albumin band. These electrophoretically distinct albumins were of approximately equal concentrations and also crossreacted immunologically. It was further shown that circulating albumin concentration may be underestimated by dye binding assays, due to the fact that the lower albumin fraction does not bind to many dyes.

Limited biochemical analysis suggested that the lower albumin fraction may be bound to different molecules than the upper albumin. This differentiation in carrying capacity results in microheterogeneity of the albumin causing differences in electrophoretic mobilities.

Possible functional differences of the two albumins were also studied during physical exercise, heat exposure and fever conditions. It was found that human subjects not participating in regular physical exercise had relatively increased lower albumin concentrations as compared to the same subjects after

undergoing a course of regular physical training. Strenuous physical exercise resulted in an increase in relative concentration of the lower albumin in the former whereas the same parameter was unchanged in the latter.

Heat exposure of rats at 37°C was found to result in a greater increase in plasma lower albumin than interstitial fluid lower albumin, by doing crossed immunoelectrophoresis. By labeling rabbit plasma albumin with 14C-tryptophan, a possible increase in the lower albumin during fever was also indicated.

Dedicated to my parents whom I dearly love.

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I INTRODUCTION

The name protein, meaning first, was given by Mulder in 1838, as suggested to him by Berzelius, to the complex organic nitrogenous component of animal and plant cells. Proteins serve as structural elements of the body, hormones, enzymes, oxygen carriers, hereditary factors, antibodies, etc. It is apparent that there is hardly an important physiological function in which proteins do not participate. Quantitatively, proteins form the main molecular component of animal tissue and plasma for they may constitute as much as 75% of the dry tissue mass.

Although the plasma proteins are diverse in their functions, they are closely related as a group, or system of proteins, due to their origin and occurrence as the major extracellular component of the circulatory system (Putnam, 1975). Because of the availability of plasma proteins, they have been the object of study for many decades. Fractional precipitation of plasma protein fractions was first achieved towards the latter part of the 19th century. This separation method was later augmented by new and more advanced methods during the mid-20th century when the identification, isolation and purification of plasma proteins were made possible with the introduction of electrophoretic and chromatographic methods. As a result of these developments, the number of plasma proteins identified rose steeply, with more being steadily added to the list (Putnam, 1975).

In addition to the hundred or so known plasma proteins, there may be many more protein fractions, minor or even minute in amount, but important in function, that remain to be discovered, purified and studied. In addition, proteins which appear to be homogeneous may in fact be found to be heterogeneous. Since many plasma proteins share one or more physical properties, such as sedimentation coefficient, molecular mass, or isoelectric point, a protein separation method

different to the well established methods, was required to achieve finer separation. A combined sephadex-acrylamide gel was thus introduced which separated some plasma proteins into more components than may be achieved with the generally used polyacrylamide gel showing that with this new development of electrophoretic separation it has become apparent that certain plasma proteins previously listed as possibly being heterogeneous are in fact so, not only in plasma, but also in other body fluids such as lymph, interstitial fluid and pericardial fluid and that the relative proportions change under certain conditions. When such a protein(s) is observed it is clear that extensive research has to be done on the isolation, identification and properties thereof. It is towards these objectives that this study aims to contribute.

At this point it is perhaps appropriate to provide a short overview of some relevant body fluid proteins and the methods generally used for the study of proteins.

This literature search was concluded in December, 1977.

1.1 BODY FLUIDS

1.1.1 Plasma

The plasma proteins can be defined as inclusive of all the blood proteins with the exception of haemoglobin. The exact number of protein components in plasma is presently unknown. Isolation and characterization of the major protein fractions are necessary steps towards a complete understanding of the nature of proteins and protein interactions. Separation and identification of blood proteins have been established as useful tools in dealing with vertebrates in establishing or confirming phylogenetic relationships and in providing identification of the physiological status of an animal (Putnam, 1975). In this respect much work has been done by many workers in the field using a variety of techniques in attempts at isolating pure samples of the various plasma protein components. The study of these components began somewhat

more than a century ago. During the first half of the present century, results pointed to the liver as the main origin of the plasma proteins with the possible exception of the immunoglobulins (Tarver & Reinhardt, 1947). It was only in 1950, however, that the first direct evidence in this regard was obtained by Peters & Anfinsen (1950).

The functions of the plasma proteins are as manifold as the diversity of the proteins themselves (Putnam, 1975). The major functions are the maintenance of colloid osmotic pressure, pH-balance, electrolytic balance, the transport of metal ions, fatty acids, steroids, hormones, drugs etc., their availability as a nutritional source of amino acids, haemostasis, the regulation of cellular activity and functions via the hormones and also as a defence mechanism against invasion by antigens.

Studies of serum protein fractions separated by electrophoresis, show variations related to sex of the individual (Nyman, 1959; Ganrot & Schersten, 1967; Stabilini *et al*, 1968; Ganrot, 1972), age (Bronte-Stewart *et al*, 1961; Weiss, 1965; Ganrot & Bjerre, 1967; Ganrot & Schersten, 1967; Laurell, 1968, 1972; Abrams & Freeman, 1969; Putnam, 1975; Watermeyer *et al*, 1977) and nutritional status (Watermeyer *et al*, 1977). These variations may be due to differences in concentration, molecular masses and isoelectric points.

Extensive work has been done on the molecular masses, isoelectric points, relative mobilities, amino acid sequences and functions of the various individual proteins of different animals. The proteins identified in this thesis are albumin, prealbumin, orosomucoid, haptoglobin, lipoproteins, α_2 -macroglobulin, ceruloplasmin and the immunoglobulins. Except for albumin the proteins are discussed very briefly. The function of each protein is the main aspect that will be referred to.

Albumin is one of the proteins separated into more components

by the sephadex-acrylamide gel mentioned previously, therefore this protein will receive the major part of our attention. It is generally accepted that albumin is one of the most important proteins as far as transport is concerned, and is the best studied of all serum proteins (Rosenoer et al, 1977). Albumin was first analyzed by Liebig and Mulder in the late 1839's when the term "protein" had just been introduced (White et al, 1968). Albumin is one of the smallest molecules and also the most abundant of the plasma protein constituents. Thus albumin occupies a paramount position in the plasma protein system, not only for the reasons already given, but also for its heterogeneity, high electrophoretic mobility at pH 8,6 and its osmotic and transport functions. Of the total osmotic effect of the plasma proteins, albumin is responsible for about 75 to 80% (Putnam, 1975). Although it constitutes slightly more than half the plasma proteins by mass, the osmotic effectiveness of albumin is greater than that of the globulins because of its lower molecular mass. Total plasma protein concentration in the human may vary between 60 and 80 g/l and albumin from 35 to 45 g/l (Ganong, 1973). Many substances which are sparingly soluble in water are readily dissolved in the presence of serum or plasma. In each instance the dissolved substance is actually bound to protein and transported in that manner. Albumin possesses this ability to a marked degree. It is known that albumin carries fatty acids (Unger et al, 1963; Kaplan & Foster, 1971; Wallevik, 1973), bilirubin (Blauer & King, 1970; Blauer et al, 1972), hormones (Corvol et al, 1971), enzymes (Wilde & Kekwick, 1964), thyroxin (Tritsch et al, 1961), steroids (Jellinck & Fletcher, 1970; Ryan & Gibbs, 1970), haemoglobin breakdown products (Odell, 1959), drugs etc. Because of albumin's ability to combine and transport various substances, it is generally accepted that serum albumin level is the best criterion of protein nutritional status. The literature on this protein is so extensive that it has prompted a number of reviews by Foster (1960), Putnam (1965), Peters (1970, 1975, 1980) and others.

Many organic dyes bind to albumin, this being useful in elec-

trophoretic studies. Such dyes are for example Bromo-phenol Blue (Bjerrum, 1968), Evans Blue (Freedman & Johnson, 1969), Bromo-cresol Green (Rodkey, 1964; Howorth, 1971), sulphobromophthalien (Baker & Bradley, 1966), methyl orange (Markus et al, 1967) and phenol red (Rodkey, 1961). All of these albumin-ligands markedly increase the electrophoretic mobility of this protein, and may even form spiked albumin peaks upon electrophoresis (Schultze & Heremans, 1966). It is known that only the faster migrating part of the albumin fraction becomes tainted when Bromo-phenol Blue is added in small quantities to the serum (Schultze & Heremans, 1966). The resulting electrophoretic heterogeneity of the albumin peak will however seldom reach the point where two separate albumin bands are found after separation on acrylamide gels.

Cases of bisalbuminemia have been reported (Merler et al, 1962; Rotbol, 1970; Zimmerman & Nejtek, 1975). Merler et al (1962) demonstrated a double urinary albumin peak using starch gels during crossed immunoelectrophoresis. Rotbol (1970) also reported two distinct albumin peaks during crossed immunoelectrophoresis using monospecific anti-albumin. A polyacrylamide gel containing Ampholine (pH 3 to 10) was used for the first electrophoretic step. Thus Rotbol (1970) described an albumin with a lower isoelectric point in addition to the main albumin. Though normal urinary albumin has been found to have the same molecular mass as its counterpart in serum (Poortmans, 1964), it has been claimed that part of the albumin occurs as a low-molecular-mass degradation product (Merler et al, 1962).

Foster introduced the term "microheterogeneity" to describe albumin species which differ with respect to the pH at which they undergo the transition from a normal or N-form to a faster or F-form (Foster et al, 1965). Sogami and Foster (1962) could distinguish the faster migrating albumin by means of electrophoresis. This transition is rapid (Brewer & De Sa, 1973) and reversible (Gutter et al, 1957). Micro-

heterogeneity is present even in the albumin of a single donor and is not the result of polymorphism (Schultze & Heremans, 1966). The disorder "double albuminemia" has been reported as early as 1957 when it was found that human serum may contain more than one class of albumin (Nennstiel & Becht, 1957).

Many other workers have also reported results indicating microheterogeneity of the albumin molecule (Swingle & Tiselius, 1951; Boman, 1955; Boman & Westlund, 1956; Sober et al, 1956; Hjertén, 1959). Denaturation, polymerization, isomerization and association are a few physicochemical changes responsible for the microheterogeneity of the albumin molecule. Rejnek et al (1963) succeeded in showing both electrophoretic and antigenic differences in highly purified albumin, but some doubt remains as to possible damage during their fractionation techniques (Schultze & Heremans, 1966). It has been discovered that only two-thirds of normal albumin can be converted to the mercaptalbumin dimer and this has been supported by Benesch and Benesch (1956) who observed that human serum albumin possesses less than one sulfhydryl group per molecule. One of the three albumins which has been isolated by Keller and Block (1959) has been found to differ from the remaining two by its cysteine content.

According to McMenamy and Lee (1967) this microheterogeneity is decreased, but not eliminated, by the removal of tightly bound fatty acids. Possible explanations for microheterogeneity of albumin are the intra-molecular disulfide bond interchange, differences in amide content or sequence (Spencer & King, 1971), or the modification or aging of circulating molecules.

Difficulty exists in proving the microheterogeneity of albumin without changing the albumin molecule as a result of fractionation techniques and the removal of tightly bound molecules being carried. Therefore a new technique had to be developed to study the heterogeneity of albumin.

Prealbumin has functions similar to that of the main albumin fraction (Harris & Kohn, 1974), but is of special importance in the binding of tetra- and tri-iodothyronine. Thyroxine is bound in serum by two major transport proteins, prealbumin and a globulin with an intermediate electrophoretic mobility between that of α_1 - and α_2 -globulin (White *et al*, 1968). At pH 8,6 prealbumin may carry up to four times as much thyroxin as does the globulins, but less at a physiological pH (White *et al*, 1968). The latter authors mentioned albumin as a third carrier for thyroxin. A progressive increase in concentration of serum prealbumin from newborn to adult has been reported by Stabilini *et al* (1968).

The remaining plasma proteins are globulins. It seems that most of the interest up to now has been centered on the β - and γ -globulins of the blood because of the presence of antibodies or immunoglobulins in these fractions, most of which are in the γ -globulin fraction. Other β -globulins function in the transport of a variety of substances, including lipids, hormones and inorganic ions. The α -globulins have the highest relative mobility in comparison to the other globulins and are responsible for the transport of lipids, as well as thyroid hormone, haemoglobin and copper ions.

The ability of the electrophoretic method to detect different proteins, varies with the dye binding capacity of the proteins under the conditions used for staining, their degree of electrophoretic homogeneity and their concentration (Laurell, 1972). For instance, plasma proteins above 0,05 to 0,1 g/l with a low content of polysaccharides and lipids appear as faint bands when stained with a general protein dye such as Amido-Black. According to Laurell (1972), orosomucoid which is an acidic α_1 -globulin, normally produces no apparent band because it stains weakly. It is also called α_1 -acid glycoprotein because of its acidic isoelectric point. Orosomucoid is one of the two mucoproteins with high carbohydrate content which can be detected in plasma after pre-

precipitation of the other proteins by addition of trichloroacetic acid or perchloric acid (White et al, 1968). In the plasma of most mammals there is normally a glycoprotein present namely haptoglobin, which forms a stable complex with haemoglobin in a one to one ratio (Laurell & Crönvall, 1962). Haptoglobin comprises about one fourth of the α_2 -globulin fractions in human plasma. This protein shows polymorphism in humans and a series of haptoglobulins of varying molecular masses have been detected (Smithies, 1955). It would appear that sheep lack a haemoglobin-binding protein (Jarrett, 1972). Haptoglobin concentrations are lower in young children than in adults (Abrams, 1971).

The majority of plasma lipids are present as lipoproteins. These migrate electrophoretically with both the α - and β -globulins. After staining, the density of the α -lipoprotein zone is more intense in children, young females and often during the first ten years after menopause (Laurell, 1972).

The α_2 -macroglobulin (α_2 -M) in humans is normally the largest single component of the electrophoretic α_2 -fraction in the serum, and in adults it represents about 4% of the total serum protein content (Schumacher & Schlumberger, 1963; Weiss, 1965). This protein has been shown to bind and transport a number of biologically active substances of protein nature such as trypsin and chymotrypsin, plasmin, thrombin and insulin, as well as the growth hormone (Haverback et al, 1962; Mehl et al, 1964; Clausen et al, 1963; Collip et al, 1964; Hadden & Prout, 1964). The α_2 -M fraction has a decreased adult appearance (Hitzig, 1961; Weiss, 1965; Ganrot & Schersten, 1967), and at the age of 70 and above the infant pattern may return (Laurell, 1972). The higher concentration in children may be related to the regulation of the growth process. It has also been observed that α_2 -M may be 20% higher in concentration in women than in men and that it increases even more during pregnancy (Hitzig, 1961; Schlagetter & Schwick, 1961; Schumacher & Schlumberger, 1963; Weiss, 1965; Ganrot, 1972).

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