COPPER AND RHEUMATOID ARTHRITIS - AN IN VITRO STUDY OF METAL-PROTEIN INTERACTIONS

by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science in the Department of Chemistry, University of Cape Town, under the supervision of Professor G. E. Jackson.

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dedicated to the loving
memory of my sister

There is no fire like lust,
no evil like hatred,
no sorrow like existence,
no happiness greater than tranquillity.

Lord Buddha
(Dhammapada)

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ABBREVIATIONS

R	no of moles of metal bound per mole of protein
V	volume
BSA	Bovine Serum Albumin
HSA	Human Serum Albumin
MW	weight-average molecular weight
K_d	apparent dissociation constant
n	number of binding sites
K	apparent association constant
HPLC	High Performance Liquid Chromatography
FMOC-Cl	9-Flourenylmethyl chloroformate
L-His	L-Histidine
L-Thr	L-Threonine
L-Try	L-Tryptophan
RA	Rheumatoid Arthritis
NSAIDs	non steroidal anti-inflammatory drugs
P.M.I.	plasma mobilizing index
ECCLES	Computer program for large equilibrium systems
D-Pen	D-Penicillamine
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
EDDA	Ethylenediaminediacetic acid
Trien	Triethylenetetramine
ß	formation constant
ESTA	Equilibrium Simulation Titration Analysis
I	Ionic strength

ABSTRACT

Acute and Chronic inflammations in Rheumatoid Arthritis are characterized by, among other features, changes in the metabolism of copper. Previous studies have shown that administration of exogenous copper, and the *in vivo* manipulation of endogenous copper may provide new ways of coping with the problem of anti-inflammatory/anti-arthritic therapies. Computer models of blood plasma have attempted to rationalize the use of copper complexes in this regard.

This thesis describes the design and commissioning of an ultrafiltration apparatus to study metal interactions with blood proteins. The results are compared with equilibrium dialysis. The systems studied using this technique includes, the binding of Cu(II) to HSA and BSA, ternary Cu(II)-HSA-amino acid systems, as well as competitive studies of the Cu(II)-HSA system with Mg(II), Ca(II) and Zn(II). The ultrafiltrate was analysed using HPLC and atomic absorption spectroscopy. Protein preparation was found to be important and the different methods used for protein purification were compared using gel electrophoresis and atomic absorption. Binding studies of chelating agents as well as several pharmaceuticals in human blood serum were carried out and compared with computer modelling studies.

PUBLICATIONS

1. ULTRAFILTRATION STUDIES OF METAL COMPLEXES IN HUMAN BLOOD SERUM.
   In *Proceedings of the South African Chemical Institute.*, Jubilee Convention, Durban, 1987.
   G. E. JACKSON and M. SINGH

2. COMPUTER MODELS OF BIOFLUIDS- AN EXPERIMENTAL VERIFICATION
   G. E. JACKSON and M. SINGH, *Polyhedron.*, **9 (4)**, 1990, 505-510.

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CHAPTER 1

INTRODUCTION

1.1 COPPER

Copper is one of the essential trace elements required for the functioning of many enzyme systems. While trace amounts of copper are essential for life, amounts in excess are toxic. Copper ions are readily absorbed from the diet in the stomach and small intestines of most mammals and are distributed throughout the body. In the blood it is largely bound to ceruloplasmin in the plasma, but it also complexes with albumin and several amino acids. The liver is the principal processing organ where it is stored as a complex with metallothionein. Here it is also incorporated into ceruloplasmin before circulation in the blood and preparation for excretion in the bile. The major pathway for excretion is via the bile and very little is excreted in the urine except in pathological conditions which affect the primary homeostatic mechanism.

Copper complexes present in pathological conditions are known to be remarkably active pharmaceutical agents [1]. Copper ions or the parent ligands on their own do not share this activity. Extensive studies have been undertaken to elucidate the transport of copper by carrier molecules, the nature of copper binding in biofluids, the kinetic behaviour of the exchange of copper between various ligands in biological systems as well as the possible mechanism of the anti-inflammatory action of copper. At present, these studies have emerged as an active area of research. A major part of the research is directed at the synthesis of novel anti-arthritic drugs and the subsequent screening of these drugs in animal and computer studies.

1.2 RHEUMATOID ARTHRITIS (RA)

The term rheumatism is generally used to refer to painful disorders of the joints and muscles,

which cannot be directly related to an identifiable infection or injury. The most common and serious of these rheumatic disorders is rheumatoid arthritis.

It affects 5% of the population of the western world [2] and is particularly prevalent among the elderly. It usually manifests itself as small nodules of inflamed fibrous tissue around the knuckles and wrists, eventually causing irreversible functional damage to the joints. The damage to the joints is due to the destructive effects of the inflamed synovial lining of the joints and is further aggravated by the penetration of the synovial tissue through cartilage and bone, giving rise to erosions on the surface of the joint. Eventually the joint may be so destroyed that deformity occurs (e.g. bent knuckles, and change in the shape of other joints, such as the wrists, elbows and knees). The situation is well represented in Fig. 1.2 and can be compared to that of a normal joint (Fig.1.1).

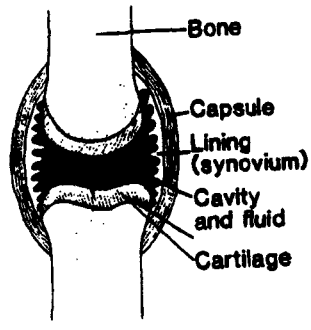


Fig. 1.1 Normal joint

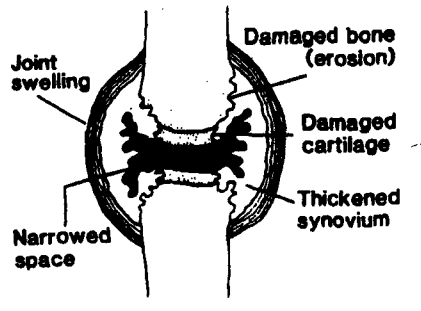


Fig. 1.2 Rheumatoid joint

The origins of rheumatoid arthritis are unknown but many suggestions centre upon a breakdown of the patients autoimmune system [3,4].

It seems, that for one reason or another, an anti-inflammatory response to physical, emotional, or hormonal stress is not properly regulated by the usual feedback mechanisms and the irritant thus continues to stimulate itself. It is clear that the disease is a complicated expression of many interrelated cellular and molecular processes. So in common with many pathologies which stubbornly resist modern medical efforts, it is difficult to remedy, because there is no unique or identifiable biochemical lesion. However, agents which suppress the inflammation interrupt the self perpetuating process and can thus be used to control the disease if not cure it.

1.3 THE INVOLVEMENT OF COPPER IN RA

Copper was believed to be of therapeutic value as long ago as 1000 years BC, in particular, copper bracelets and foods high in copper were thought to be beneficial in treating arthritic conditions [5]. In 1945, patients with rheumatoid arthritis (RA) were shown to exhibit higher than normal serum copper levels [6]. Nevertheless, during the 1940's to the early 1970's parenterally administered copper complexes were used successfully in the treatment of arthritic conditions [7]. However, the possible acceptance of this form of therapy was impeded by the concomitant development of anti-inflammatory steroids and aspirin-like non-steroid anti-inflammatory drugs.

It was not until 1960 that Bonta placed the clinical studies on a more scientific footing when he showed that copper compounds possessed anti-inflammatory effects in animals [8]. In 1976, Sorenson confirmed and extended these findings and he opened a new chapter in the copper story by claiming that copper complexes of anti-inflammatory activity were indeed the active forms of these drugs [9,10].

He also speculated that anti-arthritic agents might promote tissue distribution of elevated serum copper in RA patients, which was a physiological response to the inflammation (Fig. 1.3)

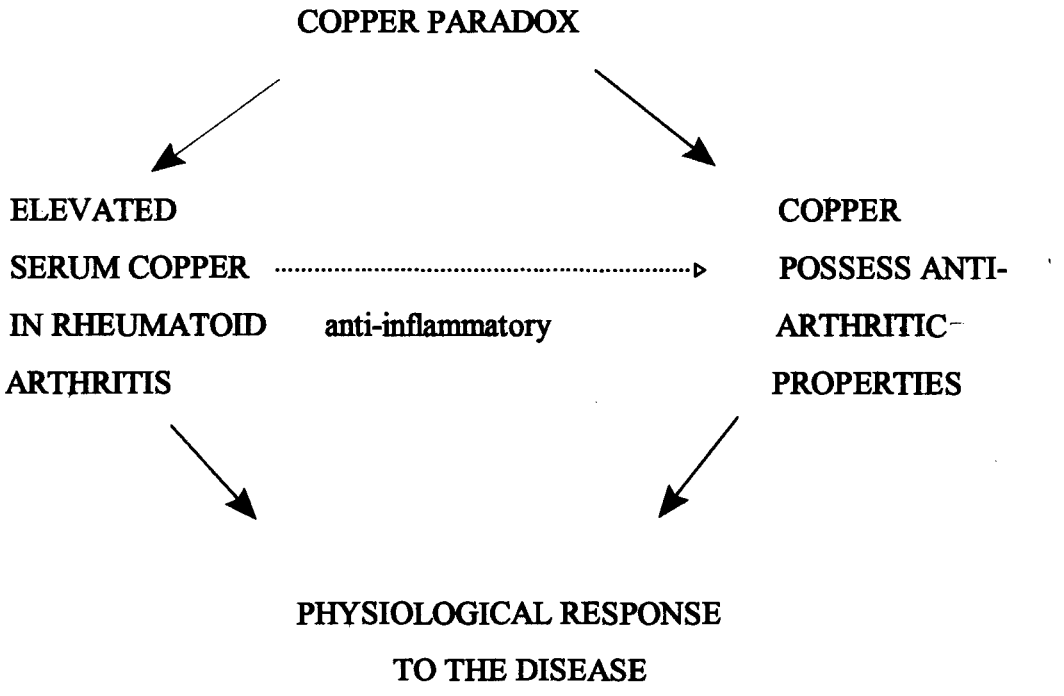


Fig. 1.3 The paradoxical role of Copper in Inflammation.

The use of copper to treat RA was rationalised by suggesting that this physiological response was insufficient to quell the ongoing inflammatory process and, consequently, exogenous help was needed. Milanino and associates who demonstrated that exacerbation of inflammation occurred in rats on a copper-deficient diet [11] supported the modulator role of copper. Numerous multi-disciplinary groups have subsequently re-examined the paradoxical role of copper in inflammation emphasising the therapeutic usefulness of copper, in inflammatory conditions. Two excellent reviews [11, 12] and two books [13, 14] covering the biochemistry and pharmacological activities of copper in the treatment of RA and other connective tissue diseases have been published covering much of this research.

It can be seen that much of the contemporary research is based on the central dogma of Sorenson [15] i.e. "the increase in copper-containing components of blood plasma, amino acid, albumin and ceruloplasmin complexes is a part of a general physiological response to disease states such as infections, arthritic diseases, epilepsy and cancers." As illustrated in Fig. 1.4, plasma copper increases with the onset of disease and returns to normal as the disease is overcome. In summary, these observations show that copper is active as an anti-inflammatory agent in chronic conditions in man and also indicates that the therapeutic effect observed may be independent of the chemical form of copper administered.

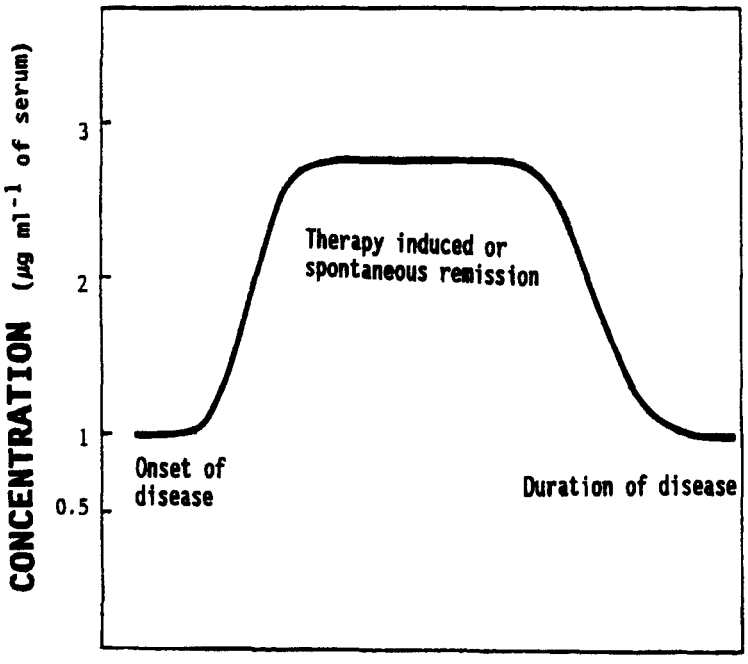


Fig. 1.4 The alteration of Serum Copper content in the general acute phase response to various Diseases [15].

1.4 MECHANISM OF ANTI-INFLAMMATION ACTIONS

Copper may exert anti-inflammatory effects through a variety of mechanisms, either directly or indirectly. These can be summarised as follows:-

(1) The induction of lysyl oxidase or lysyl oxidase mimetic activity [16]. This copper-dependant enzyme is required for collagen and elastin synthesis and is therefor essential for connective tissue maturation.

(2) The modulation of prostaglandin synthesis [17]. Copper tends to decrease the biosynthesis of pro-inflammatory PGE_2 and increases the production of anti-inflammatory PGF_2.

(3) The stabilisation of lysosymal membranes [18]. Copper stabilises lysosymal membranes and decreases their permeability by maintaining disulphide linkages in an oxidised form.

(4) The regulation of histamine activity [19]. It is possible that copper participates in the homeostatic regulation of histamine.

(5) The induction of superoxide dismutase or superoxide dismutase mimetic activity [20]. This is another enzyme involving copper whose depletion "in vivo" may lead to increased inflammation as a result of irritation of hydroxide or superoxide radicals. Another reasonable consequence of copper complexes in anti-inflammation is its ability to act as scavengers on superoxide radicals [21].

Cupric ions, copper complexes with amino acids, low molecular weight peptides and copper anti-inflammatory drug complexes which displays superoxide dismutase (SOD) activity are often greater in magnitude than endogenous SOD [21]. The superoxide anion (O_2^-) is produced and released from poly-morphonuclear neutrophiles (PMNs). There is considerable

evidence to suggest that O_2^- and H_2O_2 interact to form the hydroxyl radical, $OH^\cdot$ (Haber Weiss reaction; Fig. 1.5) which is highly reactive and is considered to be the real villain of the inflammatory scenario.

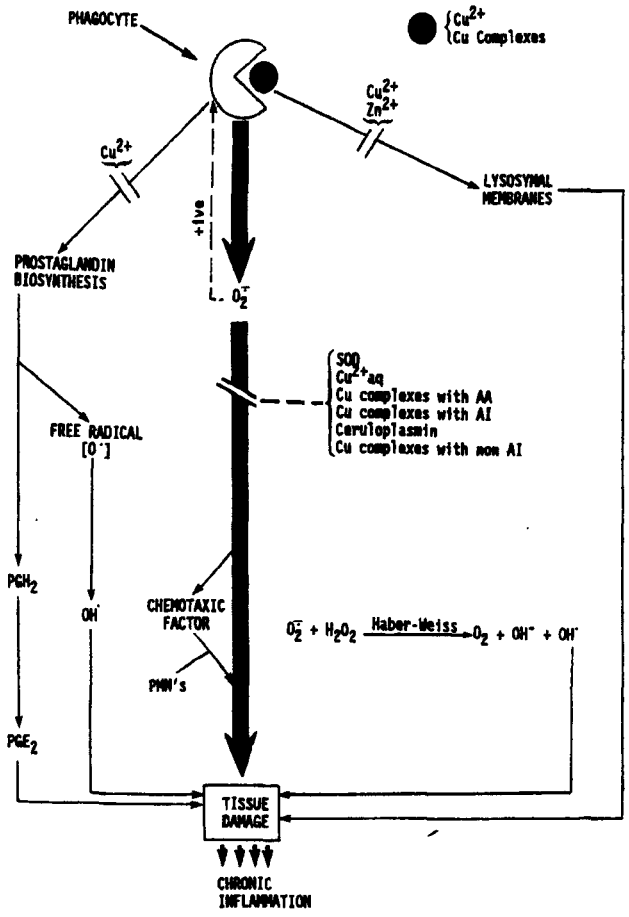


Fig. 1.5 Effects of copper compounds on phagocytic functions that may contribute to their inflammatory actions, AA= amino acid and AI = anti-inflammatory.

Interestingly no other copper protein, except SOD reacts catalytically with $O_2^{\cdot-}$. Fig. 1.5 summarises the effects of copper complexes on phagocytic functions that may contribute to their anti-inflammatory activities.

As indicated earlier, absorption, transport and distribution occurs such that ceruloplasmin and copper-albumin and copper-amino acid complexes are the major circulating forms of copper. All the above-mentioned (fig 1.5) roles for copper suggest that the metal ion is fundamentally involved in the normal processes by which the body responds to inflammatory situations. However none of them really indicate why copper administration should be more beneficial when the vast majority of rheumatoid arthritis sufferers are not overtly copper deficient. One possibility is that the metal ions, perhaps through its involvement in one of the mechanisms listed, is part of the physiological feedback by which the inflammatory response is normally controlled.

In rheumatoid arthritis, improper function of this feedback causes tissue repair processes themselves to become irritated so the copper may act by breaking this self-perpetuating cycle [22]. If this is the case, copper offers one of the most promising approaches by which permanent remissions may be achieved. Hence, the considerations for copper binding agents that may be chosen to transport the metal to the affected tissue, are of vital importance.

1.5 COMPUTER SIMULATION OF LOW MOLECULAR-WEIGHT COMPLEXING EQUILIBRIA IN BLOOD PLASMA.

The concentration of low molecular weight transition metal complexes is usually very low. Yet they are of immense biological significance because these species are orders of magnitude more abundant than their aquated metal ion counterparts and hence it is they which are involved in the transport of the metal across membranes and between biological binding sites [23].

However, their biological role is not easy to elucidate because they exist well below the limits of analytical means of detection. Furthermore, they are not amenable to isolation and concentration techniques because these would upset the labile equilibrium in which they participate.

Accordingly, computer simulation of the equilibrium reactions between transition metal ions and low molecular weight ligands was considered to be the only reliable way to determine which of the thousands of possible complexes will be important under biological conditions [24].

In the present context, computer simulation is the calculation of the equilibrium concentrations of the individual species formed in the solutions of metal ions and ligands. It requires the thermodynamic formation constants for all the complexes present in the mixture and the overall concentrations of each of the components. This task is mathematically straightforward and can be solved by a computer program. The computer program called ECCLES was developed to permit the simulation of large and comprehensive systems.

Although many attempts were made to measure equilibrium constants for metal ion binding to plasma proteins, it was considered not feasible to include these constants in the simulation models, because the numerous binding sites on protein molecules made it impossible to properly define the stoichiometry of the many individual complex species that could be produced. Nevertheless, this difficulty was to some extent bypassed by scanning the free concentration of each metal ion in plasma between its realistic upper and lower limits. It then became possible to make a number of deductions about the low-molecular-weight fractions because certain features transpired to be independent of the actual free concentration used and therefore were not affected by the exact magnitude of the metal-protein binding constants. Computer modelling using ECCLES has provided evidence in support of the hypothesis that the administration of low molecular weight complexes would be beneficial in the treatment of RA[22].

1.6. THE ROLE OF COMPUTER MODELS IN DRUG DESIGN FOR THE TREATMENT OF RHEUMATOID ARTHRITIS

Increasing the concentration of copper complexes in plasma can alleviate copper deficiencies in tissue. The most obvious way of accomplishing this would be by intravenous injection of copper solutions but because the copper binding capacity of serum albumin is easily exceeded (permitting copper to attach itself to irreversible and non specific binding sites), this approach is too hazardous to be of any therapeutic importance. Instead, a variety of ways for increasing the concentration of neutral low-molecular weight copper complexes in blood plasma have been identified [22, 23]. These are shown in Fig. 1.6. One involves copper supplementation by oral or topical administration. The other is based on liberation of endogenous reserves of the metal stored in the liver and elsewhere.

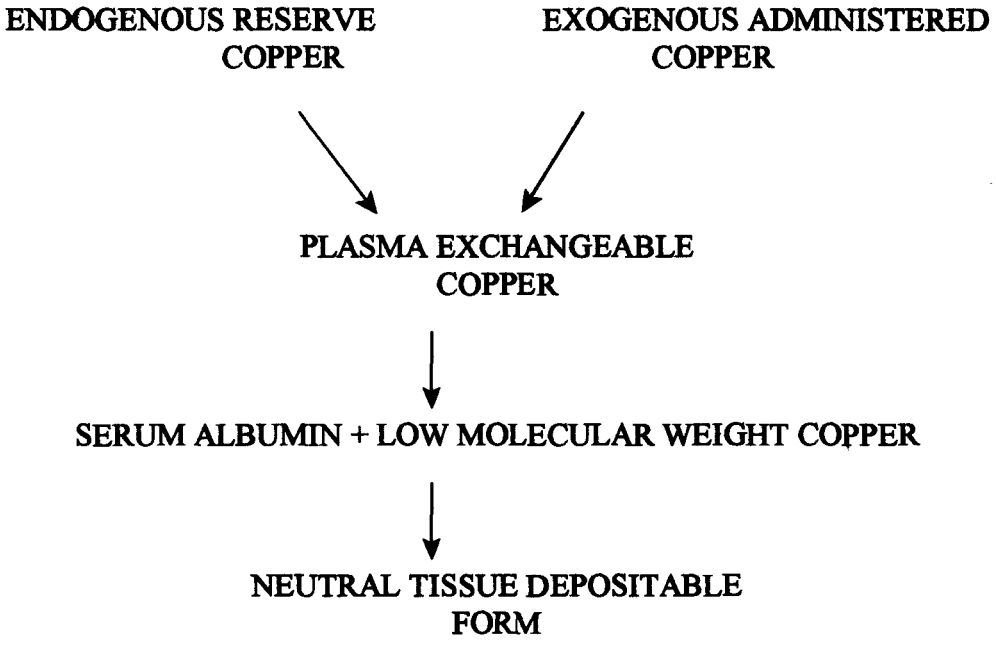


Fig. 1.6 Scheme of routes for increasing Labile Copper in Tissues [25]

1.6.1. MOBILIZATION OF ENDOGENOUS COPPER RESERVES.

The use of chelating drugs to displace the labile copper equilibrium away from plasma albumin in favour of the tissues is the most straightforward way of utilising endogenous copper reserves. This requires the drug to be able to compete effectively with the protein for the metal ion and that the predominant metal complex formed in plasma will readily diffuse into the affected synovial tissue. The extent to which administered therapeuticals may be able to fulfil these conditions can be judged by simulating their effects in plasma using the ECCLES program. In particular, a function called the Plasma Mobilizing Index (P.M.I.) is used to ascertain whether the agent is sufficiently powerful and copper specific.

Drugs that successfully binds copper "*in vivo*" in the presence of competing endogenous ligands produce large P.M.I. values which, for copper, are calculated as follows:

$$(P.M.I.)_{Cu} = \frac{\text{Total low-molecular-weight Cu(II) concentration in plasma in the presence of the drug}}{\text{Total low-molecular-weight Cu(II) concentration in normal plasma}} \quad .....1.1$$

Once again, difficulties arising from an inexact knowledge of copper-protein binding are bypassed because the free metal ion concentration appears as a factor in all terms, in both the numerator and denominator of the P.M.I. expression and can hence be cancelled. The P.M.I. values calculated by the computer thus depends only on the respective total ligand concentrations and the formation constants of the importance complex species, almost all of which have been experimentally determined under physiological conditions of temperature and ionic strength. A schematic representation of the operation of the ECCLES program is presented in Fig. 1.7.

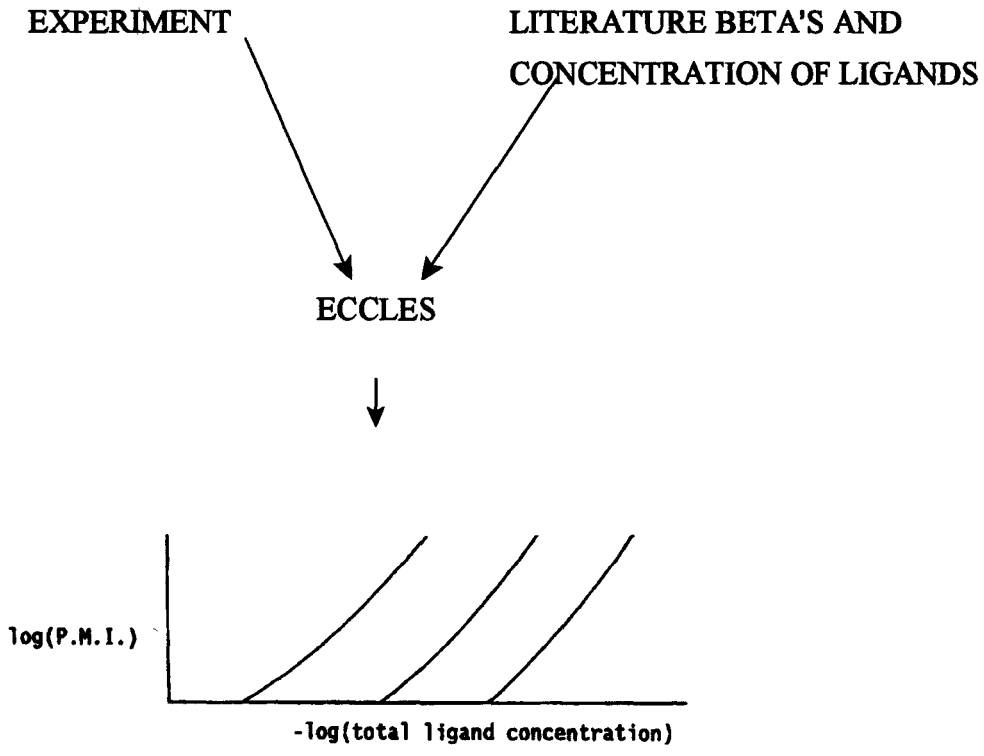


Fig 1.7 Schematic Representation of the Operation of the ECCLES program.

A completely different method of mobilising endogenous copper reserves is the extraction of copper from inert storage metalloproteins such as ceruloplasmin or metallothionein. These almost invariably represent much larger sources of copper than would be available from the labile binding fraction. Pronounced effects may be achieved by a relatively small release of stored metal.

1.6.2. ADMINISTRATION OF EXOGENOUS COPPER

The most obvious problem with oral administration of copper is that homeostatic control of transition metal absorption is highly efficient and tends to prevent a large fraction of the copper dose from being assimilated even though it is presented in a suitable complexed form. Unregulated penetration of the epithelial membrane requires that the complex be very lipophilic and extra-ordinarily stable or inert. Such species tend to be inherently toxic and are also unlikely to deliver their copper when it is required. So, attention has been increasingly drawn to the suggestion that copper may be adequately supplied by dermal application.

The topical application of copper for the treatment of rheumatoid arthritis is not a recent innovation. For many years the therapeutic value of copper bracelets has been widely believed to be part of folklore although its observed benefits tend to be regarded as psychological in nature. However, accumulating evidence concerning the permeability of skin to a host of substances [26, 27] prompted Walker and Keats to quantify the uptake of metal from copper bangles [28, 29]. Their results showed that in the presence of oxygen, copper dissolves in human sweat and is absorbed through the dermis in significant quantities. Further work by Walker et al has demonstrated that the metal can readily perfuse through the dermis as a neutral bis-complex with simple amino acids such as glycine [30]. As copper can be significantly absorbed under indeterminate conditions, it seems likely that its uptake could be greatly enhanced by careful selection of the mediating ligand. The object would be to find agents which were, in themselves, reasonably lipophilic (but not toxic) and which would form relatively strong Cu(II) complexes of low charge density under physiological conditions. This can be done by computer simulation since it is not possible to predict how well different complexes will form solely on the basis of their formation constants.

1.7 OBJECTIVES OF THE RESEARCH

From previous studies a computer model of low molecular weight species existing in blood plasma has been set up [24]. This model has been successful in explaining the therapeutic activity of several pharmaceuticals. One limitation however, is that no protein interactions have been included in this model.

The objective of this project, therefore is to measure under physiological conditions, the copper-protein binding constants. In addition the effect of other metal ions e.g. Ca^{2+}, Mg^{2+} and Zn^{2+} will also be studied. Finally, these measurements will be used to upgrade our computer model and hence to make predictions about therapeutic activities of various drugs and chelating agents that are used in the treatment of rheumatoid arthritis. The experimental method of choice for the binding studies and for checking the computer predictions, is ultrafiltration coupled with atomic absorption and HPLC. An ultrafiltration apparatus was especially designed and made, for use in this project.

The systems to be studied are as follows:

1. The effect of pH and temperature on metal ions binding to plasma proteins in human blood serum.

2. Binding of Cu(II) with human and bovine serum albumin.

3. Competitive studies of the interaction of Cu(II)-HSA with Mg(II), Ca(II) and Zn(II).

4. Studies of the interaction of binary HSA-Cu(II) complex with low molecular weight ligands to form ternary complexes where low molecular weight ligands are L-histidine, L-tryptophan and L-threonine. The binding of these amino acids with HSA is also to be investigated.

5. Computer simulation studies where all binding constants and data are put into a computer model of low molecular weight equilibria existing in blood plasma (ECCLES).

6. Finally a binding study of the chelating ligands as well as several pharmaceuticals with blood serum is to be carried out to verify the computer modelling study.

CHAPTER 2

ULTRAFILTRATION STUDIES OF METAL COMPLEXES IN HUMAN BLOOD SERUM

2.1 INTRODUCTION

The reversible interaction and transport of metal ions through biological systems usually require the formation of protein complexes, which, although labile seems to be well defined. This occurs for instance in blood plasma, in which metal ions very often form one or more protein complexes before they reach their destinations.

An understanding of the above process is of fundamental importance in the unravelling of biological reactions and control mechanisms. This requires detailed knowledge regarding the formation of labile protein complexes in terms of their composition and stability constants, which can be determined by direct binding techniques such as equilibrium dialysis, gel filtration chromatography, ultrafiltration and ultracentrifugation.

In all such studies the number of metal ions bound are directly measured by analysis of the metal protein complex as a function of the equilibrium concentration of the free metal ion. To date, equilibrium dialysis is regarded as the "classical" method of determining protein binding, while the ultrafiltration method is considered to be only an approximate method whose application is described to be limited [31] or even unsuitable [32].

ULTRAFILTRATION is the bulk passage of a fluid through a macromolecule retentive membrane by means of a pressure difference. It provides a gentle way of:

1) deproteinizing biological fluids without dilution or precipitation.
2) separating macromolecule-bound and free low molecular weight substances for binding studies, and
3) concentrating dilute protein solutions.

Among the general limitations described [31,32] is adsorption of small molecules to membrane material, the Donnan effect, increasing protein concentration during filtration and that pores become narrowed by protein molecules.

Furthermore, in estimating the extent of binding by molecular filtration, it has become an accepted practice to ulrarfilter only a small fraction of the total sample (often < 10%) to avoid disturbance of the protein building equilibria [33,34]. The subsequent difficulties in estimating extremely small amount of an ultrafiltrable metal ion or drug in the fraction of the ultrafiltrate often presents insurmountable analytical problems, particularly with strong bound, low serum concentration drugs. [35]

This chapter discusses these important aspects of ultrafiltration. An ultrafiltration apparatus was made and commissioned, in an attempt to validate the technique and extend its application to metal protein binding studies, as drug interactions in human blood serum as well as clinical evaluation of drug therapy. Thus, accurate measurement of unbound plasma drug concentrations and metal ions is essential in the therapeutic monitoring and evaluation of drugs.

2.2 THEORY

The effect of protein and metal ion concentration changes that takes place as a result of the reduction in volume and loss of metal in the ultrafiltrate is now considered.

Metal-protein interactions often can be described by equations derived from the law of mass action

$$[P] + [L] \rightleftharpoons [PL] \qquad \text{Scheme 1}$$

In Scheme I, [P], [L] and [PL] are the protein, metal, and bound metal concentrations, respectively.

Thus for a single class of binding sites:

$$r = \frac{nKm_L}{V + Km_L} \qquad \ldots\ldots\ 2.1$$

where r is the average metal-protein binding ratio and n is the number of sites on each protein molecule of association constant K. The volume of the reaction mixture is V and m_L is defined as the moles of free metal ion.

It is assumed here that the ultrafiltration membrane acts as a perfect molecular sieve, completely retaining the macromolecule and neither binding nor reflecting the smaller metal molecule, such that when centrifugal pressure is applied, its passage across the membrane, like that of the solvent, is unimpeded.

In the situation where a small volume (dV) of solution containing dm_L moles of free metal ion is ultrafiltered through the membrane, then the new binding ratio (r') can be represented by the following equation:

$$r' = \frac{nK\,(m_L - dm_L)}{(V - dV) + K(m_L - dm_L)} \qquad \ldots\ldots\ 2.2$$

This expression can be rearranged:

$$\frac{r'(V - dV)}{K} = n(m_L - dm_L) - r'(m_L - dm_L) \qquad \ldots\ldots\ 2.3$$

Hence:

$$\frac{r'}{K} = \frac{n(m_L - dm_L)}{V - dV} - \frac{r'(m_L - dm_L)}{V - dV} \qquad \ldots\ldots\ 2.4$$

If the membrane does not differentiate between solvent and ligand, then:

$$\frac{m_L - dm_L}{V - dV} = \frac{m_L}{V} \qquad \ldots\ldots\ 2.5$$

Substitution in equation 2.4 and rearrangement gives:

$$r' = \frac{nKm_L}{V(1 + Km_L / V)} = r \qquad \ldots\ldots\ 2.6$$

It is apparent that the molar binding ratio remains constant even though the protein and total metal concentrations increase markedly. Because of this constancy in r, the metal concentration in the ultrafiltrate will also be constant and will be the same as the equilibrium free concentration on the original reaction mixture. In the treatment of the ultrafiltration process, no limitation is placed on the fraction of the original reaction mixture that may be filtered.

Thus r and [L] remain constant and independent of the actual volume of a sample filtered.

2.3 ULTRAFILTRATION APPARATUS

Widespread application of ultrafiltration has been hampered by the requirement of special apparatus, labourious handling and often low processing speed . A low-cost ultrafiltration apparatus was designed in our laboratory. The apparatus is easily assembled and offers the flexibility of the reuse or disposal of components. The components of the system were constructed of materials that are known to be least reactive to low molecular weight ligand species, especially metal ions. The details of the apparatus are represented in Fig. 2.1. The ultrafiltration vessel together with a fixed angle bench centrifuge makes the ultrafiltration technique routine and rapid, without storage of sample or addition of potentially competitive buffer components and electrolytes.

2.4. EXPERIMENTAL

2.4.1 Centrifugation

After 15 minutes of standing in the water bath at 37°C, various serum aliquots (10ml each) were adjusted for pH by adding small amounts of NaOH or HCl. Temperature of 37°C was maintained by preheating the centrifuge chambers and rotor as well as the ultrafiltration vessels. Ultrafiltration was carried out at a centrifugal speed of 3000rpm. (approximately 900xg). At this speed heat production barely exceeds heat loss. The solutions were first centrifuged for 30 sec after which the ultrafiltrate was discarded so as to correct for any adsorption of water to the membrane.

These conditions described above are subsequently referred to standard ultrafiltration conditions.

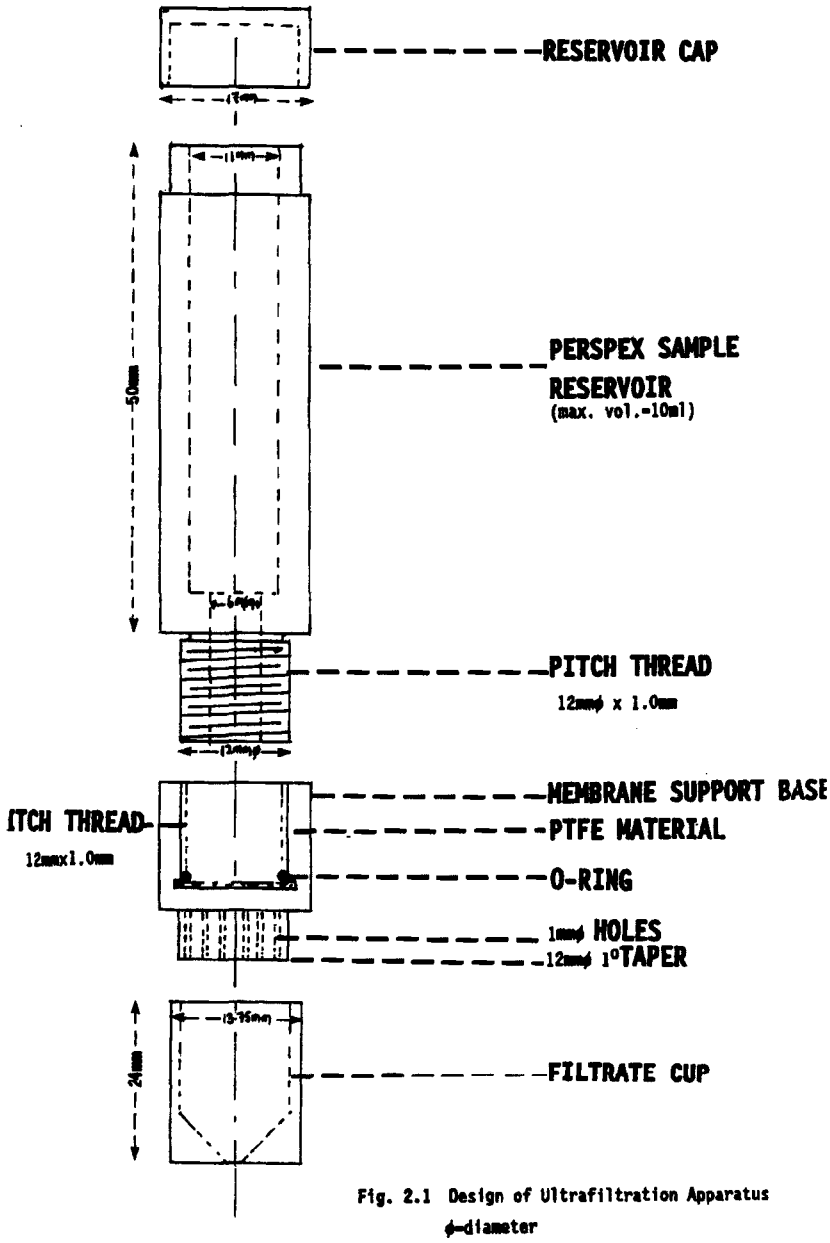


Fig. 2.1 Design of Ultrafiltration Apparatus

ϕ-diameter

2.4.2 Chemical Methods

Calcium and Magnesium were measured by atomic absorption on a Varian Techron, using a N_2O/C_2H_2 flame at 422.7 nm and 285.2 nm respectively. A 100-fold excess of lanthanum nitrate was added as a releasing agent to each of the ultrafiltrate samples.

Zinc was also measured by atomic absorption on a Varian Techron at 213.9 nm using a very lean (oxidizing) air/C_2H_2 flame

Copper was measured by atomic absorption on a Perkin Elmer 5000 Atomic Absorption Spectrophotometer (graphite furnace) coupled to an HGA 500 Programmer.

All reagents used, were of the highest purity. Standard solutions used for calibration was matrix matched, and standardized by EDTA titration. All apparatus was thoroughly cleaned by first soaking overnight in 10% CONTRAN solution, followed by soaking overnight in 10% HNO_3. This was finally followed by washing in ion free water (4X).

The protein assay used to determine the presence of protein in the ultrafiltrate was a dye-binding technique using Coomassie Brilliant Blue G-250 as the protein binding dye [36].

2.5 RESULTS AND DISCUSSION

2.5.1 MEMBRANE BINDING AND FILTERING CHARACTERISTICS

Anisotropic, hydrophilic YMT membranes with an even pore size distribution were obtained from Amicon in the U.S.A. Due to the high cost of these membranes, readily available, low-cost spectrapor membranes were also used for comparison and possible replacement of the YMT membranes. For details of the preparations of the spectrapor membranes in these studies, the reader is referred to the experimental section of Chapter 3. No preparation of YMT membranes was necessary before use.

A number of aqueous $CuCl_2$ solutions were prepared, covering a concentration range from 1×10^{-4}M to 6×10^{-4} M and all having an ionic strength of 0.15M after the addition of appropriate amounts of NaCl. Aliquots of 2ml were filled in the ultrafiltration apparatus with both YMT and spectrapor membranes and kept closed for 24 hours.

During this incubation YMT membranes showed an average concentration difference of -1.0×10^{-4}M less than the original solution which indicates that very little or no membrane binding takes place. Spectrapor membranes on the other hand, showed an average concentration difference of 3.2×10^{-7}M less than the original concentration, after incubation. This indicates that membrane binding has taken place.

The ultrafiltration rates of both membranes were then measured, using both aqueous solutions of human albumin (0.06mM) at an ionic strength of 0.15M and human serum respectively. Higher flow rates are observed in both solutions when using YMT membranes as compared to spectrapor membranes (refer to Fig. 2.2 and Fig. 2.3). This is indicative of a greater membrane surface, efficient polarization control and optimal transmembrane pressure achieved using a sample volume of 2 ml. At this stage it was decided to use only YMT membranes in further studies.

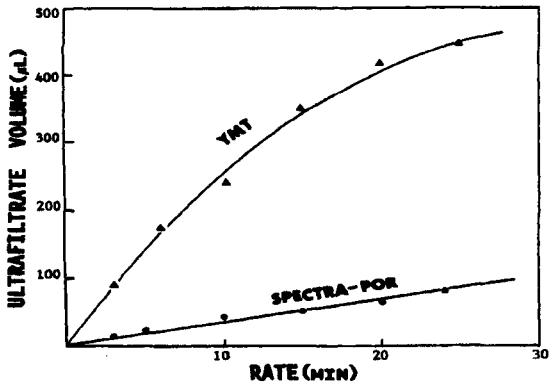


Fig. 2.2 Ultrafiltration rate using aqueous solution of human albumin (0.006mM; I=0.15M) at 3000rpm.

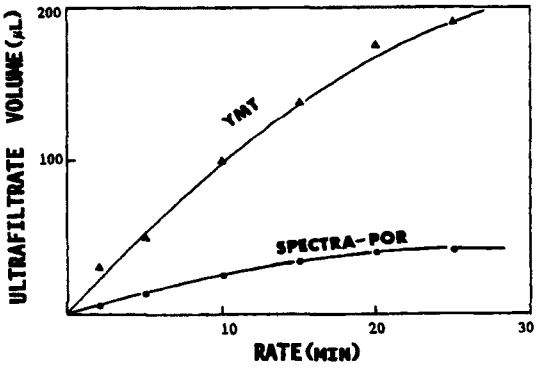


Fig. 2.3 Ultratfiltration rate using human blood serum at 3000rpm.

The same solutions used in the incubation studies were ultrafiltered under standard conditions and copper determined in the ultrafiltrates and retentates. The results are given in table 2.1 and shows that within experimental error of analysis, the membrane does not differentiate between the passage of the solvent (H_2O) and the metal ion.

Table 2.1 YMT membrane filtering characteristics of aqueous $CuCl_2$ solutions.

Original Solution (O) Cu(II) x 10^4M	Ultrafiltrate (U) Cu(II) x 10^4M	Retentate (R) Cu(II) x 10^4M	O - U 10^4M	O-R 10^4M
0.5	0.49	0.49	0.01	0.01
1.0	0.97	0.95	0.03	0.05
2.0	2.08	2.06	-0.02	-0.04
3.0	3.07	3.03	-0.07	-0.03
4.0	4.02	4.02	-0.02	-0.02
6.0	5.98	5.98	0.02	0.02

Finally, with regard to protein permeability, it must be noted that the membrane was fitted to the porous base by an O-ring. Since the sample reservoir is tightly screwed onto the O-ring, it is possible that the membrane will be subjected to circumferential stretching which will increase the porosity. However no protein permeability was observed and no membrane rapture occurred in less than 2% of the total ultrafiltration runs executed.

2.5.2. INFLUENCE OF VOLUME CHANGE DURING ULTRAFILTRATION.

Five identical solutions with a final volume of 2ml were prepared with constant human serum albumin and $CuCl_2$ concentrations of 6 x 10^{-4}M and 6.024 x 10^{-4}M respectively, in 0.1M N-ethymorpholine-HCL at constant ionic strength of 0,15M and pH = 7.5. The protein used was purified by chelex-100, the details of which will be presented in Chapter 3. The solutions were ultrafiltered under standard conditions with the duration of ultrafiltration varying from 3 to 20 minutes. The amount of ultrafiltrate collected at

each time interval was measured, and analysed for copper. The results are presented in Table 2.2 and shows that the ultrafiltrate concentration, i.e. concentration of free copper and the concentration ratio of bound copper to protein (r) remains constant and is independent of the volume fraction of the protein solution filtered up to 21% of the total volume.

Table 2.2 Effect of volume change during ultrafiltration on ultrafiltrable Copper

Volume of ultrafiltrate (μL)	**% Volume filtered**	**[Cu(II)]free x10^5 M**	$r = \frac{[Cu(II)]_{bound}}{[Protein]_{total}}$
92	4.6	5.60	0.911
176	8.8	5.51	0.912
242	12.1	5.80	0.907
348	21.0	5.39	0.914

Previous work [37] has shown that the above effect is maintained up to 42% of the protein solution filtered. Filtration beyond this limit is dangerous, as the protein solution becomes quite viscous and the protein could undergo conformational changes or formation of dimers of high order macromolecular aggregates, in which case, n and k are no longer constant. Nonetheless, the finding in our studies confirms the theory. Not only does ultrafiltration not disturb the equilibria, but a large fraction of the protein solution can be ultrafiltered, when necessary, to compensate for a lack of assay sensitivity.

2.5.3. EFFECT OF pH AND TEMPERATURE ON ULTRAFILTRABLE METAL IONS IN BLOOD SERUM

16 (10ml) Aliquots from a large serum pool were adjusted with small amounts of NaOH and HCl over a wide pH range and ultrafiltered at 37° and 22°C. pH Measurements in the retentate after ultrafiltration at 37°C disclosed an average difference of only 0.07 pH units between the original solution and the retentate indicating that the pH of the solution during ultrafiltration remains fairly constant. Serum solutions adjusted for pH at 37°C were allowed to cool to room temperature (22°C) and the pH measured. The average difference between the pH measured at 22°C and 37°C was found to be 0.22. This indicates that a change in temperature induces pH changes in the serum solution, hence, it is important that rigid control of desired temperature be maintained during ultrafiltration. The results shown in Figures 2.4, 2.5, 2.6, and 2.7 demonstrate a marked influence of pH on calcium, magnesium, zinc and copper binding to serum proteins over the pH range studied.

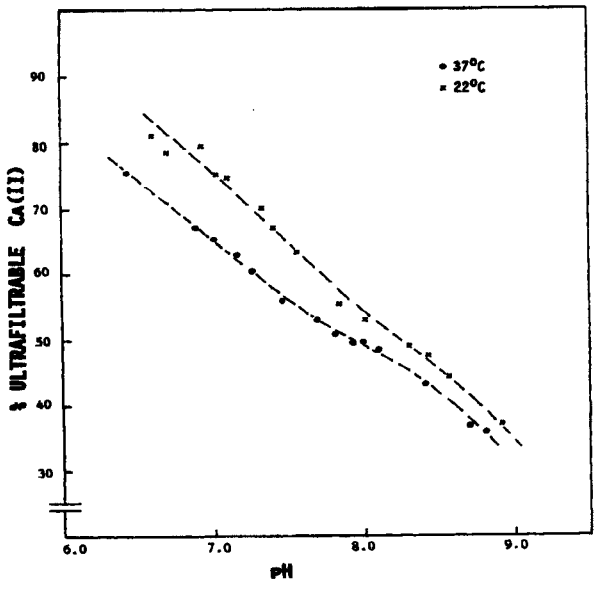


Fig. 2.4 Effect of pH on % ultrafiltrable Calcium at 37°C and 22°C.

Ultrafiltrable calcium at 37°C decreases from 76% to 36% when the pH rises from 6.4 to 9.0. This indicates an increase in calcium binding to serum proteins. The observed effect with increasing pH arises from the increase in electrostatic interaction of the more negative serum protein molecules with the calcium cation. This distribution curve for ultrafiltrable calcium over the wide pH range is very similar to the one obtained by ultracentrifugation by Locker, Havee, Garden and Whittington [38]. Figure 2.4 also demonstrates a definite lowering of ultrafiltrability (i.e. increase protein binding) of calcium at 37°C compared to 22°C. This effect of temperature on ultrafiltrable calcium is a controversial point and the lack of recognition of this variable contributes much to the variation of percent ultrafiltrable calcium found by others within the pH range encountered in the pathological states [38,39]. The results obtained here are in agreement with others [40,41], demonstrating an increase of about 6 percent in ultrafiltrable calcium at 22°C compared to 37°C.

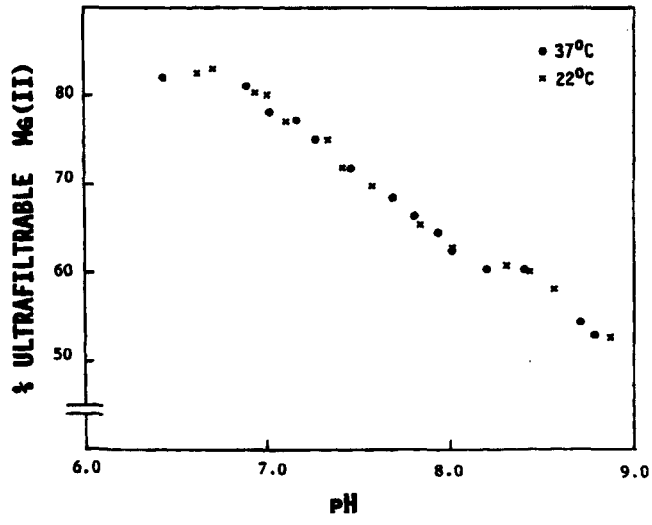


Fig.2.5 Effect of pH on ultrafiltrable Magnesium at 37°C and 22°C.

Ultrafiltrable magnesium at 37°C decreases from 84% to 52% when the pH rises from 6.4 to 9.0. The distribution curves of magnesium and calcium are rather similar (Fig. 2.4 and Fig. 2.5) and slightly curved. However, Figure 2.5 shows that there is no significant

change in the amount of ultrafiltrable magnesium at 37° compared to 22°C. Ultrafiltrable zinc at 37°C decreases from 34% to 4% when the pH rises from 6.0 to 9.0. From Figure 2.6, it can be seen that there is a sizeable increase in zinc binding from pH 6.3 to 7.2, after which the amount of zinc binding to serum protein tends to remain constant. In blood plasma zinc ions are considered to be mainly transported as albumin complexes [42]. Various studies on the binding of zinc to serum albumin [43, 44, 45] have shown that zinc binds to only one class of site, namely the imidazole group, in a ratio of one to one.

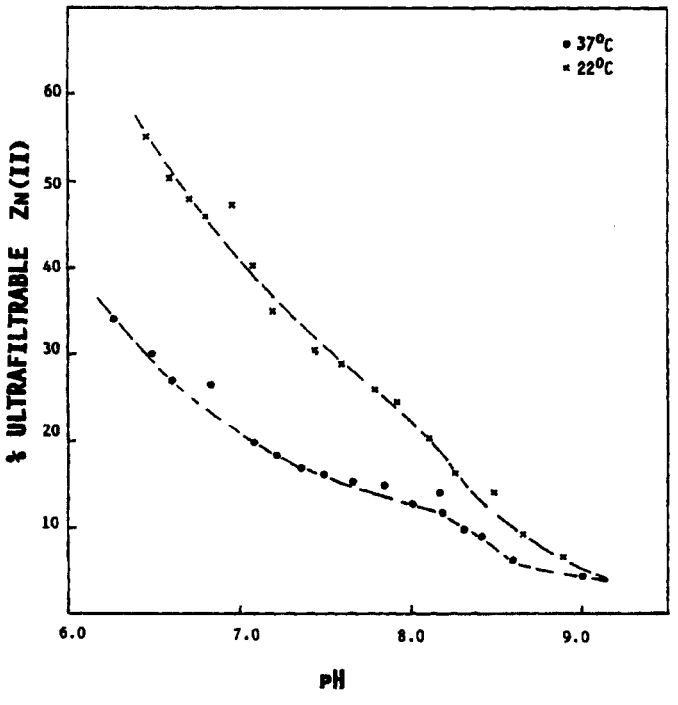


Fig. 2.6 Effect of pH on Ultrafiltrable Zinc at 37°C and 22°C.

From Figure 2.6, the large increment in binding that occurs in the pH range of 6.3 to 7.2, is the region in which imidazole groups of histidine side chains of albumin are known to change from a cationic (i.e. hydrogen ion bound) to an uncharged form [46]. Therefore, it can be suggested that the interdependence of zinc binding and pH reflects a competition between zinc and hydrogen ions for the same ligand groups on the serum proteins, especially human serum albumin. Figure 2.6, also demonstrates a definite

lowering of ultrafiltrable zinc at 37°C compared to 22°C, indicating that zinc binding in human blood serum is temperature dependant.

Ultrafiltrable Copper at 37°C decreases from 25% to 2% when the pH rises from 6.0 to 9.0. Figure 2.7 shows that this effect is marked in the pH range of 6.5 to 7.5, after which the % ultrafiltrable Cu(II) steadily decreases from 7% to 2%.

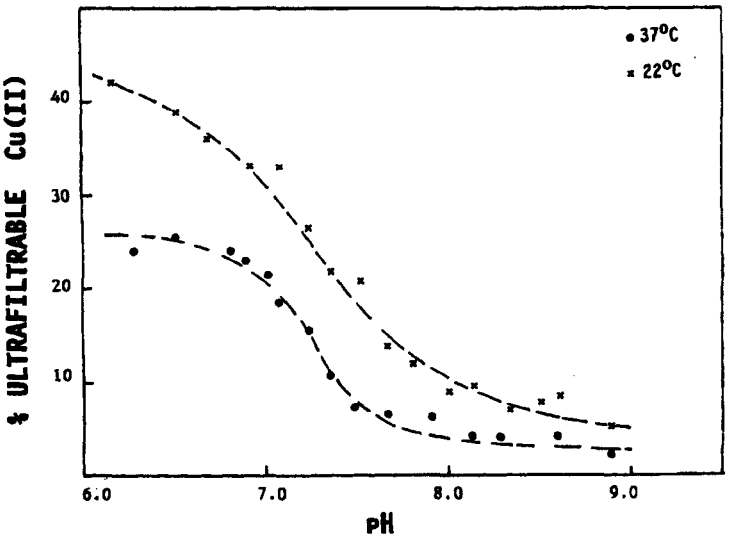


Fig. 2.7 Effect of Ultrafiltrable Copper at 37°C and 22°C.

Copper like zinc is bound most to serum albumin and it is this fraction that is considered to be in rapid exchange with tissue copper [47]. Thus, it can be assumed, for the pH range of 6.5 to 7.5, the imidazole groups of histidine side chains of serum albumin are responsible for the pH controlled binding of copper in human blood serum.

It is also evident, from Figure 2.7, that there is a definite lowering of copper binding to serum proteins at 22° compared to 37°C. It is suggested herein that the decreased binding with increased temperature can be explained by enthalpy changes and the Van't Hoff equation (log K_a = constant - $\Delta H/4.576T$ K, where K_a is the apparent association

constant, ΔH the apparent heat of association and T the absolute temperature).

This would require a detailed study of the binding of copper to the various serum proteins at different temperatures, taking into consideration, hydrogen-ion competition. Such an investigation is considered to be beyond the scope of our investigations.

2.6 GENERAL DISCUSSION

The interactions between H^+ ion and metal ions on binding to proteins in human blood serum have profound implications with respect to the availability of metal ions of different binding sites at different pH values. The consequence of a pH change is also known to have an effect on the electrostatic nature of the protein, thus affecting the binding of metal ions that form chemical bonds that are electrovalent. A classification of metal ions connected with electronegativity was proposed by Ahrland *et al* [49].

According to this classification, calcium and magnesium are termed hard metal ions whose coordination is governed mainly by electrostatic interactions existing between charges of opposite signs. The result shown in Figures 2.4 and 2.5 demonstrates temperature dependance of calcium-binding and temperature independence of magnesium-binding to serum proteins over the whole pH range studied.

The disparity of calcium- and magnesium- binding to serum proteins must be due to one or more of the globulins, since the binding of both cations to serum albumin is independent of temperature between 22° and 37°C [49]. Furthermore, calcium-protein complexes were found to be more stable than magnesium- protein complexes under physiological conditions.

Copper and zinc on the other hand are classified as soft metal ions, which form chemical bonds of covalent character. From figures 2.6 and 2.7, it can be seen that complexes formed by Zn(II) ions are less stable than those of Cu(II) ions at 37°C, indicating that the tendency of Zn(II) ions to form chelates are not very pronounced. This is further substantiated by the fact that, the dissociation constants for the serum albumin -Cu(II) binary complex and the serum albumin -Zn(II) binary complex are 6.61×10^{-17} [20] and 1.58×10^{-3} [45], respectively. Figures 2.6 and 2.7, also, demonstrates the temperature dependance of copper-binding and zinc-binding to serum proteins.

These measurements of temperature dependance in metal protein interactions are important, not only in the case of physiological interpretations, but generally, because the resulting information about energy relations gives further insight into the molecular nature of the binding process.

An analysis of all the results, indicates that under physiological conditions of pH, temperature and ionic strength, the order of complexing strength of metal ions to serum proteins is found to be:

$$Cu(II) > Zn(II) > Ca(II) > Mg(II)$$

Finally it can be stated, that ultrafiltration is an easy and reliable procedure, provided that satisfactory control of the critical variables such as pH and temperature are achieved. Experimental verification as well as mathematical simulations of the molecular separation, shows that the ultrafiltrate concentration remains constant during filtration, and, thus, binding equilibria are not disturbed by this procedure, suggesting that an arbitrary restriction on the volume filtered is unnecessary. Several investigations to date have indicated, for some drugs, that the unbound concentrations remain constant even though the proteins became concentrated in the upper reservoir [145,146]

Comparative studies by Wright *et al* [147], confirms the Ultrafiltration method to be experimentally sound and to yield adequate protein binding parameters which characterise protein-metal association, protein-drug association, the number of classes of binding sites, the number of binding sites per class of protein and the binding capacity useful for predicting unbound metal or drug concentration.

3.1 INTRODUCTION

The physiological concentration of Cu(II) ions in human blood serum is approximately 17 μM. A large fraction of this copper is bound to ceruloplasmin, and is not exchangeable *in vivo* [50,51]. A small fraction, approximately 7%, corresponding to a Cu(II) concentration of 1.5 μM, is bound mostly to albumin and to a smaller extent to amino acids [47-53]; in this fraction Cu(II) is rapidly exchanged with tissue copper, and is considered to be the immediate transport form of Cu(II) in human blood.

The physiological concentration of circulating albumin under normal conditions is 0.5 - 0.7 mM, indicating a large excess of albumin over copper, and as a result, complexation of only the first equivalent of copper ions is physiologically significant. The bulk of the studies of Cu(II)-binding to albumin was carried out with bovine serum albumin (BSA). It is indicated from these studies that the primary binding site for the Cu(II) ion is the amino-terminal end of the albumin molecule [54-59]. Within this site, the Cu(II) ion is assumed to be bound via the α-amino group, the imidazole group from the histidine residue in the third position and the two intervening peptide-bond amino nitrogens. Peters and Blumenstock [60] proposed the structure of the Cu(II)-binding site of BSA as shown in Fig. 3.A.

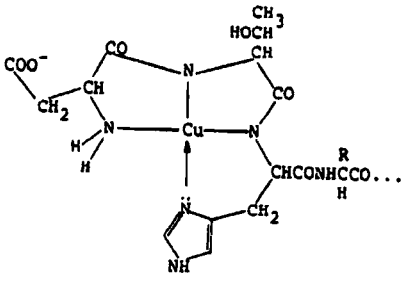


Fig. 3.A Proposed structure of the Cu(II)-binding site of Bovine Serum Albumin.

In 1981, Giroux and Schoun [61], using an ultrafiltration technique, determined the formation constant of a 1:1 complex of BSA-Cu(II) to be 1.58 x 10^{13} at a pH of 7.4 and 37^0 C . This value was found to be in excellent agreement with Ljones *et al* [62], who using a Cu^{2+} ion-selective electrode, also found the formation constant to be 1.58x10^{13} at a pH of 7.3 and 23^0 C.

The stoichiometry of Cu(II) binding to human serum albumin (HSA) was first established by Sarkar and Wigfield [63]. They studied the specificity of Cu(II) binding to HSA by ultracentrifugation using labelled copper. HSA like BSA was found to have one strong binding site for Cu(II) up to a Cu(II)/HSA molar ratio of one, after which there appears to be second and subsequent binding sites that are weaker than the primary sites. The copper (II) transport site of HSA is located at the NH_2 terminus of the polypeptide chain. In addition to the four nitrogen ligands (one amino, two peptides, and one imidazole nitrogen) at the Cu(II)-transport site, there is the involvement of the ß-carboxyl side chain of an aspartyl residue in a pentacoordinated structure [64]. It is thus suggested that the Cu(II) binds to HSA in a manner shown in Fig. 3.B.

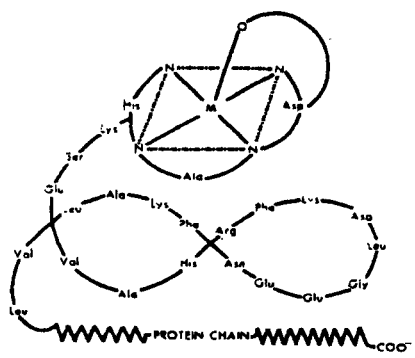


Fig 3.B Proposed structure of the Cu(II)-binding site of Human Serum albumin, where M refers to Cu^{2+}[64].

Although the primary binding site is of major importance, during some specific pathological conditions secondary binding sites, may also be involved:

(1) In Wilson's disease the concentration of ceruloplasmin is low and albumin becomes the main Cu(II)-binding protein [65];

(2) During other conditions, such as hemodialysis [66], where the blood copper concentration occasionally may reach very high values.

The Cu(II) may reach concentrations, as high as 200μM, perhaps even higher concentrations locally, especially if hemolytic crises develop. In such a situation, the complex formation of Cu(II) may extend beyond the primary site. Since the compositions and stabilities of these other Cu(II)-albumin complexes are not well understood, it is imperative that studies are undertaken to provide more detailed information regarding the Cu(II) albumin system in the form of a relatively complete equilibrium analysis. The equilibrium data of this work was therefore extended over relatively broad concentrations of Cu(II), those that occur under both physiological and pathological conditions.

However, the main objective of this study is to obtain the logß value for Cu(II)-albumin complex formation for use in computer simulated models of copper complexes in plasma of rheumatoid arthritics.

3.2 EXPERIMENTAL

3.2.1 MATERIALS

Crystalline bovine serum albumin (Fraction V, code 81-003, lot No. 385, 98% purity, MW = 69024 $gmol^{-1}$) and human serum albumin (Fraction V, code 82-302, lot No. 155, 97% purity, MW = 69000$gmol^{-1}$) were obtained from Miles laboratories. In addition a sample of HSA was obtained from the Western Province Blood Transfusion Service(Fraction V, code E83.3, purity 96%, MW = 69000$gmol^{-1}$). Chelex-100 resin and dialysis tubing (spectrapor) were generous gifts from the Red Cross Hospital, Cape Town. N-ethylymorpholine-HCl buffer was obtained from Sigma, and was passed over chelex-100 to remove trace metals. All other reagents were of analytical grade quality.

3.2.2 PREPARATION OF THE PROTEINS

In connection with the investigations on copper binding to HSA and BSA, it was desirable to obtain samples completely free from Cu(II). There is always a small amount of Cu(II) contamination even in the purest form of commercially obtained albumin [67]. Two methods were used to purify the proteins, and are herein referred to as chelex-100 treatment and EDTA treatment. In the chelex-100 treatment, a column was prepared with 14g of chelex-100 resin, after the resin had been washed with 0.2M acetate buffer. The column was then equilibrated with 1 bed volume of acetate buffer. The protein sample was dissolved in double distilled ion free water and passed through the column. The eluate was collected, dialysed and freeze dried. In the EDTA treatment, commercial HSA was dialysed against EDTA (10mM in 0.1M N-ethymorpholine-HCl buffer at pH7.5) overnight. This was followed by exhaustive dialysis against deionized water (8 changes in a 100-fold volume). The chelating agent was removed by ultrafiltration (stirred ultrafiltration cell, Amicon model 202, 75 Psi max.).

The protein solution was then freeze-dried. The amount of copper contamination, before and after cleaning was measured by atomic absorption. Albumin concentration was determined by UV spectrophotometry. The molar absorptivities at 279nm used for protein quantification was 3.6 x 10^4 $M^{-1}cm^{-1}$ (HSA) and 4.7 x 10^4 $M^{-1}cm^{-1}$ (BSA).

3.2.3. ULTRAFILTRATION

Increasing concentrations of $CuCl_2$ were added to solutions of fixed protein concentration of 6 x 10^{-4} M in 0.1 M N-ethylmorpholine-HCl buffer at pH 7.5, in a final volume of 3ml. The ionic strength was maintained at 0.15M by the addition of an appropriate amount of dry NaCl. All binding experiments and pH measurements were performed at 37°C. The three ml aliquots were ultrafiltered at standard conditions, previously defined in Chapter 2. The ultrafiltrates were collected and immediately analysed for copper by atomic absorption, using both the flame and graphite techniques. Thus, by measuring the amount of free copper, the amount of bound copper was easily calculated.

3.2.4. EQUILIBRIUM DIALYSIS

The 5-cell Spectrum Equilibrium Dialysis apparatus was chosen to perform the necessary binding experiments. The dialysis cells are made of teflon material constructed to seal in two halves with a maximum volume of 1 ml in each half. The teflon material minimizes the loss of the proteins due to wall adsorption and precision machining of the cell halves provides excellent sealing. Spectrapor semipermeable membranes are sandwiched between the two cell halves. The membranes were extensively boiled in 2% EDTA and 5% $NaHCO_3$, followed by copious rinsing and 3 repeated boils for 1hr each in double distilled deionized water. This treatment removes

all contaminating metals and nucleases from the dialysis membranes (cut from tubing before treatment). The membranes were then soaked for 15 minutes in the dialyzing buffer, after which they were considered ready for use. All dialysis cells were also washed in EDTA before use and were extensively rinsed in double distilled deionized water and air dried between subsequent experiments.

Varying concentrations of $CuCl_2$ in 0.1M N-ethylmorpholine HCl buffer were injected in one cell half and a solution of fixed concentration of Protein (6×10^{-4}M) in 0.1M buffer into the other half. The final molar ratio of Cu(II) to HSA was kept approximately one. Both solutions in each cell half were at identical pH (7.5) and ionic strength (0.15M). The familiar Donnan effect and the osmotic pressure of the protein solution were suppressed by the use of high salt and buffer concentrations. The dialysis cells were allowed to rotate at 5 revolutions/minute in a water bath equilibrated at 37 ^{0}C for 24 hours, for equilibrium to be reached. The solution in the cell containing only free copper was analysed by atomic absorption.

3.2.4 ULTRACENTRIFUGATION

Ultracentrifugation experiments were carried out in a Beckman Model L5-65 ultracentrifuge using a 65Ti rotor at a speed of 50 000 rpm at 4 ^{0}C. A 5%-20% sucrose gradient in 0.1M tris buffer at a pH of 7.5, was generated in each of the centrifuge tubes, using a gradient former. A small volume (50µl) of the protein solution to be characterized was gently layered on top of the preformed concentration gradient. The tubes were then centrifuged for 16.5 hours, allowing the molecules in the starting layer to sediment through the gradient. After centrifugation the contents of the tubes were fractionated using an ISCO density gradient fractionator Model 640, and assayed by absorption at 279 nm using an ISCO Model UA5 absorbance monitor. A plot of absorbance versus distance was obtained for each run. The S values were calculated by using the Fritsch equation [68].

3.2.5 CROSS-LINKING OF PROTEINS

Cu(II)-albumin solutions that were used in the binding studies were subjected to cross-linking with dimethyl suberimidate followed by polyacrylamide gel electrophoresis. 200µl of the above protein solution (41 mg/ml) was added to an equivalent volume of dimethyl suberimidate (41 mg/ml). After 45 minutes, 200µl of glycine (154 mg/ml) was added to the solution. The resulting mixture was placed in an ice bucket for 1 hr., after which it was dialysed against deionized water for 14 hrs. The dialysed solution was then reacted with an equivalent volume of a sample buffer with 5% mecaptoethanol. The mixture was boiled for 3 minutes, followed by spinning on a bench centrifuge for 3 minutes. 1µl of the mixture was then subjected to electrophoresis.

3.2.6. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was done according to the method of Laemli [69], except that the use of sodium dodecylsulfate (SDS) was avoided. The running gel contained 5% acrylamide, 0.133% Bis-acrylamide, 0.375 M Tris-HCl (pH8.8) and 0.133% ammonium peroxidisulfate (AMPS). The stacking gel contained 3% acrylamide, 0.08% Bis-acrylamide, 0.375 M Tris-HCl (pH 6.8) and 0.04% AMPS. The running buffer was 0.25M Tris-HCl (pH 8.3) and 1.92M glycine. The sample buffer was 0.0625 M Tris-HCl (pH 6.8), 10% glycerol, 5%β-mecaptoethanol and 0.001% bromophenol blue.

Electrophoresis was performed at 18mA until the dye front approached the bottom of the gel. The gels were removed and stained with Coomassie blue and destained with a solution of 25% tech. ethanol and 7% tech. acetic acid.

3.3 TREATMENT OF ULTRAFILTRATION AND EQUILIBRIUM DIALYSIS DATA

Previous studies on HSA and BSA have indicated the presence of a single strong binding site for Cu(II) at the amino terminal end of the protein molecules. At saturation one mole of protein (P) combines with one mole of Cu(II) to form the complex P-Cu(II).

$$P + Cu(II) \rightleftharpoons P - Cu(II) \qquad \text{..... 3.1}$$

The apparent intrinsic dissociation constant K_d for this reaction is defined as:

$$K_d = \frac{[P]\,[Cu(II)]}{[P - Cu(II)]} \qquad \text{..... 3.2}$$

Equation 3.2 indicates that the fraction of Cu(II) bound is affected by the value of [P] and [Cu(II)]. The amount of copper bound is expressed by use of a binding parameter r, which is defined as the number of moles of Cu(II) bound per mole of protein:

$$r = \frac{[Cu(II)]_{bound}}{[Protein]_{total}} = \frac{[P - Cu(II)]}{[P] + [P - Cu(II)]} \qquad \text{..... 3.3}$$

To evaluate r, a particular concentration [(Cu(II)'] of Cu(II) is added to a known concentration [P'] of the protein and then either the concentration of the bound copper [PCu(II)] or of the unbound copper [Cu(II)] is measured. Only one of these needs to be measured because [Cu(II)'] is known at the outset and [Cu(II)] + [P-Cu(II)] = [Cu(II)']. The value of [P] also need not be measured since [P] + [P-Cu(II)] = [P'].

Combining equations 3.2 and 3.3 yields:

$$r = \frac{[Cu(II)]}{K_d + [Cu(II)]} \qquad \text{..... 3.4}$$

which is known as the Langmuir isotherm. This equation is usually rewritten to allow the data to be plotted on a straight line graph:

$$\frac{1}{r} = \frac{K_d}{[Cu(II)]} + 1 \qquad \text{..... 3.5}$$

a plot of 1/r versus 1/[Cu(II)] gives a straight line whose slope is K_d. This plot is called the double reciprocal plot.

Alternatively, corresponding values of free copper concentrations [Cu(II)] and r can be plotted according to the following linearization of the mass law [70]:

$$\frac{r}{[Cu(II)]} = nK - rK \qquad \text{..... 3.6}$$

which in the case of one class of identical and independent (i.e. non interacting) binding sites yields a straight line, where the abscissa intercept, n, is the maximum number of binding sites, the negative slope of the line is the apparent intrinsic association constant, K, and the ordinate intercept, nK is equal to the apparent association constant for the first binding site. The Scatchard plot was chosen, since it gives the most relative even weight to each experimental point and readily shows how far an extrapolation is needed to obtain the desired intercepts.

It must be noted that equation 3.4 could also be rewritten to give another form:

$$\frac{r}{[Cu(II)]} = \frac{1}{K_d} - \frac{r}{K_d} \qquad \text{..... 3.7}$$

since $K = 1/K_d$ it follows:

$$\frac{r}{[Cu(II)]} = K - rK \qquad \text{..... 3.8}$$

Equation 3.8 is the same as equation 3.6 when n is equal to one.

3.4 RESULTS AND DISCUSSION

3.4.1. PURIFICATION OF SERUM ALBUMIN

The commercially available preparations of crystalline HSA and BSA that were used in these studies contained 18.86 μg Cu(II)/gHSA and 6.91 μgCu(II)/g BSA respectively, while HSA that was obtained from the Western Province Blood Transfusion Service (WPBTS) was found to contain 10.85 μg Cu(II)/g HSA (Table 3.1)

Table 3.1 Copper (II) content of HSA and BSA that were used in binding studies.

	μgCu(II)/g Protein
HSA (WPBTS)	10.85
Chelex-100 treated HSA (WPBTS)	4.02
Commercial BSA (Miles lab.)	6.91
Chelex-100 treated commercial BSA	2.62
Commercial HSA (Miles lab.)	18.86
Chelex-100 treated commercial HSA	3.68
EDTA treated commercial HSA	1.28

From Table 3.1, it can be seen that a significant amount of Cu(II) is removed when the protein is treated with either chelex-100 or EDTA. In the case of commercial HSA, treatment with EDTA resulted in a 95% removal of Cu(II) contamination, while the chelex-100 method showed a 66% removal.

It is therefore evident that the EDTA treatment was more efficient in Cu(II) decontamination than the chelex-100 treatment. However, 1.28 μgCu(II)/g HSA still remained bound to HSA even after EDTA treatment; while 2.62μgCu(II)/g BSA remained bound to BSA after treatment with Chelex-100.

It has been reported by Anderson [71] that bovine serum albumin form aggregates. In view of this finding it is considered possible that the remaining Cu(II) after treatment may somehow be bound to dimers, trimers and other polymers of HSA and BSA. It is conceivable that Cu(II) which is bound to the terminal amino group of albumin may form a bridge between two molecules of albumin. Sarkar and Wigfield [63] suggested that copper may in some way be involved in the formation of polymers of albumin *in vitro*.

Another possibility is that free sulfhydryl groups are involved in the formation of molecular aggregates and that once the dimer or polymer is formed, the copper is buried inside the aggregate in such a manner that it becomes inaccessible to Chelex-100 resin or EDTA. According to Anderson [71], one third of the dimer of albumin present in bovine serum albumin can be split by treatment with mercaptoethanol. Thus it seems that sulfhydryl bonding is only partly responsible for the formation of dimers and polymers. From our results it seems that copper may be involved here. The protein preparations were subjected to ultracentrifugation and 5% polyacrylamide gel electrophoresis. These results will be discussed later in the chapter.

3.4.2. COPPER(II) BINDING TO HSA

Figure 3.1 gives a Scatchard plot for the binding of Cu(II) to HSA (EDTA treated) at 37 ^{0}C. (See Table 3.1 in appendix for calculations). The Scatchard plot is strikingly different from the case of a single class of independent sites, in that the r/[Cu(II)] intercept actually passes through the origin with the initial slope being positive rather than negative. An interpretation of this plot seems to be rather complex.

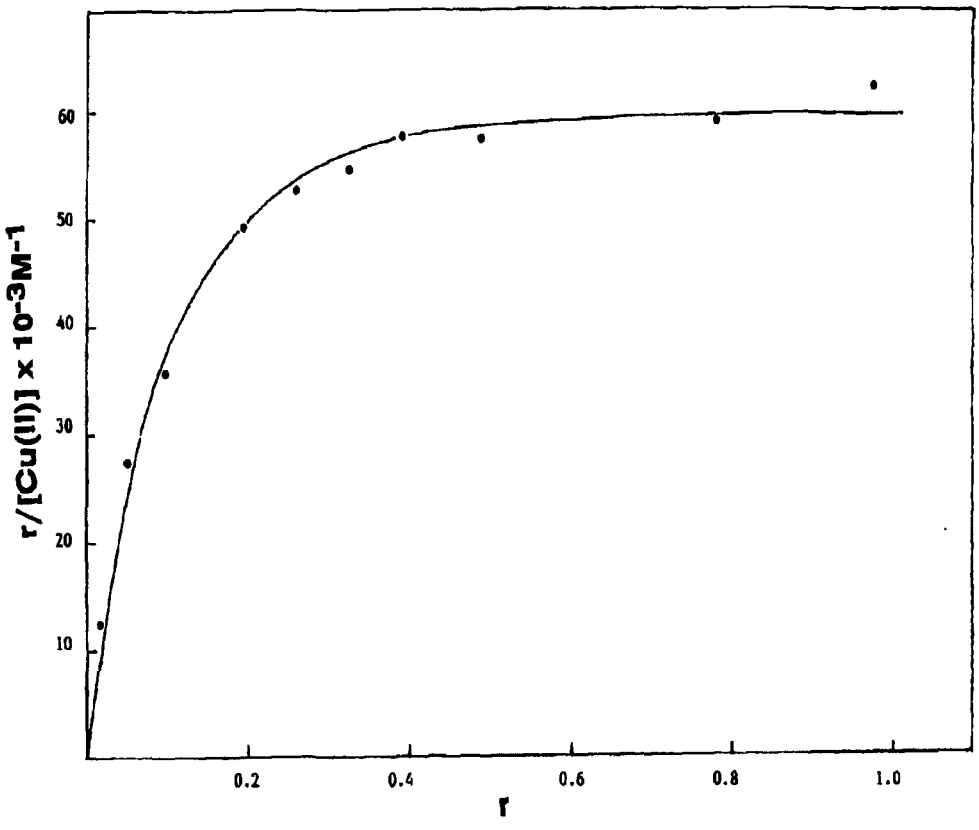


Fig. 3.1 Scatchard Plot for Copper(II) binding to HSA (EDTA treated) in 0.1M N-ethylmorpholine-HCl at pH 7.5, 37^{0}C and I =0.15M.

However, the observed behaviour could be explained by one or more of the possible suggestions:

1. That the case encountered in our present work is one in which Cu(II) ions binds to more than one class of independent sites, in which case copper(II) is considered to bind to strong and weak sites on the albumin moiety.

2. That Cu(II) concentrations encountered in our binding experiments successively induces dimers and tetramers, by forming metal bridged protein complexes.

3. That HSA has one class of interacting sites (co-operativity) together with a class of independent sites.

4. That the protein preparation contained significant amounts of molecular aggregates (polymers) that are subsequently depolymerised by increasing Cu(II) concentrations, in which case, the number of Cu(II) available sites are increased as the titration continues.

At this point, it must be noted that binding experiments alone, cannot fully elucidate the actual nature of Cu(II) binding to HSA in our system. Comparative studies with other protein preparations as well as other experimental techniques are now needed to validate any of the above-mentioned suggestions.

The occurrence of a maximum at r = 0.4 in Fig 3.1 seems to be a clear indication of co-operativity. A similar effect was observed for Mn(II) binding to yeast t RNA^{Phe} [72]. Interacting site behaviour (co-operativity) is defined here as that in which the copper(II) binding follows a greater than first power dependance on copper(II) concentration. The Scatchard parameters for a single class of interacting sites are given by Schreier and Schimmel [72]:

$$\ln[Cu(II)] = -(1/\alpha)\ln(N_i/r - 1) - \ln K \qquad \ldots\ldots 3.9$$

where K is the apparent association constant of the interacting sites, N_i the number of interacting sites and α the interaction component. A plot of ln[Cu(II)] versus $\ln(N_i/r-1)$ would yield a straight line if equation 3.9 describes the data. However, substitution of values for N_i from 1 to 16 did not yield a straight line. Since it is unlikely for a protein in a biological system to have more than 16 strong or weak co-operative sites, it can be concluded that the co-operative phase suggested by the Scatchard plot does not apply to our system.

The data is Table 3.1 (see appendix) was now plotted according to Equation 3.5 yielding the double reciprocal plot shown in Fig. 3.2. The plot is expected to yield a straight line as HSA is known to have a single strong binding site for Cu(II) up to a Cu(II)-HSA molar ratio of 1. The plot obtained in Fig. 3.2 is curved and approaches the value of 1 on the 1/r axis, asymptotically.

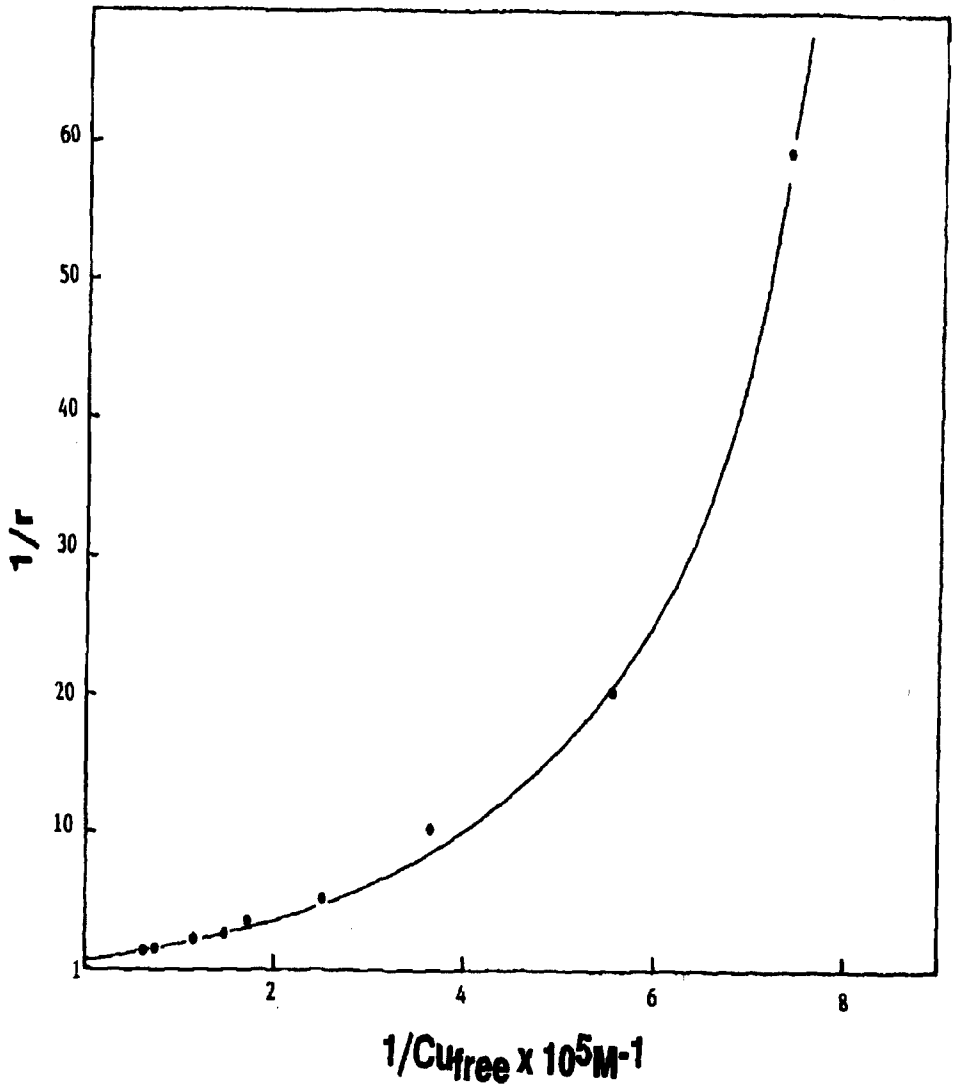


Fig. 3.2 Double reciprocal plot for Cu(II) binding to HSA (EDTA treated) in 0.1M N-Ethylmorpholine-HCl at pH 7.5, 37°C and I=0.15M.

This seems to indicate that the assumption of one mole of Cu(II) binding to one mole of HSA is invalid. The prime question at this stage is whether more than one class of independent sites do exit in our system! The amount of copper bound per mole of HSA was plotted against the Cu(II)-HSA molar ratio and is presented in Fig. 3.3. The plot obtained is strikingly similar to that obtained by Sarkar and Wigfield [63] in their study of copper binding to HSA. It is apparent that there is no abrupt change in the slope of the line, indicating that only one class of independent sites are available for Cu(II) binding to HSA.

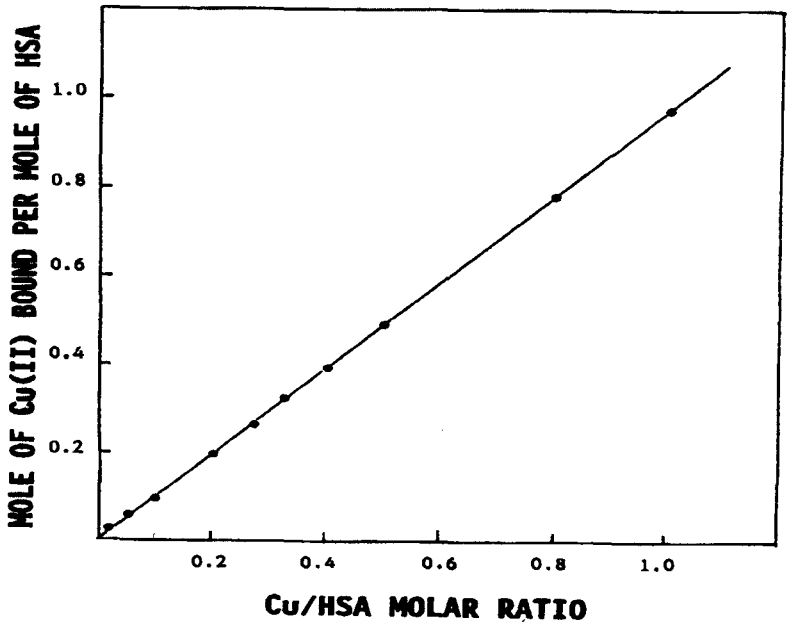


Fig. 3.3 The number of moles of Cu(II) bound per mole of HSA at different Cu(II)/HSA molar ratios.

Alternative analysis of the data where Copper bound to Albumin is examined as a function of unbound copper indicates the presence of a single class of non saturable sites. The paradoxical nature of these results will be discussed further when considering the results of the ultracentrifugation and electrophoresis experiments.

Cu(II) binding studies to other preparations of HSA viz. Chelex-100 treated

HSA(WPBTS) and Chelex-100 treated commercial HSA were carried out using ultrafiltration and equilibrium dialysis. The Scatchard plots for these binding studies are shown in Fig. 3.4. The plots obtained are similar to that obtained for Cu(II) binding to HSA(EDTA treated), in that the r/[Cu(II)] intercepts actually passes through the origin with the initial slope being positive.

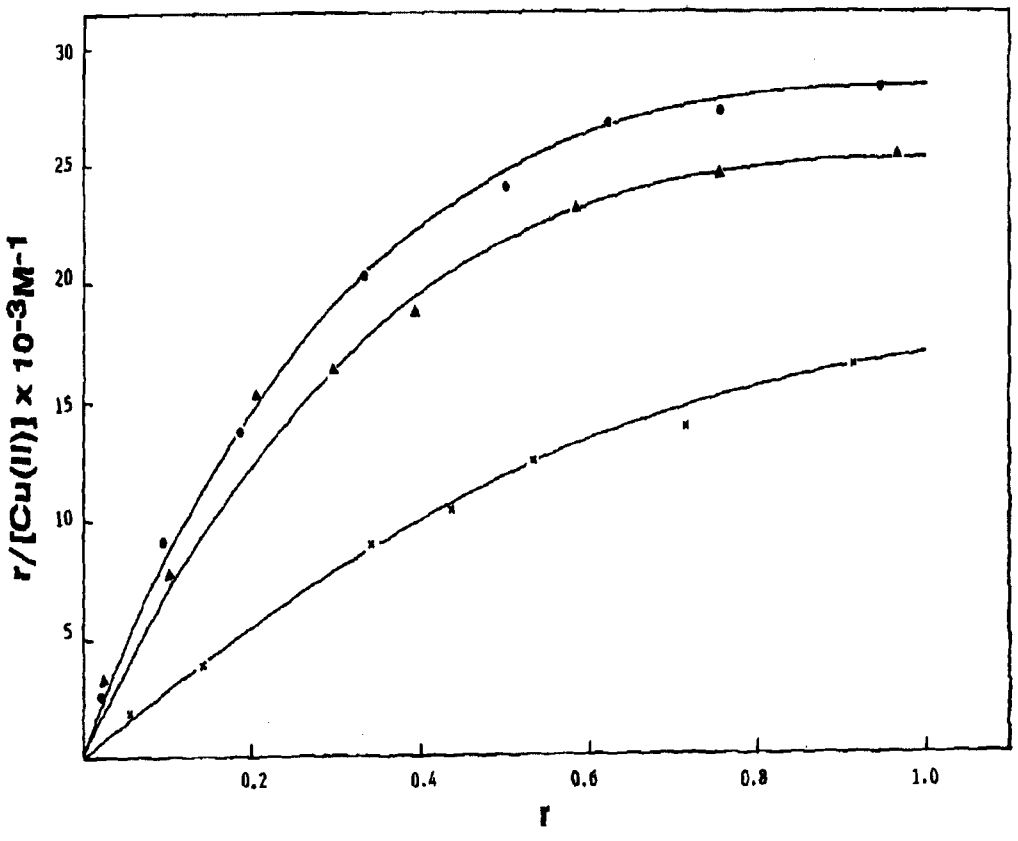


Fig. 3.4 Scatchard Plots showing the binding of Cu(II) to HSA in 0.1M N-ethylmorpholine-HCl at pH 7.5, 37°C and I=0.15M.

▲ Chelex-100 treated commercial HSA : Ultrafiltration
• Chelex-treated commercial HSA : Equilibrium dialysis
× Chelex-100 treated HSA(WPBTS) : Ultrafiltration results

However, a comparison of the amount of Cu bound to HSA shows significant differences for each protein preparation (refer to Tables 3.1, 3.2, 3.3 and 3.4 in the appendix). HSA which contained 1.28μg Cu(II)/g HSA after EDTA treatment exhibited a greater affinity for Cu(II) than HSA treated with Chelex-100 which contained 3.68lg Cu(II)/g HSA after treatment.

HSA (WPBTS) which contained the most amounts of Cu(II), after treatment with Chelex-100 showed the lowest affinity for Cu(II) ions in the binding studies. It can be concluded that the binding of Cu(II) to HSA is inversely related to the amount of Cu(II) contamination present in the protein after treatment. If the amount of Cu(II) contamination is in turn indicative of the amount of polymeric HSA species present, then it can be further suggested that Cu(II) binding to HSA is influenced by the molecular state of HSA.

Figure 3.4 shows good agreement between binding values obtained by equilibrium dialysis and those obtained by ultrafiltration. The slightly lower values of free Cu(II) measured by equilibrium dialysis as compared to ultrafiltration, can be attributed to differences in membrane properties (i.e. YMT and Spectrapor membranes) as well as adsorption of Cu(II) ions to the Spectrapor membranes used in dialysis (Refer to Chapter 2 for membrane binding characteristics).

3.4.3 COPPER(II) BINDING TO BSA

The binding values for Cu(II) binding to BSA (treated with Chelex-100) at 37°C were obtained by equilibrium dialysis and ultrafiltration. (Refer to Tables 3.5 and 3.6 in the Appendix). The Scatchard plots presented in Fig. 3.5 exhibits the same behaviour as the Cu(II)-HSA system in that the r/[Cu(II)] intercepts passes through the origin with the initial slope being positive rather than negative.

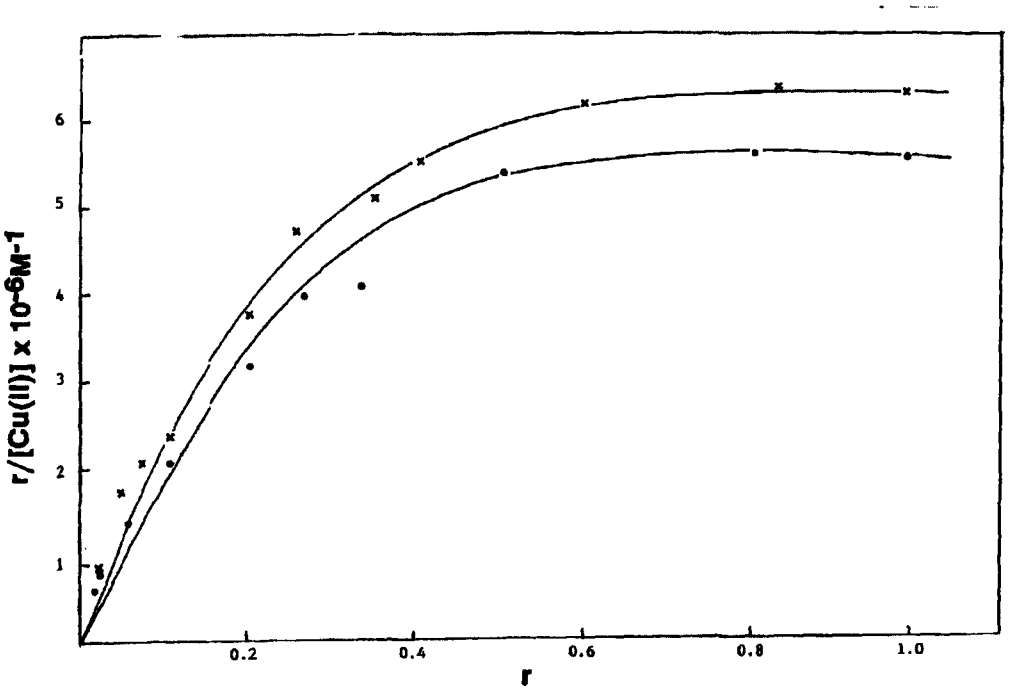


Fig. 3.5 Scatchard Plots showing Cu(II) binding to BSA in 0.1M N-ethylmorpholine-HCl at pH 7.5, 37°C and I=0.15M.
× Equilibrium dialysis results
• Ultrafiltration results

This behaviour is to be expected as the primary binding site for Cu(II) is also to be found at the amino terminal end of the albumin molecule. The amount of Cu(II) bound to BSA was found to be greater than that bound to HSA, for corresponding values of

Cu(II)-protein molar ratios. This indicates that the avidity of BSA for Cu(II) is greater than that of HSA, with BSA forming more stable binary complexes than HSA. However, published values of the formation constants for Cu(II)-HSA and Cu(II)-BSA binary complexes seems to indicate otherwise. For HSA, a formation constant of log K = 16.22 has been reported for the Cu(II)-HSA binary complex [73], as compared to a value of log K = 13.2 for the Cu(II)-BSA binary complex [62]. It is apparent from these values that the amount of Cu(II) bound to HSA is expected to be significantly greater than that bound to BSA. An explanation for our results will be afforded when considering the results of the ultracentrifugation experiments.

Figure 3.5 also shows good agreement between binding values obtained by equilibrium dialysis and those obtained by ultrafiltration . Once again it was found that values obtained for free Cu(II) measured by equilibrium dialysis were slightly lower than those obtained by ultrafiltration.

For a correlation of Cu(II) binding to the different protein preparations in our study, a plot of r versus the free concentration of uncomplexed Cu(II) on a log scale, is presented in Fig. 3.6. All titrations were followed up to a Cu(II)-protein molar ratio of one with no signs of any of the curves levelling off, indicating that Cu(II) sites in the proteins are unsaturated.

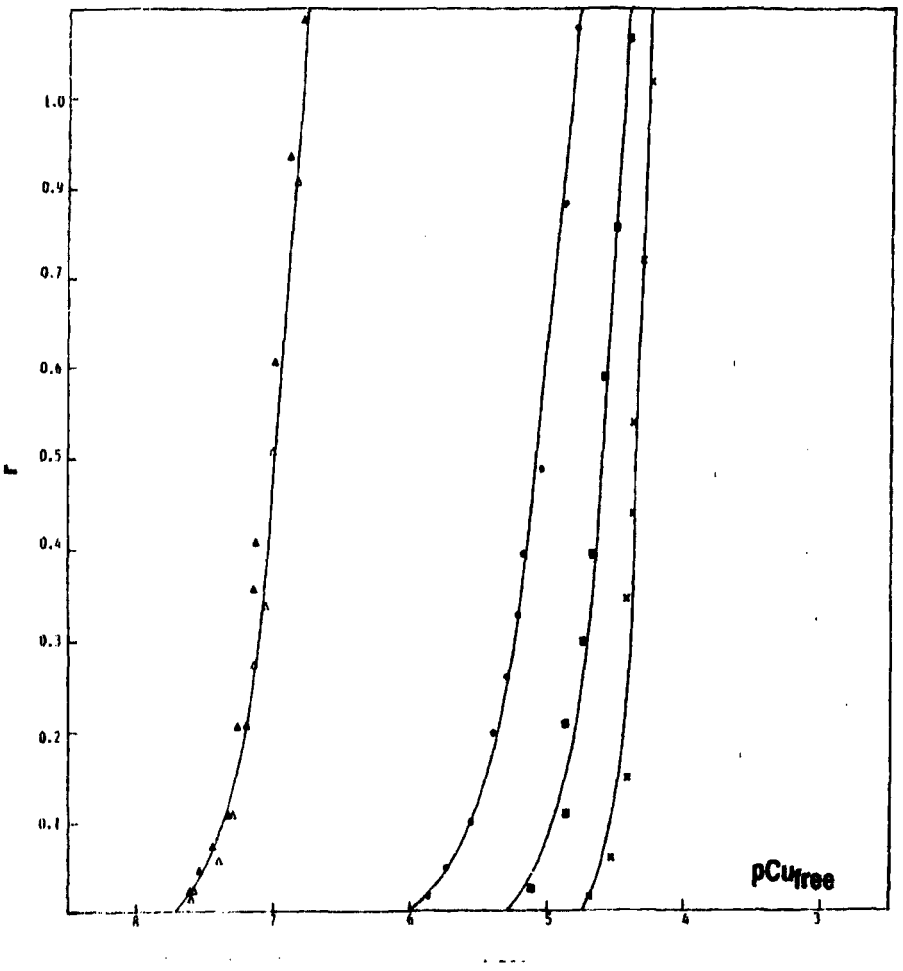


Fig. 3.6 Cu^{2+} binding to HSA and BSA under physiological conditions
× HSA-chelex treated purified (WPBTS) : ultrafiltration
· HSA-chelex-100 purified (commercial) : ultrafiltration
• HSA-EDTA purified : Ultrafiltration
▲ BSA-chelex-100 purified : ultrafiltration
△ BSA-chelex-100 purified : equilibrium dialysis

3.4.4 ULTRACENTRIFUGATION

Solutions with variable Cu(II) and fixed HSA concentrations that were used in the binding studies were subjected to ultracentrifugation experiments. The volume of each fraction separated was measured and the distance sedimented during time t for the various macromolecular species were calculated by using the Fritch equation [68].

$$r - r_m = \alpha S_{20,w} W^2 t \qquad \text{..... 3.10}$$

Where r and rm are the distance from the rotor axis to the macromolecular zone and to the meniscus respectively, W is the rotor speed, t the time and α is the proportionality constant which depends on the kind of macromolecule and the temperature. The ultracentrifugation runs are shown in Fig. 3.7 and the S values calculated for the macromolecular species are presented in Table 3.2.

Table 3.2 S values of Macromolecular Species in Solutions with variable Cu(II) and fixed HSA (0.6mM) concentrations as determined by Ultracentrifugation.

$[Cu(II)]_{total}$ mM	S Value monomer	dimer	trimer	tetramer
0.008	5.02	6.16	7.73	9.6
0.2	5.43	6.68	8.10	-
0.6	5.43	7.10	7.83	-

HSA that was prepared by EDTA treatment is found to contain at least 4 species which correspond to monomers, dimers, trimers and tetramers with S values equal to 5.02, 6.16, 7.73 and 9.6 respectively. It can be seen in Fig.3.7 that these macromolecular species constitute a dissociating system dependant on increasing Cu(II) concentrations, in which tetramers undergo successive depolymerization to form the predominant monomeric species.

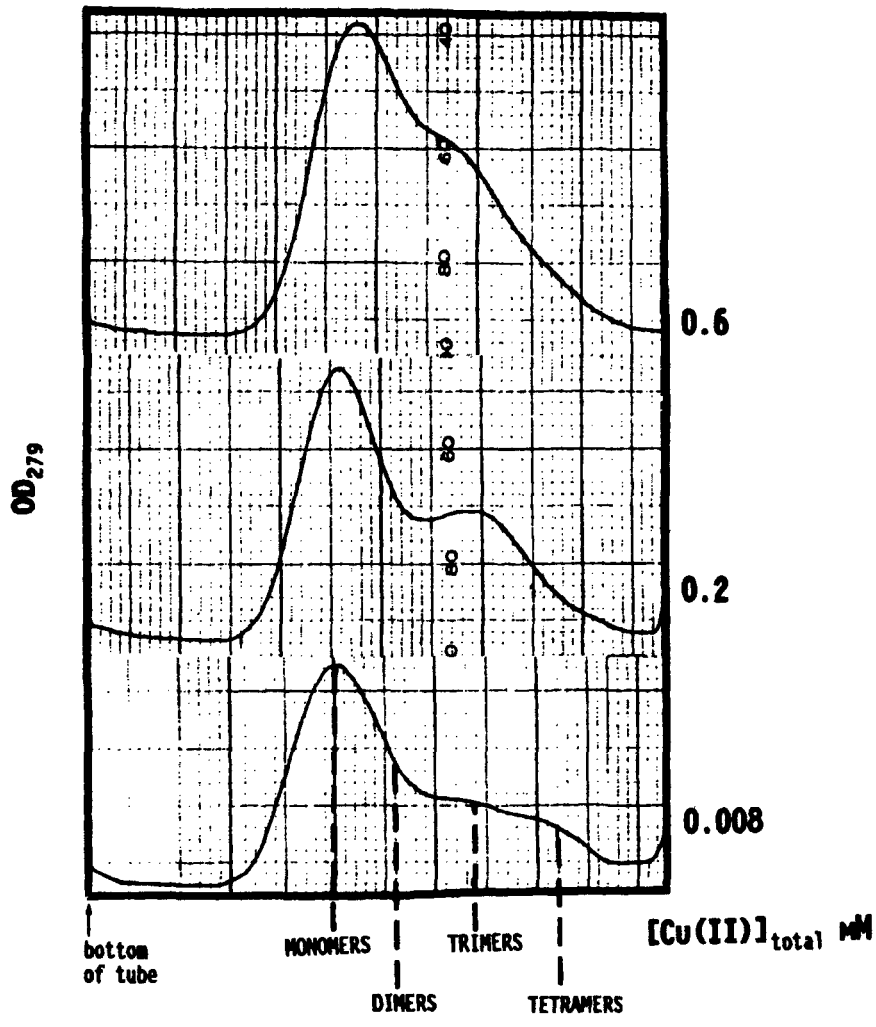


Fig. 3.7 Cu(II) induced polymerization of HSA as studied by zonal sedimentation trough a 5% to 20% gradient.

This now confirms an earlier suggestion, that the nature of the Scatchard plot (Fig 3.1) is due to polymeric species of HSA that are subsequently depolymerized by increasing Cu(II) concentrations, to yield the predominant monomeric species. This results in a situation where more binding sites are available as the titration proceeds. However it must be noted that dimers and trimers are seen to exist even at a Cu (II) concentration of 0.6mM, indicating that the binding of Cu(II) to HSA is still in an unsaturated phase, where all the binding sites are not available.

In an attempt to confirm the above results, ultracentrifugation studies were carried out on solutions containing variable Cu(II) and fixed BSA concentrations, that were used in the Cu(II)-BSA binding study. The ultracentrifugation runs are shown in Fig 3.8 and the S values calculated for the macromolecular species are presented in Table 3.3. BSA that was prepared by Chelex-100 treatment is also found to contain at least 4 species corresponding to monomers, dimers, trimers and tetramers.

Table 3.3 S values of Macromolecular Species in Solutions with variable Cu(II) and fixed BSA (0.6mM) concentrations as determined by Ultracentrifugation.

$[Cu(II)]_{total}$	S Value			
mM	monomer	dimer	trimer	tetramer
0.008	4.96	6.37	7.73	10.03
0.2	5.06	6.03	7.32	-
0.6	5.17	6.68	-	-

It is clearly evident from Fig 3.8 that increasing concentrations of Cu(II) induces depolymerization of molecular aggregates of BSA. A comparison of ultracentrifugation runs in Fig. 3.8 and Fig. 3.7 shows that at a Cu(II) concentration of 0.6mM, the concentration of monomeric species of BSA is significantly greater than that of monomeric HSA. Thus, it can be said that the effect of Cu(II) induced depolymerization for BSA is greater than the corresponding effect in HSA. This in fact explains why the free concentration of uncomplexed Cu(II) for Cu(II) binding to BSA was found to be lower than that of Cu(II) binding to HSA. It is obvious that a situation in which more monomeric species of albumin are present would be one in which more sites are available for binding to Cu(II). It is also evident by comparison that the tetramer concentration in the BSA preparation is greater than that in HSA. If this is due to the larger amount of Cu(II) contamination in BSA (see table 3.1), then it is indeed ironical that increasing Cu(II) concentrations causes depolymerization. It is suggested herein that the Cu(II) present after treatment is buried in a hydrophobic region of the protein aggregate making it inaccessible to the Chelex-100 vein or EDTA..

The notion of Cu(II) forming metal bridged protein complexes (polymers) at the amino terminal end [63] is unacceptable, in light of the fact that increasing Cu(II) concentrations were not found to induce dimers and tetramers, as evident in our results.

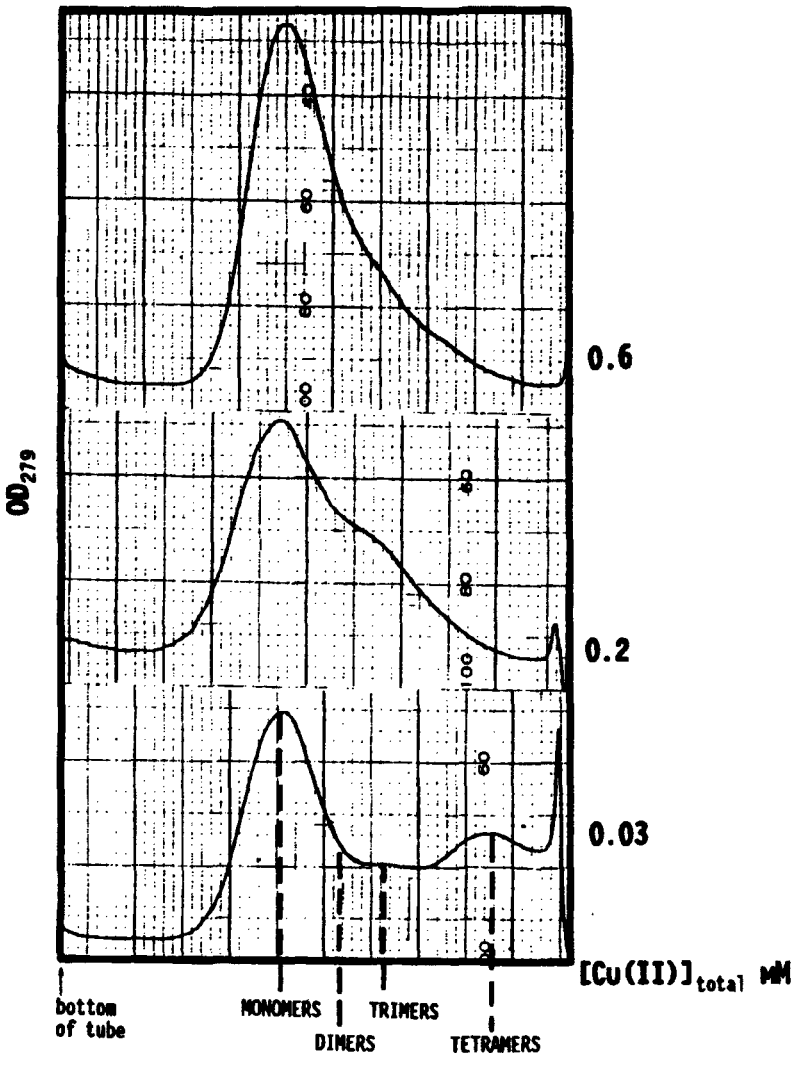


Fig. 3.8 Cu(II) induced depolymerization of BSA as studied by zonal sedimentation through a 5% to 20% sucrose gradient.

3.4.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

Solutions in which variable amount of $CuCl_2$ were added to a fixed concentration of protein (0.6mM in 0.1M N–ethylmorpholine-HCl buffer at physiological conditions were subjected to cross-linking with dimethyl suberimidate followed by 5% polyacrylamide gel electrophoresis). The electrophoresis results for Cu(II) binding to HSA and Cu(II) binding to BSA are presented in Fig. 3.9 and Fig. 3.10, respectively. Dimethyl suberimidate is known to cross-link only polymeric forms of HSA and BSA, leaving monomeric species in their native state [74].

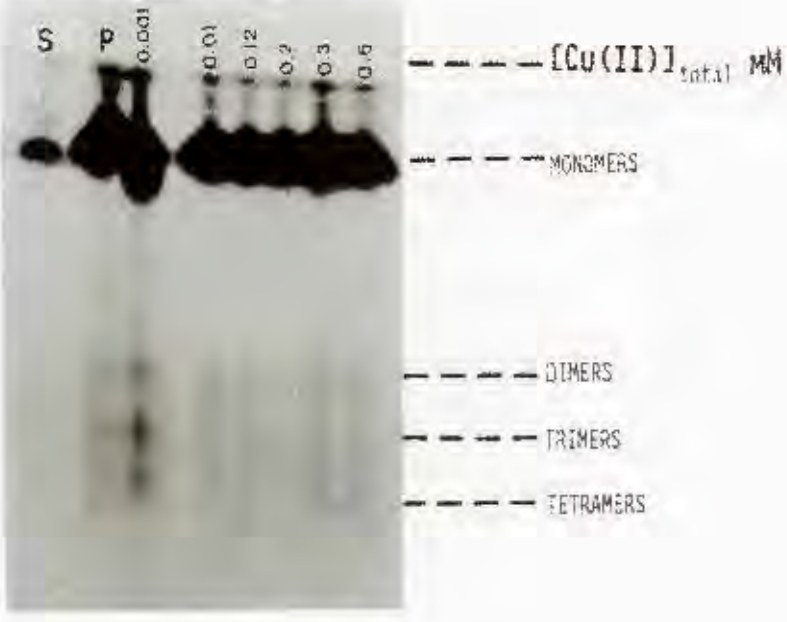


Fig. 3.9 Identification of macromolecular species in solutions of variable Cu(II) and fixed HSA(0.6mM) concentrations by 5% Polyacrylamide gel electrophoresis. S refers to the standard with a molecular weight of 69000gmol^{-1} and P refers to HSA after EDTA treatment.

Figure 3.9 confirms the finding of the ultracentrifugation experiment, in that HSA which was treated with EDTA is found to contain 4 different macromolecular species with monomers being the predominant species. The same is true for BSA that was treated with Chelex-100 (refer to Fig 3.10). The results obtained are of a quantitative nature and due to the unavailability and high cost of appropriate high macromolecular weight standards, the 3 other bands apart from the monomers, are assumed to be dimers, trimers and tetramers.

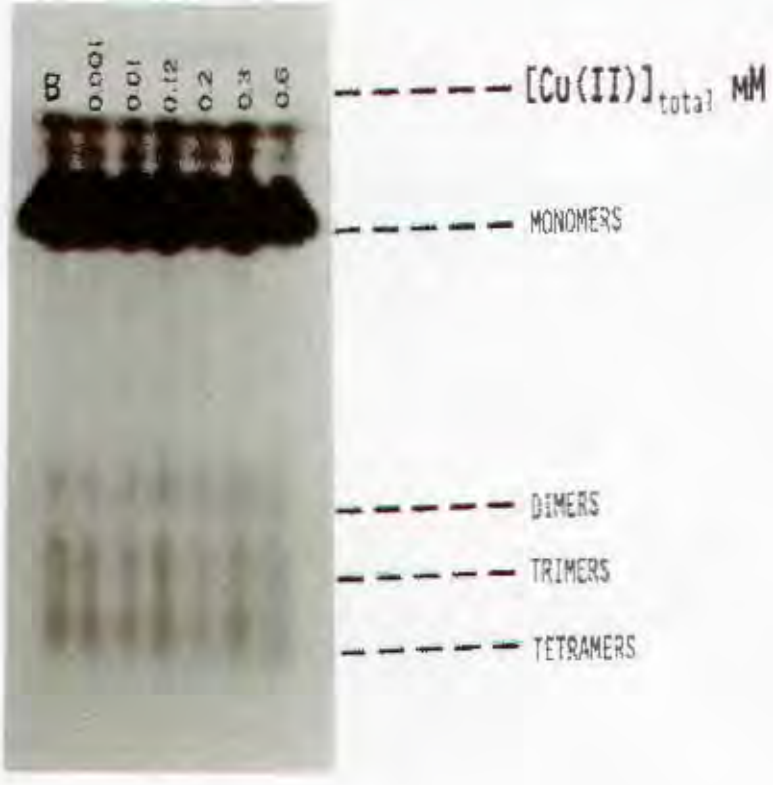


Fig 3.10 Identification of macromolecular species in solution of variable Cu(II) and fixed BSA(0.6mM) concentrations by 5% Polyacrylamide gel electrophoresis. B refers to BSA after Chelex-100 treatment.

It is evident from Fig 3.9 and Fig 3.10 that molecular aggregates of HSA and BSA do exist at high copper concentrations. However, it can be deduced that increasing concentrations of Cu(II) do not induce polymerization of HSA or BSA. In the likelihood of polymerization occurring, one would expect to see a distinct pattern in the banding zone of heavier macromolecular weight species. Therefore the results obtained by electrophoresis are significant and do lend support for the concept of Cu(II) induced depolymerization of HSA and BSA.

3.4.6 INFLUENCE OF IONIC STRENGTH ON THE BINDING OF Cu(II) TO HSA.

Increasing amounts of dry NaCl were added to glass tubes in which the concentrations of total Cu(II) and HSA(EDTA treated) were kept constant at 1.45 x 10^{-5}M and 6 x 10^{-4}M, respectively. The final solutions which had ionic strengths from 0.03 to 0.3 at pH 7.5 were then ultrafiltered at standard conditions, where after the free concentration of the uncomplexed Cu(II) was measured in the ultrafiltrate by atomic absorption. The binding results are presented in Table 3.7 (see appendix) and Fig 3.11 where the free concentration of uncomplexed Cu(II) is plotted against the ionic strength (I).

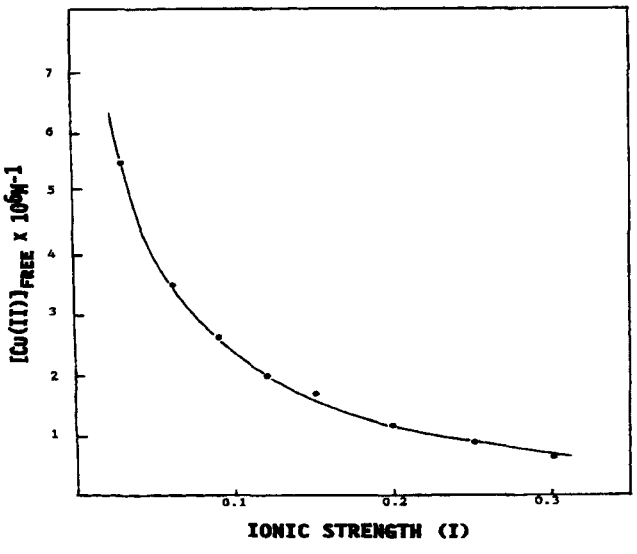


Fig. 3.11 Effect of ionic strength (I) on the amount of ultrafiltrable Copper (II) when the concentrations of total Copper (II) and HSA are 1.45 x 10^{-5}M and 6.0 x 10^{-4}M, respectively, at pH 7.5 and 37°C.

The figure shows a decrease in the amount of ultrafiltrable Cu(II) as the ionic strength increases, indicating that the binding of Cu(II) to HSA is dependant on the ionic strength. This effect is marked in the low ionic strength range and gradually diminishes as the ionic strength rises, and could be due to the interference of a Donnan-like factor

or to possible conformation changes of HSA that are induced by ionic strength.

The former possibility is not very plausible, in view of the fact, that the magnitude of the Donnan-factor is determined by the increase in protein concentration during ultrafiltration [75] which inturn has no effect on the concentration of ultrafiltrable Cu(II) up to half the original volume filtered (see Chapter 2). On the other hand, ionic strength induced conformational change of albumin has been reported [74], and in this case the effect is envisaged as one in which ionic strength induces molecular aggregates of HSA to undergo conformation changes in such a way, that more Cu(II) binding sites are made available. The results obtained shows an increase of approximately 53% in the amount of Cu(II) bound to HSA, as the ionic strength increases from 0.03 to 0.3. The possible influence of ionic strength has apparently received little attention in binding studies, and more work is needed in order to fully elucidate the effect of ionic strength on copper binding to albumin.

3.4.7. COMPETITIVE STUDIES OF THE INTERACTION OF Cu(II) WITH Zn(II), Ca(II) and Mg(II) IN THE BINDING OF HSA.

Calcium, magnesium and zinc ions are found in considerable quantities in blood serum. It is an established fact that the major transport of these ions are in the form of albumin complexes [76]. There is however, much disagreement as to the location and amino acid constitution of the primary albumin metal binding sites for Zn(II), Ca(II) and Mg(II) [77]. This coupled with the fact that the concentrations of these ions are in large excess to that of copper and albumin, prompted us to investigate the possibility of competitive binding by these ions for the primary copper binding site.

Increasing concentrations of $ZnCl_2$ were added to solutions in which the total Cu(II) and HSA concentrations were kept constant at 2.42×10^{-4}M and 6.0×10^{-4}M, respectively, at pH 7.5, 37 °C and ionic strength of 0.15M. The solutions were subjected to ultrafiltration where after the concentration of the free copper was measured in the ultrafiltrate. The results are presented in Table 3.4, which shows that there is no competition by zinc ions for the primary Cu(II) binding sites up to a zinc concentration of 0.3mM, after which the amount of Cu(II) bound to HSA is found to decrease significantly. It appears that zinc ions combine competitively to the same site as Cu(II) on the albumin molecule. However, this is not the case, as zinc ion concentration of about 0.5mM in the present concentration of albumin is known to condense the albumin molecules into polymers [78].

Table 3.4 The effects of Zn(II) on Cu(II) binding to HSA at pH 7.5, 37 °C and ionic strength of 0.15M.

[Zn(II)] total 10^4M	[Cu(II)] free 10^5M	$r = \frac{[Cu(II)]_{Bound}}{[HSA]_{total}}$
0	3.82	0.340
0.5	3.53	0.345
1.0	3.68	0.343
1.5	3.86	0.340
2.0	3.89	0.340
3.0	3.82	0.340
4.0	4.01	0.337
5.0	6.24	0.300
6.0	9.24	0.250

Therefore, the results illustrated in Table 3.4 confirms that zinc ions are unable to compete with copper for the primary Cu(II) binding site on HSA. This is to be expected as zinc complexes in blood serum were found to be less stable than copper complexes (refer to Chapter 2). These findings are supported by Salman *et al* [149], in their study of the affinity of HSA for Zn(II) and Cu(II). The intrinsic stiochiometric constants ($\log_{10}K$) as determined by Saltman *et al*, for Zn(II) and Cu(II) binding to HSA were 7.53 and 11.18 respectively and the results of Zinc-Copper competition clearly indicated that Cu(II) and Zn(II) do not share a binding site on serum albumin. The precise location of the Zinc binding site has yet to be determined. Goumakos *et al* [150] suggested that his site is at an anterior position and not at the accepted N-terminal copper site.

Similar behaviour was observed for calcium and magnesium, in that the amount of ultrafiltrable Cu(II) was found to be constant even when the concentrations of Ca(II) and Mg(II) exceeded the total copper concentration by a factor of 50. Calcium and magnesium are known to be bound with equal strength to the same type and number of binding sites on the albumin molecules [79]. Ca(II) and Mg(II) are assumed to be bound to the carboxylate groups of albumin [80].

The observed behaviour in these experiments validates the results obtained in Chapter 2, where copper complexes in blood serum were found to be more stable than the corresponding complexes of Zn(II), Ca(II) and Mg(II).

3.5 GENERAL DISCUSSION

The preparative history of albumin with its risk of denaturation or impurities has generally been held responsible for the disparate binding results [81]. This is particularly true and applicable to our studies, where several attempts to obtain the dissociation constant for the HSA-Cu(II) binary complex was unsuccessful, while similar studies conducted by Lau and Sarkar [73] yielded a dissociation constant of 6.61×10^{-17}. The significant difference between the two studies is the preparation of HSA. HSA prepared in our study was found to contain polymeric species as well as a significant amount of Cu(II) contamination, while that used by Sarkar and Lau was copper free and totally monomeric in nature. For comparison, the dissociation constant obtained by Sarkar and Lau was used to obtain a computer simulated study for Cu(II) binding to HSA.

Table 3.5 Computer simulated titration of a fixed concentration of HSA (0.6mM) with variable Cu(II) concentrations at pH 7.5, 37 ^{0}C and I = 0.15M, using the ESTA 1 computer programme.

$[Cu(II)]_{total}$	$[CU(II)]_{free}$	r
1.2×10^{-4}	2.9×10^{-15}	0.2
3.0×10^{-4}	1.17×10^{-14}	0.5
4.8×10^{-4}	4.68×10^{-14}	0.8
6.0×10^{-4}	2.65×10^{-9}	1.0

The results in Table 3.5 shows that the concentrations of free Cu(II) obtained by Sarkar and Lau are very low in comparison to those obtained in our studies (see Table 2.1 in the appendix). This is due to the fact that all the sites for Cu(II) binding on HSA were not available, as polymeric species present, prevents the exposure of reactive sites

contained therein. Nevertheless, our experiments do support the fact that Cu(II) binding to HSA involves a single class of independent sites on the albumin molecule up to an albumin-Cu(II) molar ratio of one. It was observed that increasing concentrations of Cu(II) induced successive depolymerization of molecular aggregates to yield monomeric species. At a final Cu(II) concentration of 0.6mM, depolymerization was found to be incomplete and hence the binding of Cu(II) to HSA was found to be incomplete.

On the other hand previous studies by Osterberg *et al* [82], indicate that Cu(II) induces polymerization of HSA when the Cu(II) concentration is extended beyond 0.2mM. However, a careful examination of their data shows that, induction of Cu(II)-albumin dimers is reported to occur at albumin-Cu(II) molar ratio of 1:8 while only monomeric species are predicted to exist at an albumin-Cu(II) ratio of 1, where the albumin concentration was 0.22M. Since the concentration of HSA is found to remain constant (0.6mM), even during the presence of pathological disorders [83], a Cu(II) concentration corresponding to an albumin ratio of 1 would not be expected to induce Cu(II) dimers of albumin. However, such a Cu(II) concentration would be more than 0.2mM, clearly indicating a fundamental contradiction in the findings of Osterberg *et al* [82]. Furthermore, since the induction of dimers is reported to occur at a HSA-Cu(II) ratio of 1:8, the corresponding value of Cu(II) needed to induce albumin dimers would be 4.82mM. An experiment carried out in this laboratory shows that, at this concentration of Cu(II), the albumin was found to precipitate out of solution. Cu(II) concentrations more than 0.2mM are known to exist in the pathological range, for example in Wilson's disease. To date, there has been no report to indicate the involvement of albumin dimers in the pathogenesis of Wilson's disease.

The results obtained in our studies are well substantiated by the fact that similar behaviour Cu(II) depolymerization) was observed for BSA. This is to be expected as human and bovine albumins have rather similar compositions and properties [84, 85]. Saltman *et al* [149] undertook extensive studies of Zn(II) and Cu(II) interactions with serum albumin with the similar intention of using the metal-protein binding constants in their biofluid models to predict the precise role of albumin in metal metabolism.

They also observed the disparity in the reported values for binding constants of Zn(II) and Cu(II) to serum albumin from the abundance of physicochemical data that is available. They contended that some of the ambiguities and disparities of the metal-protein studies were as a consequence of the techniques by which metals are presented as simple salts to the protein, in which case the metals hydrolyse to form metal-hydroxy and -oxy polymers resulting in a multitude of electrostatic interactions between the aquametal iron and the surface of the protein, nonspecific binding to the protein or be kinetically inert so that no reaction takes place with the protein.

They consequently developed a strategy in which metals were presented to the protein in the form of well-characterised low molecular weight chelates to prevent metal hydrolysis and nonspecific binding. By their own assertions, they were not able to eradicate nonspecific interactions between metal and protein and could only describe these interactions as being minimised. Further research is needed in the experimental strategy for Saltman and co-workers so as to place confidence in the measured binding constants obtained by this method.

The control of critical variables such as pH, temperature and ionic strength are imperative in binding studies. The ionic strength was found to have a marked influence on Cu(II) binding to HSA, i.e. increased protein binding was observed when the ionic strength was increased from 0.03 to 0.3M. It is suggested that this effect is due to ionic strength induced conformational changes of the albumin aggregates in such a way that more reactive sites become available for Cu(II) binding. Results from competitive studies found that Zn(II), Ca(II) and Mg(II) were unable to compete with Cu(II) for the primary Cu(II) binding site on the albumin molecule, even when the concentrations of these ions greatly exceeded that of Cu(II). Therefore, the result obtained herein, confirms the order of complexing strength of metal ions to serum proteins in Chapter 2.

Finally, it can be stated that in order to fully understand the structure and function of metalloproteins, it is vital that an acceptable strategy be adopted that will be able to calculate the relevant binding constants with confidence for use in computer simulated studies. This would require the convergence of different schools of thought as well as different methodologies for the appropriate compartments of the biofluid model.

CHAPTER FOUR

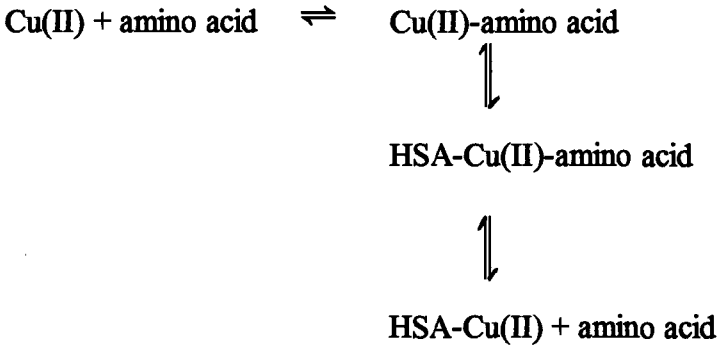
TERNARY COORDINATION COMPLEXES BETWEEN HUMAN SERUM ALBUMIN, COPPER (II) AND AMINO ACIDS

4.1 INTRODUCTION

The importance of ternary coordination complexes has long been recognized in biological chemistry and many investigators have contributed significantly in this area. Klotz and Ming [86] and Gurd [87] has demonstrated that metal ions may mediate the binding of small molecules to proteins. Malstrom [38] later studied the ternary complex formation between enolase, metal ion and D_L-2-phosphoglycerate. Since the original proposal of a bridge structure between enzyme, metal, and substrate by Helierman and Stock [89], this hypothesis has been put forward to explain the mechanism of action of many enzymes [90,92]. Recently, important contributions have been made by Mudvan and Cohn [93] to our understanding of the formation of enzyme-metal-substrate bridge structure.

Ternary complexes has also been implicated in the transport of trace metals [73]. Evidence for the existence of albumin-Cu(II)-L-histidine and albumin-Cu(II)-L-threonine ternary complexes have been reported [63]. Lau and Sarkar have pointed out that a ternary Cu(II) complex formed by histidine and human albumin may play an important biological role. It is already known that some of the Cu(II) in human blood plasma are bound to albumin and that this is considered to be the main transport form of Cu(II) in the blood. It is also known that a fraction of Cu(II) is bound to amino acid in serum, and that this fraction is in equilibrium with the fraction bound to albumin. In view of these findings, Lau and Sarkar [73], suggested that the equilibrium between HSA-Cu(II) and Cu(II)-amino acid complex is mediated through a ternary complex, HSA-Cu(II)-amino acid.

Accordingly the following equilibria was postulated to describe the situation:



The above mechanism is considered to play an importance role in the exchange of Cu(II) between a macromolecule and a low molecular weight substance which in turn can be readily transported across the biological membrane. Although L-histidine is the major Cu(II)-binding amino acid in serum [53], other amino acids could also play a similar role in formation of an intermediary ternary complex. In the present study a detailed investigation is made of Cu(II) ternary complexes with HSA and amino acids, as well as the binding of such amino acids to HSA at physiological conditions of pH and temperature.

The above equilibria was also postulated by Goa *et al* [151] in their kinetic studies of the mobilisation of Cu (II) from HSA with chelating agents. Similar to Sarkar [152], Gao *et al* suggested an Sn_2 mechanism in which the reaction may be expressed by the following scheme:

$$\text{HSA-M} + \text{L} \underset{K_{-1}}{\overset{K_{HML}.\ K_1}{\rightleftharpoons}} \text{HSA-M-L} \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} \text{HSA} + \text{M-L} \qquad \text{....4.1}$$

Where M refers to Cu(II), L the chelating ligand, and K_1, K_{-1} and K_{-2} represent the corresponding rate constants, while K_{HML} represents the stability constant of the ternary complex.

4.2 POSSIBLE ROLE OF AMINO ACIDS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

The serum and joint fluid of patients with rheumatoid arthritis characteristically, but not always contains antibodies called rheumatoid factors. These antibodies are specifically directed against immunoglobulins IgG. IgG is one of the major antibodies in human blood plasma and consists of two identical heavy chains as well as 2 identical light chains. These chains are held together by disulphide bonds. It is thought that in rheumatoid arthritis, IgG becomes inflammatory by reacting with an as yet unidentified antigen, after which it undergoes a non immunological change [95]. The proposed mechanism is a sulfhydryl-disulphide interchange reaction, in which case the structure of IgG is altered to yield a pseudoimmune complex. The human joint responds to this pseudoimmune complex as if it were true immune complex. Inflammation could then develop by the same mechanisms as are generally ascribed to true immune complex.

It is believed that there is a physiologically-meaningful amount of an endogenous sulfhydryl-blocking reaction in normal serum and joint fluid and that this reagent may be a histidine-cystine-copper complex [96]. However, patients with rheumatoid arthritis have been reported to have a low serum histidine concentration as well as a low joint fluid histidine concentration [97,99]. A subnormal histidine concentration should, by mass action, lower the concentration of the histidine-cystine-copper complex [96]. Thus, the low serum histidine concentration of rheumatoid arthritis is a possible cause of low sulphydryl-blocking reagent levels in the joint fluid of these patients. Histidine is also an unusually strong ligand of copper [100, 101] and zinc [102]. Thus the low serum histidine concentration in rheumatoid arthritis may also be relevant to other hypothesis concerning these metals and inflammation. It has also been reported that patients with rheumatoid arthritis have been shown to have an increase in the total plasma tryptophan, due to an increase in the albumin bound fraction [103].

There is a corresponding fall in the free plasma tryptophan and these changes were

found to correlate with clinical disease activity [104, 105]. It has been postulated that tryptophan may have anti-inflammatory activities and that the effect of NSAIDs is to displace tryptophan from its protein binding sites, raising free plasma levels [106]. All the NSAIDs, as well as antimalarials D-penicillamine, gold salts and alcolofenac, displace protein bound tryptophan and this displacement is dose-dependant [104, 106]. It seems that this aspect of tryptophan binding can be used as a measure to distinguish between different types of anti-inflammatory drugs.

4.3 EXPERIMENTAL

4.3.1. MATERIALS

Crystalline HSA obtained from Miles Laboratories, was subjected to EDTA treatment before use (refer to Chapter 3). L-Tryptophan (Lot. No. T9-020-4) and L-threonine (Lot. No. T3-420-7) were obtained from Aldrich Chemical Company. L-Histidine (Lot. No. 64C-5016) was obtained from Sigma Chemical Company. All solvents and reagents used in HPLC studies were of high grade purity.

4.3.2. ULTRAFILTRATION

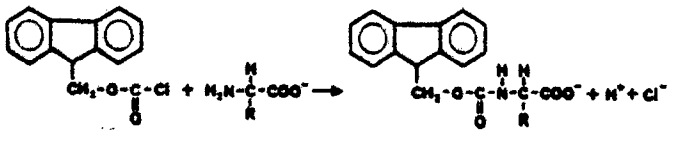
In the study of amino acids binding to HSA, varying concentrations of amino acid were added to solutions of a fixed protein concentration of 6 X 10^{-4}M in 0.1M N-etylmorpholine-HCl buffer at pH 7.5, 37°C and ionic strength of 0.15M. The solutions were then subjected to ultrafiltration under standard conditions, whereafter the ultrafiltrate was subjected to HPLC analysis for the determination of the free amino acid concentrations. For the ternary binding system, ultrafiltration was carried out on solutions where the total Cu(II) and HSA concentrations were kept constant at 6.034 x 10^{-4} and 6 x 10^{-4}M, respectively while the amino acid concentrations were varied. All these binding studies were carried out in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37 °C and ionic strength 0.15M. Ultrafiltration was performed under standard conditions and the concentration of low molecular weight Cu(II) complexes in the ultrafiltrate were determined by atomic absorption. The concentration of free amino acid in the ultrafiltrate was determined by HPLC.

4.3.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The concentrations of free amino acid concentrations were determined with 9-fluorenylmethyl chloroformate (FMOC-Cl) and reversed phase HPLC. The procedure used was based on the method developed by Einarsson et al [107]. The chromatograph consisted of two Beckman 112 solvent delivery modules linked to a Beckman 340 organizer that was equipped with a Valco injector. The recorder used was a Beckman 165 variable wavelength detector. A 250 x 4.6mm ID, 5 µl octadecylsilane column was used. The above-mentioned apparatus was linked to a microprocessor-controlled interface along with an Apple IIe computer.

The amino acid in the ultrafiltrate was derivatised by using the FMOC-Cl reagent. To 0.4ml of the sample were added 0.1ml borate buffer (pH7.7) and 0.5ml FMOC-Cl.

The reaction of FMOC-Cl with amino acids proceeds as follows:



The derivatization was complete after 30 sec, after which the sample was immediately analyzed as to minimize the formation of hydrolysis products. The separation was carried out by gradient elution. The eluent was varied linearly from acetonitrile-methanol-acetic acid buffer (10:40:50) to acetonitrite acetic acid buffer (50:50) over 7 minutes. The gradient was started approximately 6 minutes after injection, with a flow rate of 2ml/min. Thus, in reverse phase chromatography, the gradient starts from a very polar solvent and ends up with an almost organic phase. This change in solvent polarity assists in separation. Fig. 4.1 shows the gradient profile.

The amino acids were quantified by peak area measurements which were recorded by the highly sophisticated Apple system.

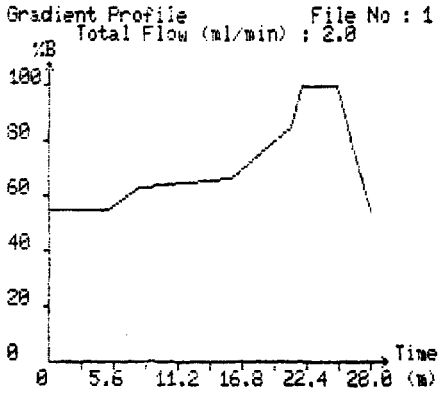


Fig. 4.1: Gradient Profile

4.3.4 COMPUTATION

The data was analyzed using the ESTA suite of computer program [108] on a UNIVAC 1108 at U.C.T. Binary or ternary association constants were varied iteratively and the simulated species concentrations compared with the experimental results.

4.4 RESULTS AND DISCUSSION

4.4.1 BINDING OF AMINO ACIDS TO HSA

Fig. 4.2 and Table 4.1 (see Appendix) show the binding of L-tryptophan to HSA in 0.IM N-ethylymorpholine-HCl at pH 7.5, 37°C and ionic strength of 0.15M. The experimental points were best fitted by a binding constant of 1.12×10^4 and a value of n, and the number of binding sites on the albumin molecule equal to 0.83.

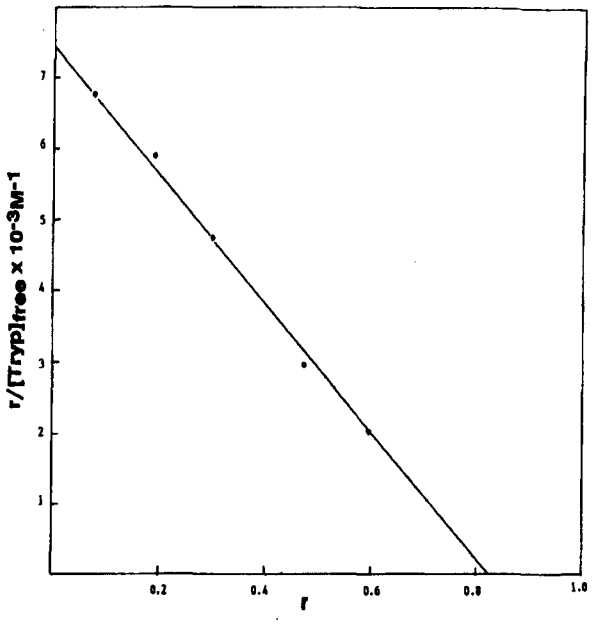


Fig. 4.2 Scatchard Plot for the binding of L-Tryptophan to HSA in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M

Since this was a protein preparation of high purity, the value of n, indicates that less than one site per albumin molecule was available for binding. It is suggested that there is a maximum of one site on the albumin binding, but that this one site was partially blocked. Similar results were obtained by McMenamy an Oncley in their study of L-tryptophan binding to HSA [109].

It is suggested that this blockage is due to aggregate forms of HSA inherent in the protein preparations (Refer to Chapter 3).

The binding of L-tryptophan to albumin was unchanged when cupric ions were added, at a molar concentration equivalent to that of HSA. This indicates that L-tryptophan and Cu(II) are not bound to the same site, such as the N-terminal end of the protein. The binding constant obtained (1.12×10^4) is in good agreement with the constant of 0.62×10^4 obtained for the binding of L-tryptophan in human plasma at pH 7.4, 37 ^{0}C and ionic strength of 0.1M [110]. The similarity of these binding constants implies that L-tryptophan in blood plasma is bound to serum albumin and that other plasma proteins cause no appreciable interference with tryptophan binding. In similar binding studies, L-histidine and L-threonine were found not to bind to HSA at physiological conditions.

4.2.2. INTERACTION OF Cu(II) AND HSA WITH AMINO ACID IN A TERNARY SYSTEM

Fig. 4.3 shows that the affinity of L-His for Cu(II) is high enough to compete with the first copper binding site on albumin. This is more evident when one considers that as the L-His/HSA ratio rises as the amount of Cu(II) bound per mole of protein (r) decreases significantly from 0.93 to 0.34 (refer to Fig. 4.4) The same effect was observed for L-threonine and L-tryptophan, except that the amount of low molecular weight Cu(II) mobilized by these amino acids is less than that of L-His. This is indicative of the fact that the affinity of these amino acids for Cu(II) is less than that of L-His.

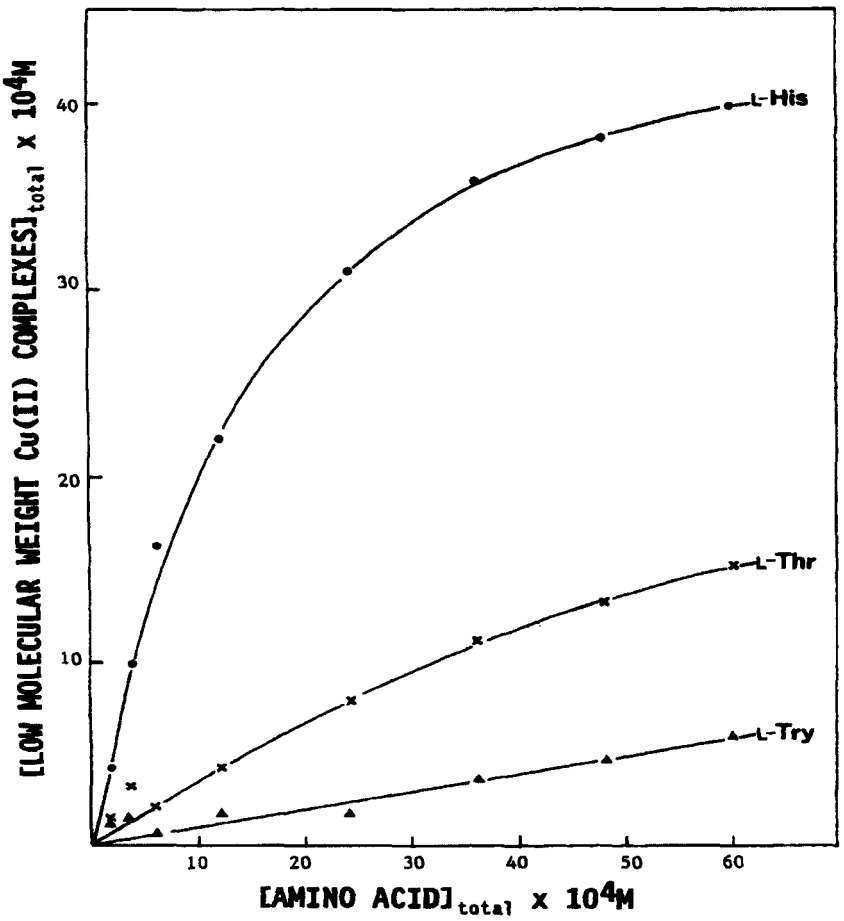


Fig. 4.3 Ultrafiltration studies of the ternary binding systems in which the total Cu(II) and HSA concentrations are kept constant at 6.034 x 10^{-4}M and 6.0 x 10^{-4}M respectively, while the amino acid concentration was varied.

ultrafiltrable Cu(II) in the form of low molecular weight complexes increases proportionally to the amount of amino acid added. The results obtained in our experiments shows a saturation phase, in which the amount of ultrafiltrable Cu(II) tends to level off.

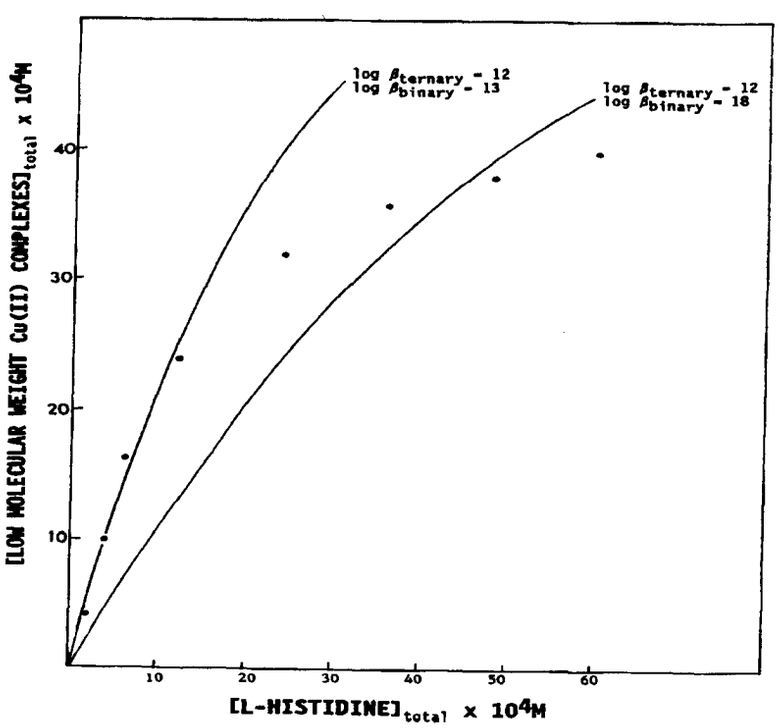


Fig. 4.5 A comparison of experimental data(•) for the HSA-Cu(II)-L-His system with computer simulated data obtained by varying the binary and ternary dissociation constants in the ESTA 1 program. [ternary= HSA-Cu(II)-L-His; binary = HSA-Cu(II)]

In order to determine whether the concentration of free amino acids obtained by HPLC analysis, represented free amino acids (including protonated species) or amino acid species which included low molecular weight complexes with Cu(II), titrations were carried out in which fixed amounts of amino acids were titrated against varying amounts of Cu(II). One such titration is represented in Fig. 4.6 in which varying amounts of $CuCl_2$ were titrated against a fixed concentration of L-histidine (40 x 10^{-4}M)

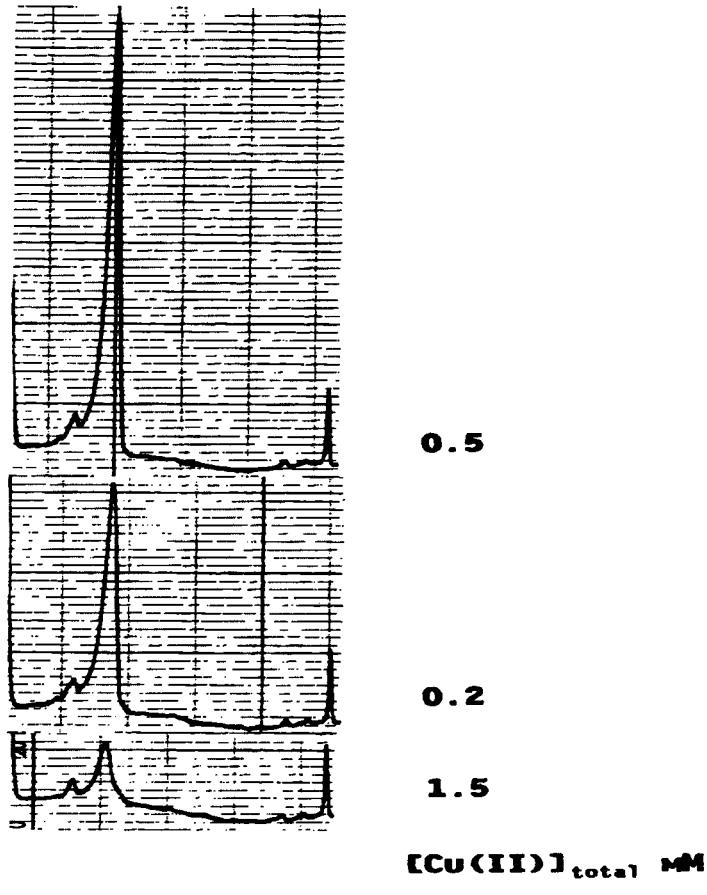


Fig. 4.6 Detection of L-His by HPLC in solutions where varying amounts of $CuCl_2$ were added to a fixed concentration of L-His (40 x 10^{-4}M) at pH 7.5 and I=0.15M

It can be seen that decreasing amounts of L-histidine are detected as the $CuCl_2$ concentration in solution is increased. This indicates that the derivatization agent, FMOC-CI, reacts only with free histidine (including protonated species) and is unable to displace the Cu(II) that is bound to the imidazole groups of L-histidine in a square-planar structure. Similar results were obtained for L-threonine and L-tryptophan. It can be concluded that the concentration of amino acid determined, represents the free amino acid determined by HPLC analysis total low molecular weight amino acid fractions. Fig. 4.7 shows the concentration of free amino acid in the ultrafiltrate as amount of amino acid is added to solutions in which the Cu(II) concentrations are kept constant (refer to Tables 4.2, 4.3 and 4.4 in the Appendix) Fig. 4.7 confirms the results in Fig.4.4, where the amount of Cu(II) bound per mole of protein is greater for L-histidine followed by L-threonine and L-tryptophan.

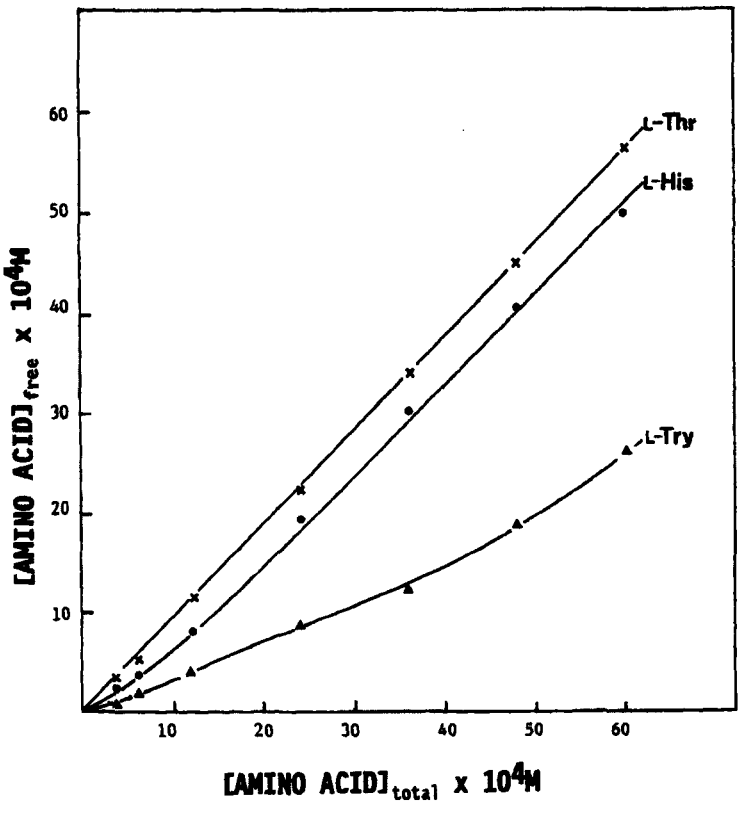


Fig. 4.7 HPLC measurements of the concentrations of free amino acid in ternary systems consisting of Cu(II), HSA and amino acid.

L-Tryptophan is different from L-histidine and L-threonine in that L-tryptophan binds to HSA. It is suggested that the low values of L-tryptophan are due to both strong and weak binding sites that are present on the albumin moiety. An examination of Table 4.2 (See Appendix) reveals that an average of 8% of the total histidine that was added to the system is unaccounted for. Similarly in Table 4.3 (See Appendix) an average of 4.5% of the total threonine is unaccounted for. Since it is unlikely that these amino acids will bind to HSA alone, it can be assumed that the remaining amount of amino acids that are unaccounted for could be bound in a HSA-Cu(II)-amino acid ternary complex.

If the above-mentioned assumption is valid, we can now calculate the species distribution from the presently available experimental data. Since the amount of albumin under normal physiological conditions is in excess of the amount of copper and amino acids found in blood plasma, only the first equivalents of these substances binding to HSA are significant. The species distribution for each ternary system studied is presented in Table 4.2. In the case of the L-histidine ternary system at physiological conditions, the predominant form of Cu(II) is in the form of the HSA-Cu(II) binary complexes in contrast to the expectations of Sarkar and Lau [73] who pointed out the predominant formation of HSA-Cu(II)-L-His complex from their solution equilibria.

The results obtained in our studies are substantiated by the fact that similar results are obtained for the L-threonine and L-tryptophan ternary systems. The results in Table 4.2 also confirms an earlier finding that L-His is the major Cu(II)-binding amino acid in blood serum [53]. It can be further suggested that the major fraction of low molecular weight Cu(II) complexes that are rapidly exchanged with tissue copper is in the form of Cu(II)-L-His_2 complexes.

Table 4.2 Calculation of the concentration of Cu(II) binding species in a 1:1 amino acid: HSA-Cu(II) system at pH 7.5, 37^{0}C and ionic strength of 0.15M

TERNARY SYSTEM	SPECIES	% DISTRIBUTION OF Cu(II)
L-His	HSA-Cu(II)	63.9
	Cu(II)-L-His_2	27
	HSA-Cu(II)-L-His	9.3
L-Thr	HSA-Cu(II)	91.7
	Cu(II)-L-Thr_2	3.5
	HSA-Cu(II)-L-Thr	4.8
L-Try	HSA-Cu(II)	95.3
	Cu(II)-L-Try_2	1
	HSA-Cu(II)-L-Try	3.7

4.5 GENERAL DISCUSSION

L-Tryptophan has been observed to bind to serum albumin. Comparisons with other binding studies [109, 110] show that serum albumin is the only plasma protein which binds L-tryptophan appreciably. L-Tryptophan is bound to HSA predominantly at one site at physiological conditions. In view of the fact that tryptophan has been suggested to have anti-inflammatory activities, the binding and transport of tryptophan in blood serum seem to have important clinical implications in patients with rheumatoid arthritis. On the basis of our experimental results, the major form of tryptophan transport is seen to be in the form of the HSA-L-tryptophan binary complex from which L-tryptophan is most likely to be exchanged with tissue tryptophan. Hence, the albumin bound fraction is considered to be the physiologically important transport form of tryptophan.

The existing mechanism for the transport of Cu(II) in blood serum postulates that the transport of Cu(II) is mediated by ternary complexes in the form of HSA-Cu(II)- amino acid [73]. The majority of work in this area has been performed by Sarkar and Co-workers. It is evident that there are apparent contradictions in their studies concerning the albumin-Cu(II)-amino acid ternary systems. In independent experiments Sarkar and Wigfield found that significant amounts of L-histidine and L-threonine were bound to HSA when copper was added in the form of Cu(II)-amino acid complexes [63]. It is highly unlikely that the attachment of Cu(II) to the first binding site changes the structure of HSA in such a way as to open up an entirely new site for a free amino acid to bind to form the ternary complex. In a later study Lau and Sarkar [73] suggested that in the ternary complex, histidine binds the Cu(II) ion in a tridentate chelate and that the fourth position may be occupied by the amino group of the albumin. This suggestion is in contradiction, by their own findings, in that the number of protons set free at the complex formation did not indicate that a peptide bond nitrogen is involved. On the basis of our results, it seems unlikely that the transport of Cu(II) is mediated by ternary complexes in the form of HSA-Cu(II)-amino acid.

From the above-mentioned facts, it is now clear that the evidence led by Sarkar and co-workers for the existence of ternary complexes, is questionable. Furthermore, an examination of their experimental data shows that an appreciable amount of ternary complex was formed when the total Cu(II) ion concentration was 1.2×10^{-4}M. Under physiological conditions the concentration of Cu(II) in human blood plasma is not high enough to allow the formation of an appreciable amount of ternary complex. It is indeed strange that Sarkar and Wigfield [53] in an earlier study, reported the value of $K_{HSA\text{-}Cu(II)\text{-}His}$ to be 2.3×10^{3}, while the value obtained in a later study [73] is reported to be 7.5×10^{21} and that of the HSA-Cu(II) binary complex was found to yield a formation constant of 1.51×10^{16}. If one considers the first value (2.3×10^{3}) to be true then the mechanism for Cu(II) transport is to be seen in a different light. If the second value of the constant (10^{21}) is true, the ternary complex should be the major species in an Albumin-Cu(II)-histidine system. This is unlikely as other studies [61 &111] have reported the presence of the ternary complex as a minor component of the metal concentrations in the experiments.

Saltman and Masouka [153] make similar observations and noted that the analysis of the spectroscopic data of Lau and Sarkar is complicated by the fact that all complexes contribute to the observed spectra, in which case the addition of free histidine to a Cu(II)-albumin system will necessarily add a Cu(II)-His contribution to the spectra. In this complex system it is difficult to separate the individual components and to know with confidence, if any are due to a specific ternary complex. Saltman and Masouka concluded that while the data of Lau and Sarkar suggested the presence of a ternary complex, they do not by themselves unambiguously demonstrate its existence.

Hence, it follows that the mechanism suggested by Sarkar and Lau [73] for the transport of Cu(II) is contradictory and contentious. Further support for the above contention comes from the work conducted by Sakurai and Nakahara [111]. Attempts to disclose the mechanism of copper transport in blood were made by employing glyclglycl-L-histidine (GlyHis) as a model for the primary copper binding site of serum albumin.

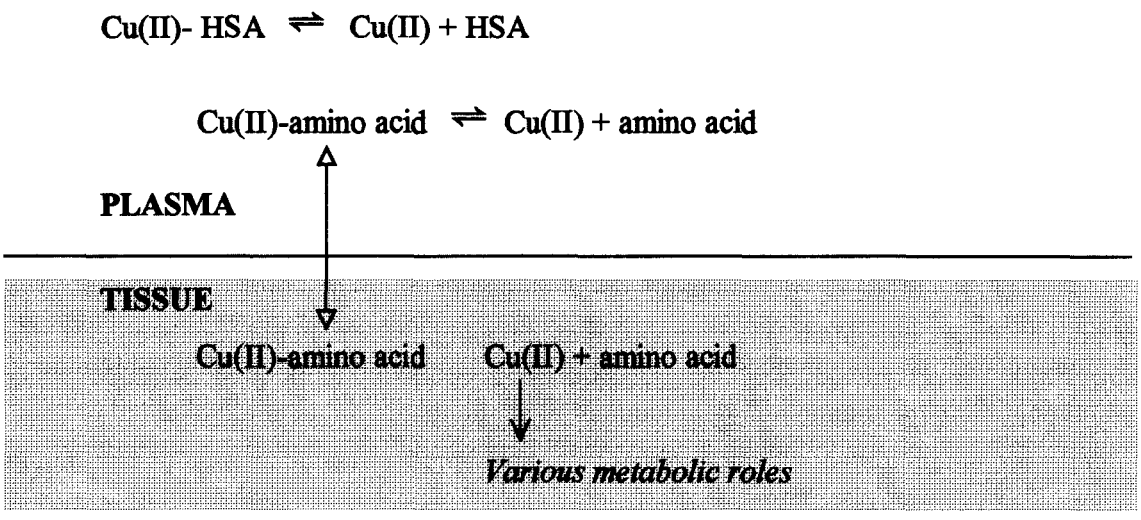
Previous studies using this model included the work done by Kruck and Sarkar [112]. It was clearly evident from these studies that the understanding of the solution equilibrium of the binary Cu(II)-GlyGlyHis was in confusion and that of the equilibrium of the ternary Cu(II)-GlyGlyHis-L-His, was even more so.

Sakurai and Nakahara, showed in their potentiometric study of the Cu(II)-GlyGlyHis-L-His system, that the Cu(II)-H_2-GlyGlyHis species were dominant (56%) at physiological pH(7.4) while the ternary species formed only 6% of the total species present. These results contradicted the display by Kruck and Sarkar [112] who suggested the predominant formation of the ternary species Cu(II)-H_{-1}-GlyGlyHis-L-His and Cu(II)-H_2-GlyGlyHis-L-His over neutral to alkaline pH regions. However, Sakurai and Nakahara were able to substantiate their results by CD and ESR spectral behavior of the ternary system. The results of Sakarai and Nakahara are in excellent agreement with our experimental results (refer to Table 4.2).

In an attempt to extract some support for the case of the ternary complex, the burden of proof or explanation must rest on the findings of Gao *et al* [151], who have also postulated the transport process to be mediated through ternary complex formation. In their spectrophotometric studies, no new peak emerged in all studies and the authors could only submit that the ternary complex cannot be excluded, as all the competing reactions studied followed the displacement mechanism (Sn_2) which they added was related to the process of ternary complex formation.

In view of the above-mentioned facts, it is suggested that Cu(II) binds predominantly in the form of the Cu(II)-HSA binary complex in contrast to the predominant formation of the ternary HSA-Cu(II)-amino acid complex.

Accordingly, the transport of copper from plasma through biological membranes into tissue can be represented by the following equilibria:



From our experimental results, the order of mobilization strength of amino acids for Cu(II), across the biological membrane is found to be:

L-His > L-Thr > L-Try

These results are in excellent agreement with the findings of Naughton *et al* [154], in their investigation of the molecular nature of low-molecular-mass copper ions in isolated rheumatoid knee-joint synovial fluid. Of significant importance to our studies are the low levels of low-molecular-mass Cu(II) species that were demonstrated to be present in 'fresh' rheumatoid synovial fluid and the preferential complexation of Cu(II) by histidine thereby reflecting the important role played by histidine in Cu(II) homeostasis *in vivo*. Copper(II)histidinate complexes have been shown to exercise SOD-mimetic activity [155]. The rate constant for the dismutation of O_2 by Cu(II)-His species is a similar order of magnitude to that of SOD itself.

The above findings coupled with the fact that patients with rheumatoid arthritis are reported to have a low serum histidine concentration, makes it plausible to suggest that treatment of these patients with oral or injected L-histidine would not only raise the serum histidine concentration, but also mobilize more Cu(II) to the site of inflammation. Patients with rheumatoid arthritis have also been reported to have elevated serum Cu(II) levels [7], in which case an excess of Cu(II) ions can be transported via histidine complexes to the tissues. It is known that such excess concentrations of Cu(II) are toxic and have very deleterious effects on living tissues [113]. However, the results in our studies indicate that L-histidine is only able to mobilize a maximum of two thirds of the Cu(II) bound to HSA (refer to Fig 4.3).

A possible explanation for the observed behavior is that as more Cu(II) is removed the albumin molecule undergoes polymerization in such away that the copper is unavailable for interaction with L-His. This an be construed as an homoeostatic mechanism which regulates the amount of Cu(II) transported to the tissue compartments via amino acid complexes. If the above postulation holds true *in vivo,* then the treatment of rheumatoid arthritis with L-His holds much promise, in so far as the control of inflammation is concerned.

CHAPTER 5

ULTRAFILTRATION STUDIES OF THERAPEUTIC AGENTS IN HUMAN BLOOD SERUM

5.1 INTRODUCTION

According to the accepted scheme in medicine, the treatment of a pathological process, either disease or syndrome, leads to cancel the causal agent(s), second the pathological mechanisms, and finally, when the first two possibilities are lacking, the consequences, the signs, and the symptoms. In the case of rheumatoid arthritis (RA) the causal factor(s) of the disease is not yet known, therefore a causal treatment is not possible. There is general agreement on the mechanism of the pathological process, which is considered due to chronic inflammation and it is agreed that an immunological disorder plays a pivotal role in the onset and in the recruitment of the inflammatory process.

A wide variety of drugs are available which alleviate the pain and inflammation associated with RA, but many are unsuitable for long term prescription and few, if any, are permanently effective [114-116].

5.1.1. DRUGS USED IN THE TREATMENT OF RA.

The various groups of drugs used in the treatment of RA, can be classified as follows: -

- Simple analgesics
- Nonsteroidal anti-inflammatory drugs (NSAIDs)
- Second-line antirheumatic drugs
- Corticosteroids
- Immunosuppressives

Simple analgesics are used to relieve pain caused by the degenerative conditions, but have no anti-inflammatory effects.

The NSAIDs are the drugs of first choice to relieve pain and stiffness associated with active inflammation in rheumatoid arthritis. A universal effect of all NSAIDs is the ability to inhibit prostaglandin synthesis from fatty acid precursors via an effect on the enzyme cyclo-oxygenase [117]. Although NSAIDs are the most extensively used drugs in the treatment of RA, there is much morbidity and mortality associated with these drugs. This is due to the adverse side effects which most commonly include gastrointestinal ulceration and micro bleeding rashes, fluid retention and headaches.

Second line antirheumatic drugs, e.g. D-Penicillamine, are slow-acting disease suppressing drugs and are given to patients who do not respond to NSAIDs. As these drugs have no immediate anti-inflammatory effects, they are usually given in combination with an NSAID. The uses of corticosteroids in RA are usually reserved for patients with progressive forms of arthritis that have failed to respond to second-line agents. The side effects of steroid use are usually severe and these include muscle wasting and osteoporosis.

Immuno-suppressive drugs are drugs initiated by the specialist physician and are reserved for serious forms of RA that are unresponsive to other agents. These drugs have potentially serious side effects and require careful monitoring for their effect on the bone marrow. Considering the above-mentioned facts, the most serious impediments in the use of any of the above-mentioned drug groups for long term therapy, are the adverse side effects. Accordingly, there are strong grounds for seeking better anti-arthritic agents and in this respect an urgent need to engage in more fundamental drug design has become apparent.

5.1.2 POSSIBLE RELEVANCE TO THERAPY OF THE COPPER APPROACH

As previously noted, the use of copper containing agents in the therapy of inflammatory disorders in man is by no means a new one. For example, a copper-salicylate mixture, marked until 1971 with the commercial name of Permalon, was used in uncontrolled trials in 600 arthritic patients obtaining, in the opinion of rheumatologists, "very successful" results [118]. The administration of copper preparations, however, is not the only way to tackle the problem.

The other obvious way is to exploit the rise of circulating copper, which characterizes inflammation, through the use of suitable administered ligands, in the attempt to improve the anti-inflammatory activity naturally exerted by endogenous copper. Potential ligands that could be used in the treatment of RA are being designed and screened, with the aid of a computer model of blood plasma called ECCLES [119].

Considering the assumptions employed in any model study, there is no guarantee that the therapeutic predictions by ECCLES are valid, due to the fact that simulated conditions, are more often than not far removed from conditions in biological fluids *in vivo*. In order to check the observations of ECCLES, we have conducted ultrafiltration experiments of therapeutic agents in human blood serum, under physiological conditions. Since ultrafiltration is now known to be a reliable technique in binding studies (refer to Chapter 2), the data arising from such experiments can be treated with much confidence and can be compared to theoretical predictions. In doing so, the blood plasma model, ECCLES, that has been constructed to yield such predictions can be validated.

5.2 EXPERIMENTAL

5.2.1 MATERIALS

Human blood serum was obtained from the Western Province Blood Transfusion Service. Drugs involved in this study were kindly donated by pharmaceutical companies and used without further purification. These drugs were Prednisolone, Piroxicam and Mefanamic Acid (Warner Lambert), Aspirin (May and Baker), Indomethacin (Beecham Pharmaceuticals), Tiaprofenic Acid (Roussel Laboratories) and D-Penicillamine (Lilly Laboratories USA). EDDA, DTPA and Trien were obtained from Sigma while EDTA was obtained from Macdonald Adams and Co. All other solvents and reagents used, were of analytical grade.

5.2.2 ULTRAFILTRATION

Varying concentrations of drugs/ligands were added to various serum aliquots (10ml each). The pH of these solutions was then adjusted to 7.5 at 37 °C, by the addition of small amounts of NaCl or HCl. The solutions were then subjected to ultrafiltration under standard conditions (defined in Chapter 2). The ultrafiltrates were collected and immediately analysed for Cu(II), Zn(II) and Ca(II) by atomic absorption (For details refer to Chapter 2). The ability of a ligand to liberate a metal ion from labile protein-binding sites is calculated as a plasma mobilizing index (PMI) at different ligand concentrations (refer to Equation 1.1)

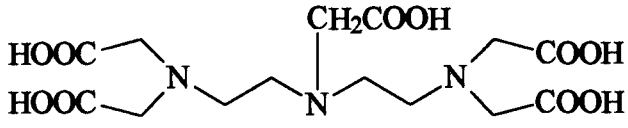
5.2.3 COMPUTATION

Theoretical predictions for the mobilization of metal ions in blood plasma by chelating agents and drugs were obtained by using the ECCLES program and data base [120]. In the cases of drugs, when equilibrium constants were unavailable, they were estimated from analogous model compounds in the literature [121] and, where necessary, adjusted to 37 °C and an ionic strength of 0.15.

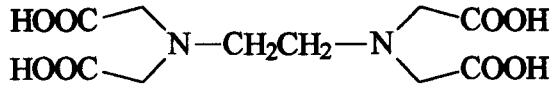
5.3 STRUCTURE FORMULAE OF LIGANDS INVESTIGATED

5.3.1 CHELATING AGENTS

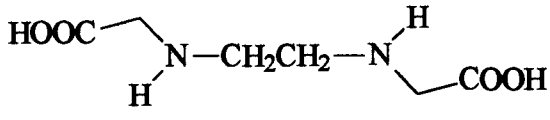
5.1 Diethylenetriaminepentaacetic acid



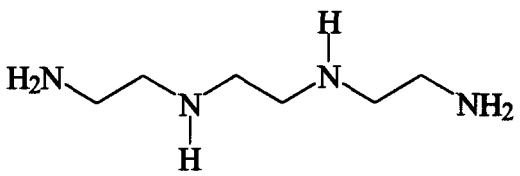
5.2 Ethylenediaminetetraacetic acid (EDTA)



5.3 Ethylenediaminediacetic acid (EDDA)

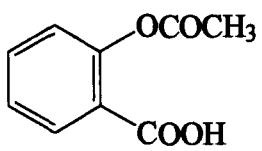


5.4 Triethylenetetramine (Triene)

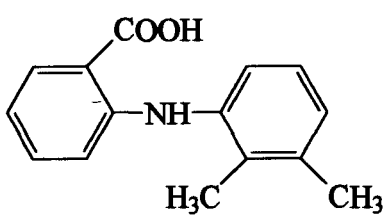


5.3.2 NSAIDs

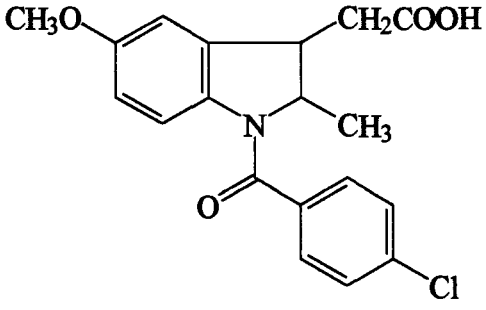
1. Acetyl Salicylic Acid (Aspirin)



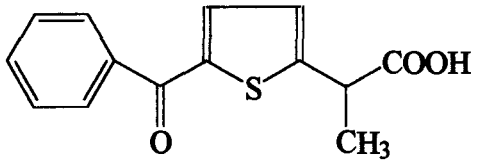
2. Mefanamic Acid (Ponstan)



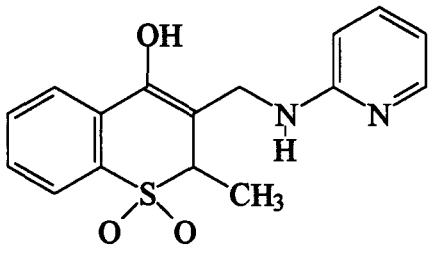
3. Indomethacin



4. Tiaprofenic Acid (Surgam)

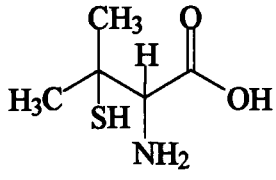


5. Piroxicam (Feldene)



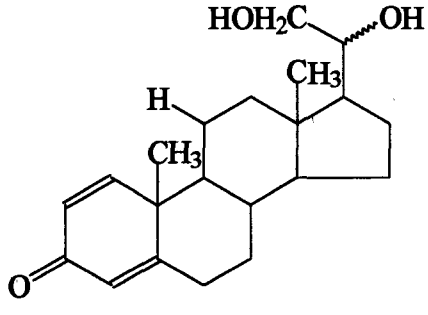
5.3.3 SECOND-LINE ANTIRHEUMATIC DRUG

D-Penicillamine (Penicillamine)



5.3.4 CORTICOSTEROID

Prednisolone



5.4 RESULTS AND DISCUSSION

5.4.1 MOBILIZATION OF ENDOGENOUS COPPER BY THERAPEUTIC AGENTS IN HUMAN BLOOD SERUM

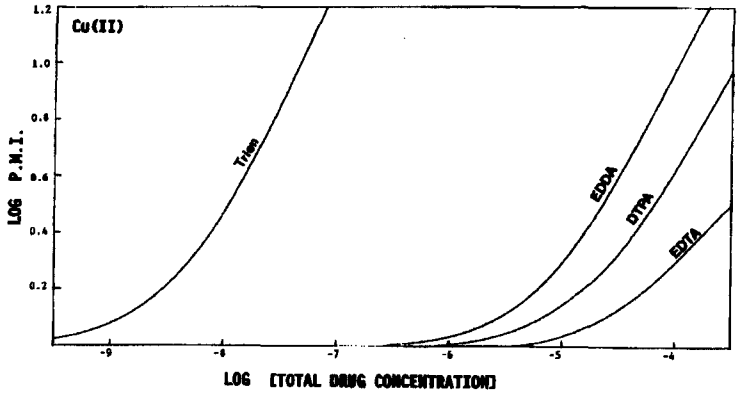


Fig. 5.1 P.M.I curves calculated for Cu(II)-chelating agents in blood plasma by using computer simulation (ECCLES) at pH 7.5, 37°C and I=0.15M.

Figure 5.1 shows the PMI curves calculated for Cu(II)-chelating agents in blood plasma by using ECCLES. The order of ability of these agents to mobilize Cu(II) from serum albumin at pH 7.5 and 37 °C is found to be:

Trien > EDDA > DTPA > EDTA

Apart from structural considerations, the mobilizing order can be rationalized by taking into account the mobilization of Zn(II) and Ca(II) by these ligands. The computer model, ECCLES, predicts that both, Trien and EDDA are unable to mobilize Ca(II) (see Fig. 5.8), and that Trien is a weaker mobilizer of Zn(II) than DTPA and EDTA (see Fig. 5.5). Therefore, the high mobilization of Cu(II) by Trien is a consequence of its limited calcium(II) and Zn(II) complex formation and hence, most of the Trien in blood plasma is available for Cu(II) mobilization.

Simarlarly, although EDDA forms weaker Cu(II) complexes than DTPA and EDTA, it has a greater ability to mobilize Cu(II). Hence, the powerful Cu(II) binding abilities of EDTA and DTPA are suppressed in plasma by competition with zinc and calcium. The higher mobilization of Cu(II) by DTPA is expected as DTPA forms more stable Cu(II)-complexes than EDTA. This was confirmed by Gao *et al* [151] from their spectrophotometric studies of the mobilization of Cu(II) from HSA with chelating agents.

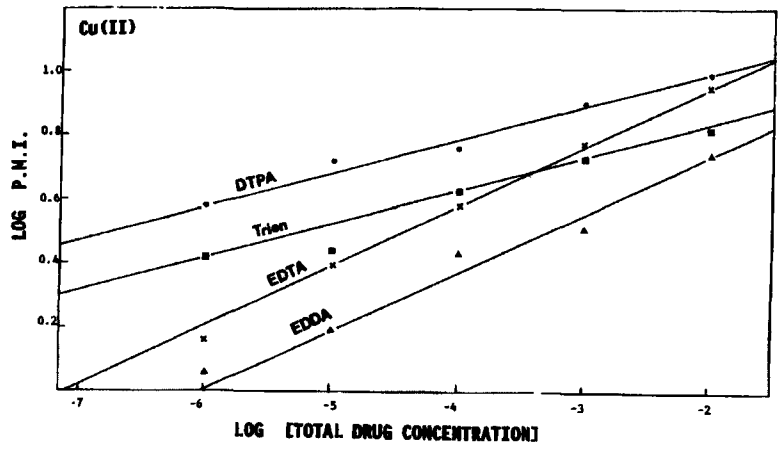


Fig. 5.2 Experimental P.M.I curves for Cu(II)-chelating agents in human blood serum under physiological conditions.

Fig. 5.2 shows the experimental PMI curves for Cu(II)-chelating agents in human blood serum under physiological conditions of pH and temperature (refer to Table 5.1 in the appendix for raw data and calculations). By comparison of Fig. 5.1 with Fig. 5.2 it is evident that there are a number of discrepancies between the experimental and theoretical results. Firstly, the order of ability of the chelating agents to mobilize Cu(II) from serum albumin in blood serum is now found to be:

DTPA > Trien > EDTA > EDDA

Secondly, the shapes of the experimental PMI curves are distinctly different from the theoretical curves. On the basis of these observations it would seem logical to conclude,

that there are no correlations between the experimental and theoretical results. This in turn would seriously question the validity of the computer model.

However, a careful examination of the data on Fig. 5.1 and 5.2, shows that, with the exception of Trien, the amount of Cu(II) mobilizations by the polyaminopolycarboxylic acids are similar to the amount of Cu(II) predicted to be mobilized by ECCLES. In both the theoretical and experimental plots, above a threshold value of PMI = 0.2, the curves are linear, with the slopes of the theoretical curves being much greater than the experimental ones. Thus, the model is partially correct in that, it predicts the right order of magnitude of PMI values, but fails to predict the finer details with respect to the order of metal mobilization by the chelating agents. Support for the computer model, comes from the fact that apart from comparing equilibrium constants of different ligands, the model makes allowance for competition between metal ions for ligand complexation in blood plasma [119].

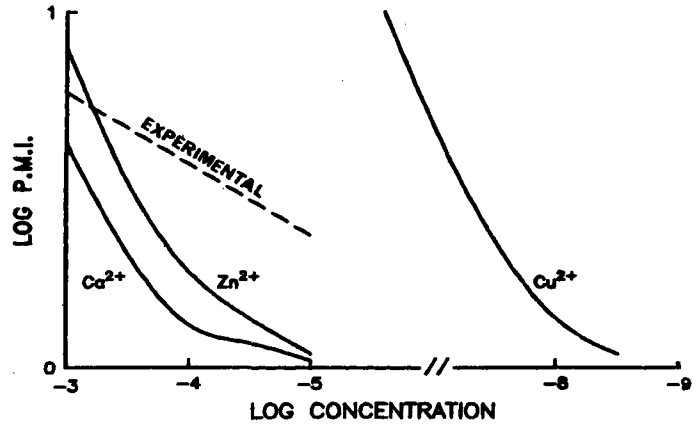


Fig. 5.3 The effect of competitive metal ions in the mobilization of Cu(II) by EDTA. The solid line represents the theoretical predictions obtained by using the ECCLES program. The dashed line represents the experimental P.M.I. curve for Cu(II) mobilization by EDTA in blood serum.

Fig. 5.3, shows the effect of competing metal ions in the blood plasma model of EDTA. If no account is taken of Ca(II) and Zn(II), then EDTA is predicted to be extremely efficient in mobilizing Cu(II). With the introduction of either Ca(II) and Zn(II) into the

model, the efficiency of EDTA for Cu(II) mobilization decreases dramatically. The reason being, while Cu(II)-EDTA complexes are more stable than Ca(II)-EDTA and Zn(II)-EDTA complexes, the concentration of calcium and zinc are much higher than copper in vivo. On the basis of these findings, the prediction by the computer model seems to be well substantiated. The dashed line in Fig. 5.3 represents the experimental results for Cu(II) mobilized by EDTA in blood serum.

It can be seen that the experimental results do not compare favourably with computer predictions, with respect to the shape of the plot as well as the magnitude of the PMI values. The need now arises to validate the experimental results. Experimental PMI curves for Cu(II) with NSAIDs, D-Pen and prednisolone in blood serum are shown in Fig. 5.4 (refer to Table 5.2 in the Appendix for details). It is clearly evident that these drugs are capable of mobilizing endogenous copper. These results are supported by the findings of Fiabane and Williams [122], who reported that NSAIDs are capable of releasing copper ions to a low molecular weight pharmacoactive form. Contrary to these findings, the computer model predicts that these drugs are incapable of mobilizing Cu(II) in blood plasma. The inability of the computer model to predict the therapeutic activities of these drugs, highlights the apparent deficiencies inherent in the model. These deficiencies will be discussed at a latter stage.

The order of ability of the various drugs to mobilize Cu(II) from serum albumin in blood serum is found to be:

D-Penicillamine > Aspirin > Mefanamic Acid >
Indomethacin > Piroxicam > Tiaprofenic Acid >
Prednisolone.

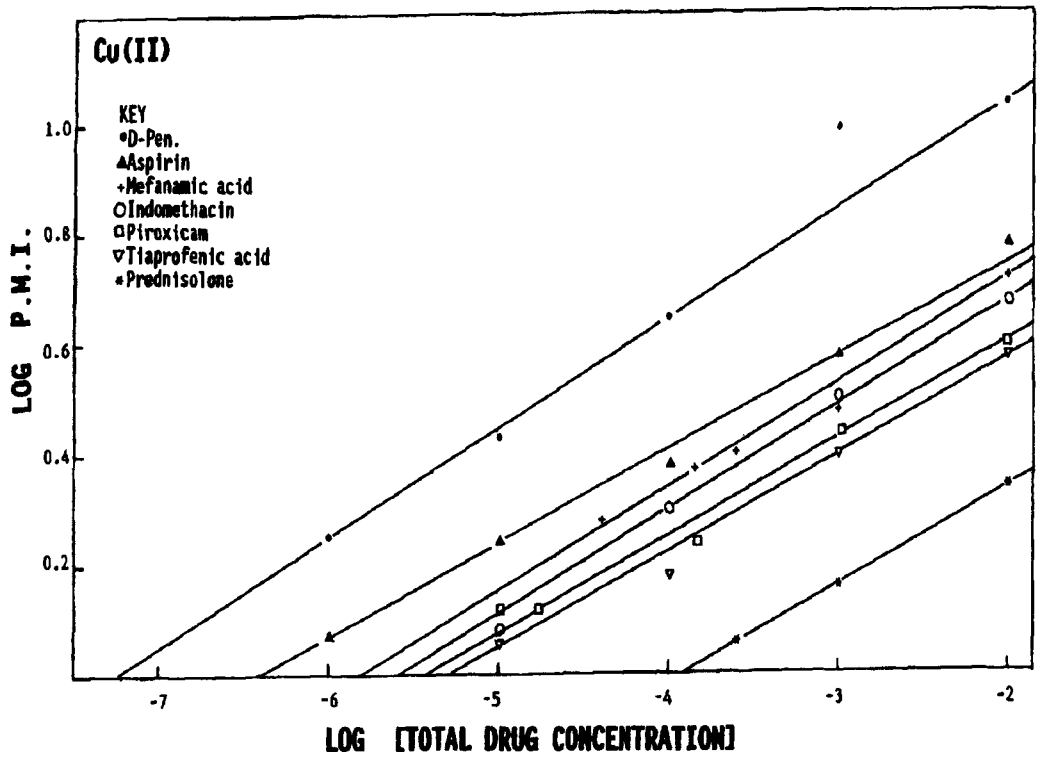


Fig. 5.4 Experimental P.M.I. curves for Cu(II) with NSAID's, D-Pen and Prednisolone in blood serum under physiological conditions.

By comparison of Fig. 5.4 with Fig 5.2, NSAIDs are found to be approximately equivalent to EDDA, with respect to Cu(II) mobilization in blood serum. The NSAIDs are all acidic and are known to bind to serum albumin [122]. These properties may be essential to allow their penetration into inflammed tissue. On the basis of the experimental evidence, part of the anti-arthritic activities of these agents can be suggested to occur through a mechanism which encourages the release of albumin-bound copper and in doing so enhances the formation of neutral low molecular weight pharmacoactive copper complexes that can be transported across the biological

membranes to the site of inflammation, where they can exercise their anti-inflammatory activities. The corticosteroid, prednisolone, is seen to be a weak mobilizer of Cu(II), indicating that the therapeutic effects of this drug could be due to a mechanism other than the mobilization of endogenous copper to the inflammed tissue.

The ability of aspirin to mobilise Cu(II) in comparison to other NSAIDs used deserves special mention. Traditionally, RA was treated with aspirin which has been largely superceded by prescription NSAIDs because of pre-marketing studies suggesting lesser toxic effects for NSAIDs than for aspirin. A study undertaken by J.F. Fries *et al* [161] to evaluate the above-mentioned toxic effects, established that the toxicity index was only 1.37 for aspirin as compared to 1.87 to 2.90 for selected non-salicylate NSAIDs. The above findings coupled to our results warrants a reevaluation of aspirin therapy which has become a forgotten drug in the management of RA.

The case of D-penicillamine also deserves special attention. D-Penicillamine is extensively used in the treatment of RA and is frequently capable of effecting remission in patients suffering from this disorder. However, an unequivocal explanation of how the drug modifies the course of rheumatoid diseases has not yet emerged.

Previous studies by Micheloni *et al* [123] have reported that D-penicillamine is unlikely to act as a chelating agent which increases the low molecular weight Cu(II)-fraction in blood plasma, by displacing the equilibrium away from the labile protein bound species. These authors suggested that D-penicillamine affects copper metabolism by liberating Cu(II) from metalloprotein stores such as ceruloplasmin and in doing so, violates the structural integrity of the protein, irreversibly transferring the copper ion to the exchangeable metal ion pool. Several reports are in accord with such a destructive role for penicillamine [124-126].

If the theory of Micheloni and co-workers is to hold true, then the amount of copper released into the labile equilibrium, would in practice be expected to be significantly greater than the amount of Cu(II) mobilized by DTPA, Trien or EDTA. This is to be

expected as 90% of Cu(II) in blood serum is bound to ceruloplasmin. On the basis of our experimental results, it seems highly unlikely that D-penicillamine acts by the mechanism as suggested by Micheloni *et al* . Support for this contention, comes from the fact that several reports have indicated that it is unclear whether the metalloprotein in question can be ceruloplasmin [127-129].

Due to the problem of reductive chelation, D-penicillamine could not be included in the computer modelling studies, but experimentally D-penicillamine is found to mobilize Cu(II) to the same extent, if not, better than EDTA (compare Fig.'s 5.2 and 5.4). Hence, it can be deduced that D-penicillamine acts as an efficient chelating agent and much of its therapeutic activities can be related to this property. The relationship of these properties to its mode of actions in RA is unknown. One possible mode of action of D-penicillamine and other slow-acting anti-rheumatic drugs (SAARDs) involves the participation of their copper complexes in plasma and, more particularly, in synovial fluid as superoxide dismutase mimetics which destroy extracellular superoxide radicals, $O^{\bullet}_2$ with a rate constant virtually identical to that of superoxide dismutase (SOD) [156]

$$2\,O_2^{\bullet-} \xrightarrow[SOD]{2H^+} O_2 \;+\; H_2O_2$$

This mode of anti-rheumatic activity is consistent with other findings [157] which reported SOD mimetic activities of other copper complexes including $Cu(II)_2$(indomethacin)$_4$[158], $Cu(II)_2$ (aspirinate)$_4$ [159] and Cu(II)(salicylate)$_2$ [160], to mention but a few.

Arising from the above, it is reasonable to suggest that the pharmacological effect of the NSAIDs and SAARDs used in our study can be attributed to the formation of small molecular copper complexes that exercise their anti-inflammatory activities by way of SOD mimetic activity. This would not apply to all drugs used in this study as there are alternative mechanisms, eg immuno suppression which could lead to clinical anti-rheumatic activity.

5.4.2. MOBILIZATION OF ENDOGENOUS ZINC BY THERAPEUTIC AGENTS IN HUMAN BLOOD SERUM.

PMI curves calculated for Zn(II)-chelating agents in blood plasma by using ECCLES are shown in Fig. 5.5. The order of ability of these agents to mobilize Zn(II) from serum albumin at pH 7.5 and 37 °C is found to be:

DTPA > EDDA > EDTA > Trien

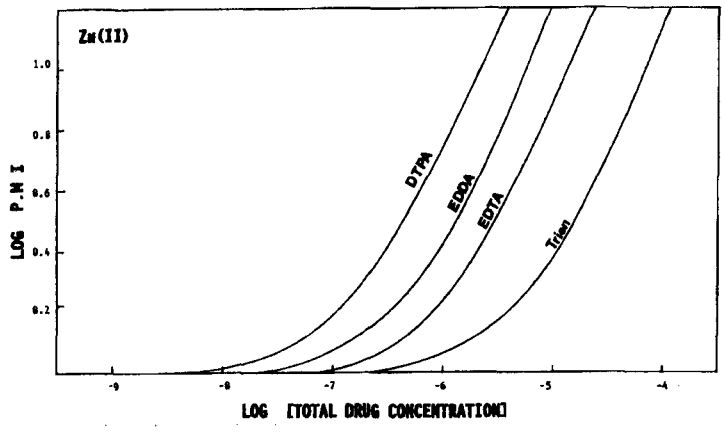


Fig. 5.5 PMI curves calculated for Zn(II)-chelating agents in blood plasma by using ECCLES at pH 7.5, 37°C and I = 0.15M

The experimental result confirms the computer predictions, in that the chelating agents are found to mobilize Zn(II) in blood serum, but once again there are discrepancies between the theoretical and experimental results with respect to the order in which these agents are predicted to mobilize Zn(II), as well as the shape of the plots.

The major point of discrepancy that needs to be resolved is the order of sequence in which the chelating agents are predicted to mobilize Zn(II). By comparing Fig. 5.2 with Fig. 5.6, the chelating agents are found to mobilize Zn(II) and Cu(II) in the same order, i.e. DTPA > Trien > EDTA > EDDA. This observation can be rationalized by the fact that, both Zn(II) and Cu(II), are similar with respect to being soft metal ions which forms chemical bonds of covalent nature (refer to Chapter 2). Furthermore, on closer examination of the above-mentioned plots, the amount of Zn(II) mobilized is found to be less than the amount of Cu(II) mobilized by the corresponding chelating agents.

It could be argued that since the concentration of Zn(II) ions in blood plasma are approximately three times greater than that of Cu(II) ions, and one would expect a significantly greater mobilization of Zn(II) ions. However, it is now known that the tendency of Zn(II) ions to form chelates is not as pronounced as Cu(II) ions (Refer to Chapter 2).

This is further substantiated by the fact that Cu(II)-complexes with chelating agents have larger stability constants than the corresponding Zn(II)-complexes [77]. In addition to these findings, it must be noted, that on the basis of structural considerations and the competitive equilibria that exists between metal ions and ligands in blood plasma, it is highly unlikely that EDDA is more effective in mobilizing Zn(II) than EDTA (as predicted by ECCLES).

It is now clear that the predictions of the computer model are indeed questionable. This is further exemplified by the inability of the computer model to predict the mobilization of Zn(II) in blood plasma, by the various drugs used in this study. On the other hand, ultrafiltration studies show that these drugs are capable of mobilizing endogenous Zn(II) in human blood serum.

The experimental PMI curves for Zn(II) with NSAIDs, D-penicillamine and prednisolone in blood serum are shown in Fig. 5.7. (See Table 5.4 in Appendix). The order of ability of these drugs to mobilize Zn(II) from serum albumin under physiological conditions is found to be:

D-Penicillamine > Aspirin > Piroxicam > Mefanamic Acid > Indomethacin > Prednisolone > Tiaprofenic Acid.

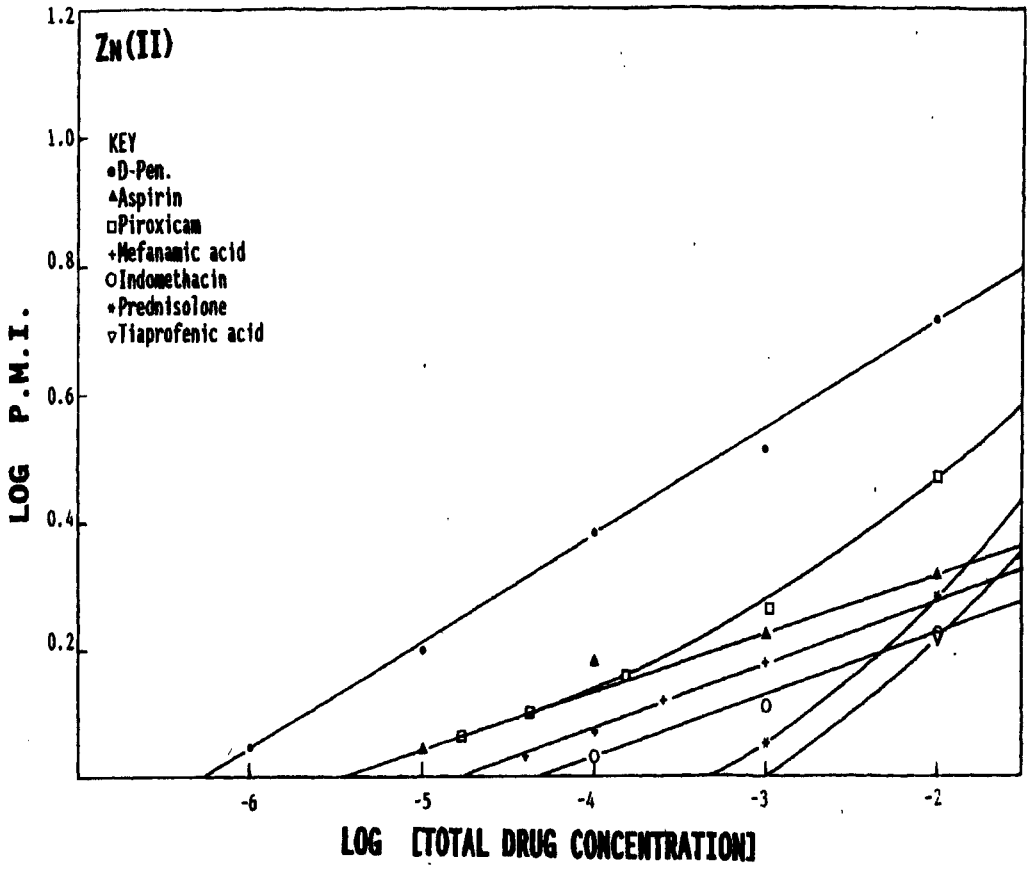


Fig. 5.7 Experimental P.M.I. curves for Zn(II) with NSAID's, D-Pen and Prednisolone in blood serum under physiological conditions.

By comparison of Fig. 5.7 and Fig. 5.6, D-pencillamine is found to mobilize Zn(II) to the same extent as EDDA, indicating that this drug also acts as a Zn(II)-chelating agent in vivo. This observation is substantiated by the fact that D-penicillamine has been implicated in zinc excretion [130]. The NSAIDs and prednisolone are found to be weaker mobilizers of Zn(II) than the cheating agents. It is interesting to note that, with the exception of piroxicam and prednisolone, the order in which these drugs mobilize Zn(II) is remarkably similar to the order in which Cu(II) is mobilized in blood serum (compare Fig. 5.7 and 5.4). The slight disparity can be attributed to the difference in propensities of these drugs for different metal ions.

5.4.3 MOBILIZATION OF ENDOGENOUS CALCIUM BY THERAPEUTIC AGENTS IN HUMAN BLOOD SERUM.

With the exception of Aspirin and EDTA, all other drugs and chelating agents are theoretically predicted to be unable to mobilize Ca(II) bound to serum albumin (refer to Fig. 5.8). Experimentally, only DTPA and EDTA are found to mobilize endogenous Ca(II) (Fig. 5.8). D-Penicillamine and Trien exhibit very low PMI values, signifying that these ligands, also do not compare effectively with serum albumin for Ca(II) ions (refer to Table 5.5 in Appendix).

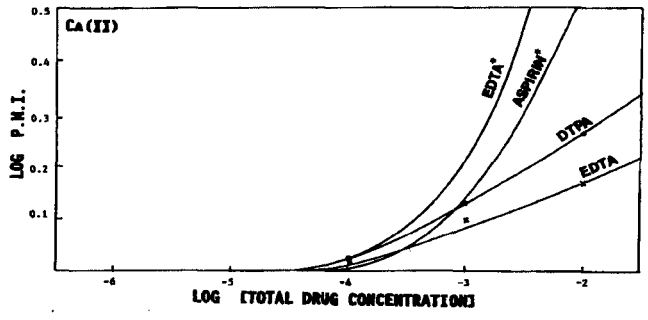


Fig. 5.8 Experimental P.M.I. curves for Ca(II) binding by EDTA and DTPA in human blood serum
* Theoretical P.M.I. curves for Ca(II) binding by Aspirin and EDTA, obtained by using ECCLES.

The experimental results are well supported by the fact that DTPA and EDTA are known to form very stable complexes with calcium ions in blood serum [131]. This properties of the chelating agents have been exploited for their use as antidotes for heavy metal poisoning [132]. Experimentally, DTPA is found to be more efficient in mobilizing Ca(II) than EDTA. This is to be expected as DTPA is a higher homolog of EDTA and the stability constant of the Ca-DTPA complex is higher than the corresponding EDTA complex [130]. DTPA is the most effective known chelating agent for the removal of internally deposited transuranium elements in humans [133].

The deficiencies inherent in the ECCLES computer model, are now confirmed, in that the model is unable to predict the therapeutic activity of DTPA with respect to Ca(II) mobilization. Furthermore, the PMI values predicted for EDTA are found to be an overestimation of the actual experimental values.

Aspirin is known to possess several therapeutic properties, some occurring in the brain (analgesic and antipyretic effects) and some at peripheral sites [134] (anti-inflammatory effects). Aspirin has been postulated to exert their biological actions through the ability to bind metal ions [135]. The ECCLES program suggests that aspirin is able to mobilize Ca(II) into the low molecular weight fractions. However, the anti-inflammatory effects of these low molecular weight fractions in rheumatoid arthritis are yet to be reported.

5.5 GENERAL DISCUSSION

Comparison between experimental and theoretical results has shown, that the computer model, ECCLES, is unable to explain the finer details of metal mobilization by therapeutic agents in blood plasma. The inadequacy of the model may be attributed to the following limitations:

(1) The model does not take into consideration ligand binding by protein or low molecular weight complexes binding to protein.

(2) A basic assumption in the model is that the protein buffers the free metal ion concentration. We have shown in the ternary studies that a simple metal-protein interaction is not valid and that as you remove metal ions from the protein, it becomes progressively more difficult to remove the remaining metal ions bound to the protein. This explains why the limiting slopes of the experimental curves are much lower than the theoretical ones.

(3) The model does not take into account the interactive effects between the various binding sites on the serum proteins.

Serum albumin is known to be a versatile carrier protein not solely for copper ions, but also for long chain fatty acids and for smaller molecules. In addition, Brown's recent structural elucidation of serum albumin [136. 137], suggests that serum albumin binds to drug molecules of the type used in our studies. If this binding is not taken into account in the computer model, then an overestimation of the amount of metal ion mobilized would occur. This is well illustrated in the comparison between experimental and theoretical results, where the magnitude of PMI values predicted by ECCLES, are very much greater that the values measured by ultrafiltration studies. It can now be said that the criterion taken into consideration when omitting protein-metal/ligand interactions in

the model study, needs to be re-evaluated. In the previous study, Jackson *et al* [25], also noted the limitations of the computer model, with respect to the availability of formation constants required by the model.

The absolute precision of the computer model thus directly depends on: -

1. The precision of the free metal ion concentration in normal blood plasma

2. The availability of formation constants and the degree of reliability of the formation constants on which the model is based

As far as the first point is concerned, the plasma concentration of the free copper ions used in the present study is 1×10^{-20}M, a quantity used in a previous study [119]. Agarwal and Perrin [138], established a value in the order of magnitude of 3.1×10^{-16}M. It is obvious that a more precise value would be highly desirable and such a value be derived from the stability constants of copper protein complexes. The second point of concern was raised by Duffield *et al* [139], who showed that the current knowledge concerning the interaction of polyaminopolycarboxylic acids with metal ions, under biological conditions, is imperfect, mainly due to the interaction of the background electrolyte. This is well substantiated by our own findings, which indicates that ionic strength has a marked influence on metal-binding to serum albumin. These are indeed, strong grounds for further research in this area.

The ultrafiltration studies show that NSAIDs, D-penicillamine and prednisolone are capable of mobilizing Cu(II) and Zn(II) in blood serum, while the ECCLES program predicts otherwise. This could be due to the fact that all considerations in the ECCLES program are based upon an equilibrium model, in which case, the slow pharmacokinetics or active transport processes of these agents cannot be explained by this model. Hence, a serious limitation of the computer program is brought to the attention of the model designers.

Apart from the inhibition of prostaglandin synthesis, it can be suggested on the basis of experimental evidence that NSAIDs, D-penicillamine and prednisolone may also function by releasing Cu(II) ions from serum albumin by a direct competitive complexing mechanism or through a remote mechanism whereby the drug becomes bonded to a site some distance away from the copper ion and facilitates copper ion release through allosteric effects. The order in which these drugs are found to mobilize metal ions in blood serum, does not necessarily represent the order of potency with respect to anti-inflammatory effects in patients with rheumatoid arthritis. It remains to be clarified whether the low molecular weight species of these drugs are neutral and can be transported across the biological membranes. The half lives of these drugs will undoubtedly play an important part in the above mechanism.

Zinc is involved in many biological processes related to inflammation. A decrease of plasma zinc levels has been found in patients with rheumatoid arthritis [140]. Sinkin [141], has reported clinical improvements in patients with rheumatoid arthritis treated with zinc sulphate, but another study failed to confirm this [142]. Zinc has also been reported as beneficial in patients with psoriatic arthritis [143]. On the basis of our experimental evidence, it seems possible that the anti-inflammatory actions of NSAIDs, D-penicillamine and prednisolone may be linked to their effect on tissue zinc. However, more work needs to be done, so as to correlate the elevated levels of copper with the reduced levels of zinc in the blood plasma of patients with RA.

The rationale for administering ligands selective for copper to beneficially influence the inflammatory process can be summarized in two points: -

1. The rise in circulating copper may represent a significant part of the natural anti-inflammatory response elicited by the organism to control development and remission of the inflammatory process.

2. The widespread responsiveness of inflammatory disorders to a copper supplementation therapy suggests that the control exerted by endogenous

copper on inflammation is susceptible to improvement by means of pharmacological manipulations.

The design of anti-inflammatory chelating agents is conditioned by many variables, often unknown. Nevertheless, a satisfactory chemical solution may probably be attained and Jackson *et al* [139] have listed a number of features that could suitably orient the synthesis of new ligands potentially capable of manipulating endogenous copper *in vivo*. Such molecules would be extremely interesting tools in elucidating the role played by copper in inflammation and could be screened at industrial level to discover new lines of anti-inflammatory or anti-arthritic drugs. In the latter case, some further considerations may be noteworthy.

In the first place the ability of a molecule to effectively compete with known endogenous ligands of copper, such as albumin, may be studied *in vitro*. If negative results are obtained one cannot expect the molecule to be able to "strip" *in vivo*, endogenous copper from its ligands and hence it could not be convenient to promote the compound to the *in vivo* assay program. Secondly, additional information about the possible mechanism of action *in vitro* of the complex between copper and the ligand (for example, scavenging activity at different pH values) could help to discriminate amongst different chemical structures, the most promising ones.

In addition to the above-mentioned suggestions, it can be concluded that ultrafiltration studies of drugs in human blood serum, appears to be a good screening criteria for the mobilization of endogenous copper *in vivo*. This in turn could be related to the anti-inflammatory potency of these drugs.

CONCLUDING REMARKS

Ultrafiltration is not only theoretically equivalent to equilibrium dialysis but much simpler to carry out. The results obtained using ultrafiltration are in excellent agreement with that obtained by equilibrium dialysis. Apart from metal-protein binding studies, the ultrafiltration technique was extended to binding studies of therapeutic agents in human blood serum.

Our inability to obtain logβ values from either ultrafiltration or equilibrium dialysis experimental data, is indicative of the gross inadequacies of *in vitro* studies, that more often yields only semi-quantitative information, that can only be treated with a limited amount of confidence. Simulations of *in vivo* conditions, especially the concentration of metal ions and low molecular weight ligands, present the greatest challenge to solution chemists, who are only able to investigate interactions between species under in vitro conditions, at manageable concentrations. This is due to the fact that concentration of metal ions and complexes present as low molecular fractions of body fluids cannot as yet, be monitored *in vivo* by instrumental techniques because such concentrations are several orders of magnitude below those identifiable by even the most sophisticated research equipment.

Presently, powerful computer programs are used to simulate the equilibrium conditions *in vivo* as well as aid in design of anti-inflammatory drugs. It is evident from comparison with experimental studies, that the data base of the computer program, ECCLES, needs to be supplemented with the critical stability constants of the various metal-protein complexes that are known to exist *in vivo*. The existing computer model is inadequate to make reliable therapeutic predictions or select drugs for the treatment of rheumatoid arthritis. However, in the meantime, the ECCLES program is significant, in that a basic understanding of biological components can be achieved by correlating biological activities with species concentration.

PROGNOSIS

Further trends of research development that most urgently needs to be explored include:

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1. The accurate determination of formation constants for important complexes in blood plasma viz. metal-protein complexes, mixed ligand complexes, metal ion-amino acid complexes and ternary metal-protein-amino acid complexes.

2. The improvement of estimates of the free metal-ion concentrations in plasma, especially that of copper.

3. It is important to identify the low molecular weight copper complexes present, at least in that of serum, of both normal subjects and those suffering from rheumatoid arthritis. Despite the considerable efforts made by using the computer-based approach, we lack a credible map of non-ceruloplasmin-bound copper among different low molecular weight endogenous ligands in biofluids. Information on this topic is relevant for the identification of natural pharmacologically-active forms of copper possibly present in serum and inflammatory exudates. Moreover, they are of major importance for the design of chelating agents able to "strip" copper *in vivo* from endogenous chelates such as serum albumin.

   A modern approach to the study of metal-derivatives in solution (hence *in vivo*) will be the use of nuclear magnetic resonance (NMR), due to the actual increasing availability of high-field multinuclear spectrometers. This technique has the potentiality to identify and quantitate different complexes of a metal present in samples of biological origin. Many metallic nuclei can be detected, among which is copper, although at present, only Cu(I) leads to high resolution nmr spectra[161].

4. Last but not least, our knowledge about the mechanism(s) of action through which the inflammatory/anti-arthritic activities of both exogenous and endogenous copper are achieved is inadequate. A better understanding of this point of the problem would lead to a more rational choice of *in vitro* assays to be carried on as preliminary tests, for the selection of anti-inflammatory drugs.
However, measurements of drug-metal interactions using *in vivo* ultrafiltration may be more clinically relevant, as they are carried out in physiologic conditions and maybe more useful when measurement of the free drug or metal is impossible because a sensitive assay is unavailable or when protein binding to metals or drugs rapidly degrade in vitro.

Recent developments in Ultrafiltration have successfully led to the application of the technique *in vivo* for the study of protein binding to drugs [148]. *In vivo* ultrafiltration has demonstrated potential advantages over conventional methods as it does not require a highly sensitive assay. This is due to the fact that only total plasma concentration of the drug needs to be quantified as opposed to the concentration of the unbound drug.

More *in vivo* ultrafiltration studies of protein binding to drugs and metal ions are necessary for the method to be validated, after which the method can be extended to explore the behaviour and mode of action of drugs in conditions of unknown etiology such as Rheumatoid Arthritis, thereby creating a body of knowledge that can help analyse the disorder and promote appropriate drug design that would be able to effectively treat the disorder if not cure it.

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APPENDIX

Table 3.1 Ultrafiltration study of Cu(II) binding to HSA (EDTA treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37^{0}C and I = 0.15M. $[HSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total}$ x 10^4M	$[Cu(II)]_{free}$ x 10^6M	r
0.115	1.35	0.0169
0.315	1.80	0.49
0.62	2.75	0.98
1.22	4.00	0.196
1.61	4.93	0.259
2.02	5.90	0.326
2.41	6.76	0.391
3.01	8.55	0.488
4.82	13.28	0.779
6.02	15.84	0.976

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.2 Ultrafiltration study of Cu(II) binding to HSA (WPBTS, chelex-100 treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37^{0}C and I = 0.15M.
$[HSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total}$ x 10^4M	$[Cu(II)]_{free}$ x 10^5M	r
0.324	2.6	0.011
0.63	2.85	0.056
1.23	3.75	0.142
2.43	3.80	0.341
3.03	4.15	0.435
3.63	4.28	0.533
4.83	5.25	0.717
6.03	5.55	0.912

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.3 Equilibrium Dialysis of Cu(II) binding to HSA (Chelex-100 treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M. $[HSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total} \times 10^{4}M$	$[Cu(II)]_{free} \times 10^{5}M$	r
0.22	0.86	0.022
0.68	1.07	0.096
1.23	1.35	0.183
2.16	1.65	0.333
3.21	2.09	0.501
3.96	2.34	0.621
4.83	2.81	0.758
6.03	3.37	0.945

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.4 Ultrafiltration study of Cu(II) binding to HSA (Chelex-100 treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M. $[HSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total} \times 10^{4}M$	$[Cu(II)]_{free} \times 10^{5}M$	r
0.02	0.73	0.022
0.76	1.36	0.104
1.36	1.33	0.205
1.95	1.79	0.295
2.56	2.10	0.392
3.76	2.54	0.584
4.83	3.08	0.753
6.16	3.81	0.963

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.5 Equilibrium Dialysis of Cu(II) binding to BSA (Chelex-100 treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M. $[BSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total} \times 10^{4}M$	$[Cu(II)]_{free} \times 10^{8}M$	r
0.13	2.45	0.021
0.29	2.82	0.048
0.44	3.55	0.073
0.64	4.63	0.107
1.23	5.48	0.205
1.55	5.52	0.259
2.13	7.0	0.355
2.45	7.43	0.408
3.61	9.83	0.601
5.00	13.15	0.831
5.91	15.83	0.985

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.6 Ultrafiltration study of Cu(II) binding to BSA (Chelex-100 treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37^{0}C and I = 0.15M. $[BSA]_{total} = 6 \times 10^{-4}M$

$[Cu(II)]_{total} \times 10^4M$	$[Cu(II)]_{free} \times 10^8M$	r
0.08	2.40	0.014
0.13	2.60	0.021
0.34	3.98	0.055
0.63	5.17	0.105
1.23	6.47	0.205
1.63	6.85	0.271
2.03	8.41	0.338
2.43	7.76	0.405
3.04	9.48	0.506
4.83	14.48	0.804
5.94	18.00	0.990

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 3.7 Influence of Ionic Strength on Cu(II) binding to HSA (EDTA treated) in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M.
$[HSA]_{total} = 6 \times 10^{-4}M$

Ionic Strength M	$[Cu(II)]_{free} \times 10^{6}M$	r
0.03	5.50	0.015
0.06	3.50	0.018
0.09	2.65	0.020
0.12	2.00	0.021
0.15	1.70	0.021
0.20	1.18	0.022
0.25	0.90	0.023
0.30	0.65	0.023

where $r = [Cu(II)]_{bound}/[HSA]_{total}$

Table 4.1 Binding of L-tryptophan to HSA in 0.1M N-ethylmorpholine-HCl buffer at pH 7.5, 37°C and I = 0.15M.

$[Try]_{total}$ x 10^4M	$[Try]_{free}$ x 10^4M	r
0.56	0.11	0.074
1.46	0.32	0.19
2.35	0.61	0.30
4.42	1.58	0.473
6.58	2.99	0.598

where $r = [Try]_{bound}/[HSA]_{total}$

Table 4.2 Ultrafiltration studies on solutions in which the total Cu(II) and HSA concentrations were kept constant at 6.023 x 10-4 and 6.0 x 10-4M, respectively, while the concentration of L-His was varied.

$\frac{\text{L-His}}{\text{HSA}}$	$[\text{L-His}]_{total}$ x 10^4M	$[\text{Cu(II)}]_{lmw}$ x 10^5M	$[\text{L-His}]_{free}$ x 10^4M	r
0.3	1.8	4.2	1.21	0.933
0.6	3.6	9.9	2.39	0.841
1.0	6.0	16.4	3.80	0.732
2.0	12	24.0	8.14	0.606
4.0	24	32.0	19.50	0.476
6.0	36	35.8	30.02	0.410
8.0	48	38.10	40.81	0.370
10.0	60	39.90	49.90	0.340

where $r = [\text{His}]_{bound}/[\text{HSA}]_{total}$

Table 4.3 Ultrafiltration studies on the HSA-Cu(II)-L-Thr ternary system

$\frac{\text{L-Thr}}{\text{HSA}}$	$[\text{L-Thr}]_{total}$ x 10^4M	$[\text{Cu(II)}]_{lmw}$ x 10^5M	$[\text{L-Thr}]_{free}$ x 10^4M	r
0.3	1.8	0.15	1.70	0.98
0.6	3.6	3.24	3.50	0.952
1.0	6.0	2.10	5.50	0.971
2.0	12	4.20	10.88	0.935
4.0	24	7.80	22.20	0.876
6.0	36	11.20	34.00	0.820
8.0	48	13.30	44.90	0.780
10.0	60	15.20	56.70	0.750

where $r = [\text{Thr}]_{bound}/[\text{HSA}]_{total}$

Table 4.4 Ultrafiltration studies on the HSA-Cu(II)-L-Try ternary system

$\frac{\text{L-Try}}{\text{HSA}}$	$[\text{L-Try}]_{total}$ x 10^4M	$[\text{Cu(II)}]_{lmw}$ x 10^5M	$[\text{L-Try}]_{free}$ x 10^4M	r
0.3	1.8	1.2	0.5	0.986
0.6	3.6	1.5	1.0	0.980
1.0	6.0	0.6	2.0	0.996
2.0	12	1.68	4.3	0.977
4.0	24	1.68	8.9	0.977
6.0	36	3.60	12.2	0.945
8.0	48	4.6	19.0	0.928
10.0	60	5.9	26.1	0.907

where $r = [\text{Try}]_{bound}/[\text{HSA}]_{total}$

Table 5.1: Ultrafiltration studies of chelating agents in blood serum under physiological conditions.

Cu(II) ultrafiltrable in blood serum = 6.8×10^{-7}M

log[drug]	Abs	$[Cu(II)]_{lmw} \times 10^6$ M	PMI	log PMI
EDTA				
-2.0	1.750	6.15	9.0	0.96
-3.0	1.285	4.10	6.03	0.78
-4.0	0.868	2.6	3.82	0.58
-5.0	0.581	1.70	2.51	0.40
-6.0	0.345	0.99	1.46	0.16
EDDA				
-2.0	1.197	3.75	5.5	0.74
-3.0	0.740	2.20	3.24	0.51
-4.0	0.623	1.82	2.69	0.43
-5.0	0.379	1.05	1.55	0.19
-6.0	0.274	0.78	1.15	0.06
DTPA				
-2.0	1.843	6.61	9.72	0.99
-3.0	1.625	5.52	8.12	0.9
-4.0	1.235	3.58	5.76	0.76
-5.0	1.153	3.92	5.25	0.72
-6.0	0.860	2.58	3.80	0.58
TRIEN				
-2.0	1.379	4.5	6.61	0.82
-3.0	1.179	3.66	5.37	0.73
-4.0	0.949	2.84	4.17	0.62
-5.0	0.635	1.86	2.75	0.44
-6.0	0.611	1.78	2.63	0.42

Table 5.2: Ultrafiltration studies of chelating agents in blood serum under physiological conditions.

log[drug]	Abs	$[Cu(II)]_{lmw} x10^6 M$	PMI	log PMI
Aspirin				
-2.0	1.283	4.1	6.1	0.78
-3.0	0.672	2.30	3.8	0.58
-4.0	0.565	1.65	2.43	0.38
-5.0	0.415	1.18	1.74	0.24
-6.0	0.282	0.8	1.17	0.07
Mefanamic Acid				
-2.0	1.152	3.56	5.25	0.72
-3.0	0.701	2.05	3.02	0.48
-3.6	0.590	1.71	2.51	0.40
-4.4	0.454	1.3	1.9	0.28
-3.85	0.548	1.6	2.35	0.37
Indomethacin				
-2.0	1.49	3.18	4.68	0.67
-3.0	0.731	2.15	3.16	0.50
-4.0	0.478	1.36	2.0	0.30
-5.0	0.294	0.82	1.20	0.08
-6.0	0.237	0.68	1.0	0.0
Piroxicam				
-2.0	0.901	2.70	3.97	0.60
-2.97	0.639	1.87	2.75	0.44
-3.83	0.404	1.18	1.74	0.24
-4.77	0.32	0.90	1.32	0.12
-5.0	0.320	0.90	1.32	0.12
Prednisolone				
-2.0	0.520	1.5	2.2	0.34
-3.0	0.345	0.99	1.47	0.16
-3.6	0.274	0.78	1.15	0.06
-4.6	0.172	0.5	0.735	-0.13
D-Penicilamine				
-2.0	2.130	7.61	11.19	1.04
-3.0	1.598	5.40	7.94	0.90
-4.0	1.077	3.01	4.43	0.65
-5.0	0.635	1.85	2.72	0.43
-6.0	0.378	1.20	1.76	0.25
Tiaprofenic Acid				
-2.0	0.880	2.65	3.90	0.59
-3.0	0.586	1.70	2.50	0.40
-4.0	0.280	1.03	1.52	0.18
-5.0	0.274	0.78	1.15	0.06
-6.0	0.237	0.68	0.99	0

Table 5.3: Ultrafiltration studies of chelating agents in blood serum under physiological conditions.

Zn(II) ultrafiltrable in blood serum = 3.75×10^{-6}M

log[Drug]	Abs	$[Zn(II)]_{lmw} \times 10^5$M	PMI	log PMI
EDTA				
-2.0	0.220	3.10	8.27	0.92
-3.0	0.136	1.46	3.88	0.59
-4.0	0.114	1.17	3.12	0.49
-5.0	0.080	0.76	2.04	0.31
-6.0	0.051	0.47	1.27	0.10
EDDA				
-2.0	0.150	1.65	4.4	0.64
-3.0	0.118	1.21	3.23	0.51
-4.0	0.084	0.85	2.16	0.34
-5.0	0.066	0.63	1.67	0.22
-6.0	0.041	0.40	1.05	0.022
DTPA				
-2.0	0.235	3.42	9.12	0.96
-3.0	0.195	2.48	6.60	0.82
-4.0	0.168	1.94	5.17	0.72
-5.0	0.150	1.65	4.40	0.64
-6.0	0.123	1.30	3.44	0.54
TRIEN				
-2.0	0.163	1.85	4.93	0.69
-3.0	0.138	1.49	3.98	0.60
-4.0	0.129	1.36	3.63	0.56
-5.0	0.093	0.91	2.41	0.38
-6.0	0.077	0.73	1.93	0.28

Table 5.4: Zinc ultrafiltration studies of various drugs in blood serum under physiolopgical conditions

log[Drug]	Abs	$[Zn(II)]_{lmw} x10^5 M$	PMI	log PMI
Aspirin				
-2.0	0.082	0.80	2.13	0.32
-3.0	0.065	0.63	1.67	0.22
-4.0	0.059	0.57	1.51	0.18
-.50	0.045	0.41	1.10	0.04
-6.0	0.038	0.38	1.00	0.00
Mefanamic acid				
-2.0	0.082	0.79	2.10	0.32
-3.0	0.059	0.57	1.52	0.18
-3.6	0.054	0.52	1.38	0.14
-4.4	0.043	0.41	1.08	0.03
-3.85	0.046	0.45	1.18	0.07
Indomethacin				
-2.0	0.065	0.63	1.67	0.22
-3.0	0.052	0.48	1.28	0.11
-4.0	0.039	0.40	1.07	0.028
-5.0	0.035	0.33	0.87	-0.06
-6.0	0.034	0.33	0.87	-0.06
Piroxicam				
-2.0	0.110	1.11	2.96	0.47
-2.97	0.0	0.68	1.82	0.26
-3.83	0.057	0.54	1.44	0.16
-4.77	0.046	0.44	1.17	0.07
-4.36	0.049	0.48	1.27	0.10
Prednisolone				
-2.0	0.077	0.73	1.93	0.28
-3.0	0.044	0.42	1.12	0.05
-3.8	0.038	0.36	0.94	-0.02
-4.6	0.033	0.32	0.85	-0.06
D-Pen				
-2.0	0.167	1.92	5.12	0.71
-3.0	0.118	1.23	3.27	0.51
-4.0	0.093	0.91	2.41	0.38
-5.0	0.063	0.60	1.60	0.20
-6.0	0.053	0.50	1.33	0.12
Tiaprofenic Acid				
-2.0	0.066	0.61	1.61	0.22
-3.0	0.038	0.38	1.0	0
-4.0	0.038	0.38	1.0	0
-5.0	0.035	0.34	0.89	-0.05
-6.0	0.036	0.34	0.91	-0.04

Table 5.5: Calcium ultrafiltration studies of various drugs in blood serum under physiological conditions

Ultrafiltratable Ca(II) in blood serum = 7.18×10^{-4}M

log[Drug]	Abs	[Ca(II)]x10^{4}M	PMI	log PMI
DTPA				
-2.0	0.365	13.1	1.82	0.26
-3.0	0.268	9.62	1.34	0.13
-4.0	0.211	7.57	1.05	0.023
-5.0	0.197	7.07	0.98	0
-6.0	0.200	7.18	1	0
EDDA				
-2.0	0.206	7.30	1.02	0.007
-3.0	0.193	6.90	0.96	-0.017
-4.0	0.200	7.18	1	0
-5.0	0.203	7.20	1	0
-6.0	0.203	7.20	1	0
EDTA				
-2.0	0.294	10.6	1.47	0.169
-3.0	0.255	9.15	1.27	0.10
-4.0	0.210	7.53	1.05	0.02
-5.0	0.183	6.57	0.92	-0.04
-6.0	0.226	8.10	1.13	0.05
TRIEN				
-2.0	0.230	8.30	1.15	0.060
-3.0	0.221	7.92	1.10	0.042
-4.0	0.218	7.80	1.08	0.035
-5.0	0.216	7.75	1.08	0.023
-6.0	0.213	7.60	1.06	0.025

Table 5.6: Calcium ultrafiltration studies of various drugs in blood serum under physiological conditions

Ultrafiltratable Ca(II) in blood serum = 7.18×10^{-4}M

log[Drug]	Abs	[Ca(II)]x10^4M	PMI	log PMI
Aspirin				
-2.0	0.201	7.20	1.0	0.0
-3.0	0.195	7.00	0.98	-0.01
-4.0	0.203	7.28	1.01	0.006
-5.0	0.206	7.30	1.02	0.008
-6.0	0.202	7.20	1.0	0.0
Mefanamic acid				
-2.0	0.201	7.20	1.0	0.0
-3.0	0.201	7.20	1.0	0.0
-3.6	0.207	7.35	1.02	0.01
-4.4	0.205	7.35	1.02	0.0
Indomethacin				
-2.0	0.234	8.40	1.17	-0.07
-3.0	0.237	8.50	1.18	0.07
-4.0	0.226	8.10	1.13	0.05
-5.0	0.225	8.10	1.13	0.05
-6.0	0.216	7.70	1.07	0.03
Piroxicam				
-2.0	0.205	7.30	1.02	0.007
-2.97	0.212	7.60	1.06	0.02
-3.83	0.202	7.20	1.0	0.0
-4.77	0.202	7.20	1.0	0.0
-5.0	0.201	7.20	1.0	0.0
Prednisolone				
-2.0	0.205	7.30	1.02	0.007
-3.0	0.202	7.20	1.0	0.0
-3.6	0.189	6.78	0.94	-0.02
-4.6	0.210	7.50	1.04	0.018
-5.0	0.214	7.68	1.07	0.030
D-Pen				
-2.0	0.225	8.10	1.13	0.052
-3.0	0.225	8.10	1.13	0.052
-4.0	0.224	8.10	1.13	0.052
-5.0	0.232	8.32	1.16	0.064
-6.0	0.232	8.32	1.16	0.064
Tiaprofenic acid				
-2.0	0.205	7.30	1.02	0.007
-3.0	0.231	8.32	1.16	0.06
-4.0	0.217	7.65	1.06	0.03
-5.0	0.231	8.32	1.16	0.06
-6.0	0.215	7.63	1.06	0.026